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Upper Airway Collapsibility during Dexmedetomidine and Propofol Sedation

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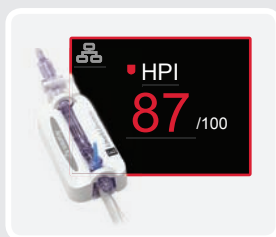The Journal of the American Society of Anesthesiologists, Inc.

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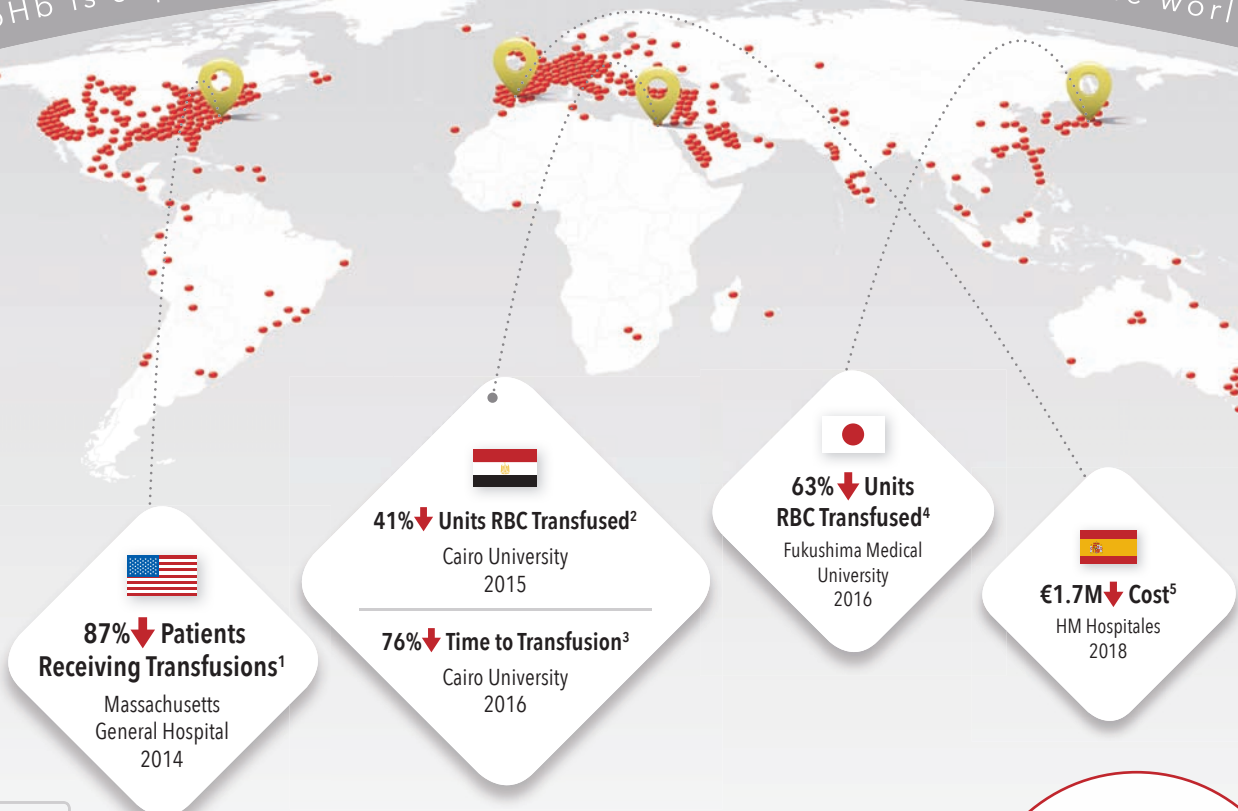American Society of **Anesthesiologists**®

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[1] Ehrenfeld et al. *J Blood Disorders Transf*. 2014. 5:9. [2] Awada WN et al. *J Clin Monit Comput*. DOI 10.1007/s10877-015-9660-4. Study Protocol: In each group, if researchers noted SpHb trended downward below 10 g/dL, a red blood cell transfusion was started and continued until SpHb trended upward above 10 g/dL. The transfusion threshold of 10 g/dL was predetermined by the study protocol and may not be appropriate for all patients. Blood sampling was the same for the control and test group. Arterial blood was drawn from a 20 gauge radial artery cannula into 2 mL EDTA collection tubes, mixed and sent for analysis by a Coulter GEN-S Hematology Analyzer. [3] Kamal A, et al. *Open J of Anesth*. 2016 Mar; 6, 13-19. [4] Imaizumi et al. *Proceedings from the 16th World Congress of Anaesthesiologists*, Hong Kong. Abstract #PR607. [5] Ribed-Sánchez B, et al. *Sensors* (Basel). 2018 Apr 27;18(5). pii: E1367. * Data on file.

THIS MONTH IN
ANESTHESIOLOGY
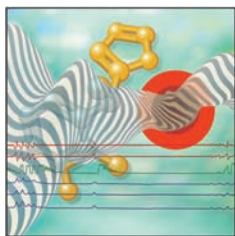
962 Upper Airway Collapsibility during Dexmedetomidine and Propofol Sedation in Healthy Volunteers: A Nonblinded Randomized Crossover Study

Dexmedetomidine is used for sedation in both children and adults, with and without obstructive sleep apnea, in the belief that both upper airway patency and ventilatory drive are less compromised with dexmedetomidine than they are with other sedatives. This belief has been challenged by findings from several recent studies. The hypothesis that dexmedetomidine sedation would have less effect on upper airway collapsibility than propofol sedation was tested in a randomized crossover study of nine volunteers. Pharyngeal critical pressure, which assesses the intraluminal pressure at which airway closure occurs, was used as a measure of collapsibility. Apnea episodes occurred during sedation with both drugs. Pharyngeal critical pressure values indicative of total obstruction at atmospheric pressure (*i.e.*, values of 0 cm H_2O or higher) were observed in five of the nine subjects during dexmedetomidine infusion at low and/or moderate infusion rates. There was no difference in pharyngeal critical pressure value thresholds during sedation with dexmedetomidine or propofol at either low or moderate infusion rates. *See the accompanying Editorial View on* **page 953.** *(Summary: M. J. Avram. Image: A. Johnson, Vivo Visuals.)*

1004 Pharmacodynamic Interaction of Remifentanil and Dexmedetomidine on Depth of Sedation and Tolerance of Laryngoscopy

Patients sedated with standard clinical doses of dexmedetomidine can be readily aroused. Dexmedetomidine doses producing mild to deep sedation lack significant analgesic effect. Although remifentanil is an opioid analgesic with only modest sedative properties, addition of remifentanil to propofol sedation reduces the propofol concentration required to reach tolerance of shaking the patient while shouting their name and tolerance of laryngoscopy. This three-phase crossover trial was designed to study the pharmacodynamic interaction between remifentanil and dexmedetomidine and quantify their expected synergy in 30 age- and sex-stratified healthy volunteers on two occasions. On day one volunteers were administered stepwise increasing target-controlled infusions of dexmedetomidine while on day two they were administered target-controlled infusions of remifentanil alone and remifentanil with a fixed background dexmedetomidine concentration. Despite falling asleep, most subjects remained arousable by calling their name, shaking them while shouting their name, or a trapezius squeeze, even after reaching supraclinical dexmedetomidine concentrations. The addition of remifentanil to dexmedetomidine sedation did not affect the likelihood of subject response to graded stimuli. *(Summary: M. J. Avram. Image: J. P. Rathmell.)*

983 An Assessment of Penetrance and Clinical Expression of Malignant Hyperthermia in Individuals Carrying Diagnostic Ryanodine Receptor 1 Gene Mutations

Malignant hyperthermia is a rare life-threatening disorder triggered in genetically predisposed individuals by exposure to certain anesthetics. The ryanodine receptor 1 (*RYR1*) gene, which encodes the Ca^{2+} release channel of skeletal muscle sarcoplasmic reticulum, is the major malignant hyperthermia-associated locus. Malignant hyperthermia diagnostic mutations are more prevalent than the reported incidence of clinical malignant hyperthermia episodes because many mutation carriers are never exposed to anesthetic triggers and some may have several uneventful anesthetics before developing malignant hyperthermia. In a multicenter case-control study of 229 genotype-positive subjects with previous recorded exposure to trigger anesthetics, there were 93 malignant hyperthermia cases, for an overall penetrance for the analyzed *RYR1* mutations of 41%. The probability of developing malignant hyperthermia on exposure to triggers was 0.25 among all *RYR1* mutation carriers and 0.76 in survivors of malignant hyperthermia reactions (95% CI of the difference, 0.41 to 0.59). Young age, male sex, and the use of succinylcholine were major nongenetic risk factors influencing expression of the *RYR1* genotypes conferring malignant hyperthermia susceptibility. *See the accompanying Editorial View on* **page 957.** *(Summary: M. J. Avram. Image: J. P. Rathmell.)*

974 Genetic Analysis of Patients Who Experienced Awareness with Recall while under General Anesthesia

The incidence of explicit recall of intraoperative events, or awareness with recall, is less than 0.2%. Anesthetic dosing is apparently adequate in 10 to 25% of patients with awareness with recall. The awareness with recall phenotype only reveals itself when patients are exposed to anesthesia; typically, awareness with recall patients display no other identified phenotypic disturbance in day-to-day life. A preliminary study in 12 patients who had suffered awareness with recall in the presence of apparently adequate anesthesia sought to determine whether there is evidence that awareness with recall is caused by a few rare genetic variants with high penetrance. Whole exome sequencing was conducted and identified variants were filtered and prioritized to identify a candidate list that might be suitable for further investigation of causes of awareness with recall. No candidate gene(s) suggestive of a monogenic etiology were identified, possibly because of the application of a filtering strategy, the small sample size, or use of exome sequencing, which does not interrogate potentially important regulatory noncoding sequences. *See the accompanying Editorial View on* **page 955.** *(Summary: M. J. Avram. Image: ©gettyimages.)*
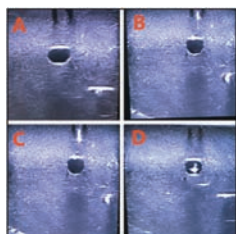
1018 Acoustic Shadowing Facilitates Ultrasound-guided Radial Artery Cannulation in Young Children

The success rate of radial artery puncture has improved with the ultrasound-guided technique but depends on the operator's experience and skills due to the two-dimensional nature of the imaging. The acoustic shadowing ultrasound with double developing lines produced by metal-containing strands taken from x-ray detectable surgical gauze and bound in parallel 2 mm apart to the ultrasound probe helps locate the projection point of the midpoint of the radial artery on the skin surface to enable quick and accurate determination of the puncture point. The hypothesis that ultrasound-guided vascular puncture with double developing lines could help increase the success rate of radial artery puncture was tested in a randomized controlled trial of 79 young children. Radial artery cannulation was successful at the first attempt in 35 of the 39 (90%) patients in the novel ultrasound group and in 24 of the 40 (60%) patients in the traditional ultrasound group (difference 30%; 95% CI, 12 to 48%). *(Summary: M. J. Avram. Image: From original article.)*
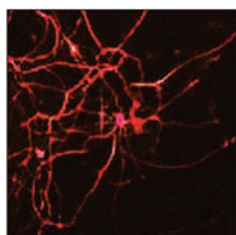
1063 Nitrous Oxide Impairs Axon Regeneration after Nervous System Injury in Male Rats

Methionine is the single carbon donor in mammalian cells. Methionine synthase requires 5-methyl-tetrahydrofolate as its single carbon source and is irreversibly inactivated by nitrous oxide with oxidation of its cobalamin cofactor. The hypothesis that single and serial *in vivo* nitrous oxide exposures impair axon regeneration was tested in four experimental male rat models of nervous system injury. *In vitro* axon regeneration 48 h after a single *in vivo* 70% nitrous oxide exposure was less than half that in the absence of nitrous oxide. One exposure to 80% nitrous oxide fully inhibited the beneficial effects of folic acid on *in vivo* dorsal root ganglion axon regeneration after sharp spinal cord injury. After sharp optic nerve injury, serial 80% nitrous oxide administration reversed the regenerative benefits of folic acid on *in vivo* retinal ganglion cell axon regeneration. The marked beneficial effects of folic acid on *in vivo* scores of behavioral recovery after direct spinal cord contusion were reversed by coadministration of serial 80% nitrous oxide exposure. *(Summary: M. J. Avram. Image: From original article.)*
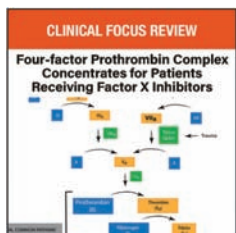
1153 Four-factor Prothrombin Complex Concentrate for the Management of Patients Receiving Direct Oral Activated Factor X Inhibitors (Clinical Focus Review)

Direct oral anticoagulants, which achieve anticoagulation by inhibiting specific coagulation factors, have been approved for the prevention of stroke and systemic embolism in atrial fibrillation, treatment and secondary prevention of venous thromboembolism, and thromboprophylaxis after major orthopedic surgery. In cases of severe or life-threatening bleeding or for patients undergoing urgent surgery, restoration of hemostasis requires prompt reversal of anticoagulation in addition to a multimodal approach using hemostatic agents. Prothrombin complex concentrates contain factors II, IX, and X, with or without factor VII, and, depending on the formulation, similar proportions of coagulation inhibitors such as protein C, protein S, and low doses of heparin. Current data support the use of prothrombin complex concentrate for the reversal of activated factor X inhibitors in bleeding patients and suggest that prothrombin complex concentrate could become a useful and relatively affordable option for management of direct oral anticoagulant-associated bleeding. Further studies are needed to investigate the optimal dosing of prothrombin complex concentrate to maintain the balance between procoagulant effectiveness and low thrombotic risk. *(Summary: M. J. Avram. Image: J. P. Rathmell.)*
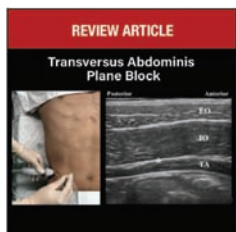
1166 Transversus Abdominis Plane Block: A Narrative Review (Review Article)

Transversus abdominis plane blocks have been used to provide postoperative analgesia for open and laparoscopic abdominal surgery as well as inpatient and outpatient surgical procedures. This review discusses the anatomy, nomenclature, history, approaches (posterior, lateral, and subcostal), techniques, pharmacology, and complications of transversus abdominis plane blocks. It also reviews the evidence supporting their clinical use for common open and laparoscopic surgical procedures and explores possible alternative truncal blocks as well as areas requiring further investigation. Despite contradictory findings, scarcity of evidence, and shortcomings afflicting some randomized controlled trials, certain clinical suggestions can be made. Overall transversus abdominis plane blocks appear most beneficial in the setting of open appendectomy (posterior or lateral approach). Lateral transversus abdominis plane blocks are not suggested for laparoscopic hysterectomy, laparoscopic appendectomy, and open prostatectomy. Transversus abdominis plane blocks could serve as an analgesic option for Cesarean delivery (posterior or lateral approach) and open colorectal surgery (subcostal or lateral approach) if there exist contraindications to intrathecal morphine and thoracic epidural analgesia, respectively. *(Summary: M. J. Avram. Image: From original article.)*

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At comparable levels of light to moderate sedation, dexmedetomidine and propofol exhibit similar degrees of pharyngeal collapsibility and reductions in ventilatory drive. The findings suggest that sedation with dexmedetomidine does not offer inherent protection against upper airway obstruction or ventilatory depression.

A preliminary study sought to determine whether there is evidence that awareness with recall is caused by a few rare variants with high penetrance in 12 patients who had experienced awareness with recall in the presence of apparently adequate anesthesia. Whole exome sequencing was conducted and identified variants were filtered and prioritized to identify a candidate list that might be suitable for further investigation of causes of awareness with recall. No candidate gene(s) suggestive of a monogenic etiology were identified, possibly because of the application of a filtering strategy, the small sample size, or use of exome sequencing, which does not interrogate potentially important regulatory noncoding sequences.

In a multicenter case-control study of 229 genotype-positive subjects with previous recorded exposure to trigger anesthetics, there were 93 malignant hyperthermia cases, for an overall penetrance for the analyzed *RYR1* mutations of 40.6%. The probability of developing malignant hyperthermia on exposure to triggers was 0.25 among all *RYR1* mutation carriers and 0.76 in survivors of malignant hyperthermia reactions (95% CI of the difference 0.41 to 0.59). Young age, male sex, and the use of succinylcholine were major nongenetic risk factors influencing expression of the *RYR1* mutations conferring malignant hyperthermia susceptibility.

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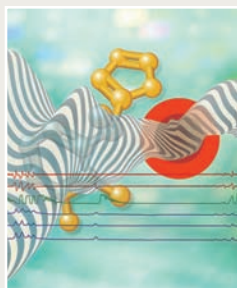
ON THE COVER: Dexmedetomidine is a sedative promoted as having minimal impact on ventilatory drive or upper airway muscle activity. However, more recent study has demonstrated impaired ventilatory drive and induction of apneas in sedated volunteers. In this issue of ANESTHESIOLOGY, Lodenius *et al.* measured upper airway collapsibility during dexmedetomidine sedation and related it to propofol. In an accompanying Editorial View, Ward and Karan review previous comparative studies with this new trial and conclude that light to moderate sedation with dexmedetomidine does not appear to offer any protection from central ventilatory apneas and airway obstructions over propofol. Cover illustration: A. Johnson, Vivo Visuals.

• Lodenius *et al.*: Upper Airway Collapsibility during Dexmedetomidine and Propofol Sedation in Healthy Volunteers: A Nonblinded Randomized Crossover Study, p. 962

• Ward and Karan: Dexmedetomidine and the Upper Airway: Not as Simple as We Hoped, p. 953

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Results from standardized physical function questions and hip accelerometers were compared in 522 participants. Physical function questions were sensitive but nonspecific. Other approaches to assessing physical functional status should be considered.

This three-phase crossover trial to study the pharmacodynamic interaction between remifentanil and dexmedetomidine in 30 age- and sex-stratified healthy volunteers found that, despite falling asleep, most subjects remained arousable by calling their name, shaking the subject while shouting their name, or a trapezius squeeze, even after reaching supraclinical concentrations. Adding remifentanil to dexmedetomidine sedation did not affect the likelihood of response to graded stimuli. Dexmedetomidine potency increased with increasing age.

This prospective, randomized trial in young children shows that a modified ultrasound-guided approach, using focused acoustic shadowing, results in a higher success rate and shorter cannulation time of the radial artery when compared with traditional ultrasound guidance.

Among elderly patients undergoing emergency hip fracture surgery in Ontario, Canada, peripheral nerve blocks may be associated with slightly decreased postoperative lengths of stay and health system costs. The use of peripheral nerve blocks was not associated with a difference in postoperative pneumonia rates.

The investigators tested the hypothesis that a cognitive aid to guide selective use of sugammadex reduced use. They conducted a segmented regression (interrupted time series) retrospective analysis before and after implementing the cognitive aid and informational meetings for their department. Sugammadex use and associated costs, which were increasing, decreased substantially after introduction of the cognitive aid.

In this retrospective analysis, the intraoperative ventilation bundle was associated with a lower rate of postoperative pulmonary complications. Lower modified driving pressure was independently associated with fewer pulmonary complications.

BASIC SCIENCE

In *in vitro* and *in vivo* experimental models of male rats, nitrous oxide exposure impairs folic acid-induced axonal regeneration of dorsal root and retinal ganglion neurons. The beneficial effects of folic acid on functional recovery following spinal cord contusion in male rats are hindered by co-administration of nitrous oxide. These experiments suggest that nitrous oxide can interfere with axonal regeneration and functional recovery following central nervous system injury.

Exposure of 7-day-old mouse pups to isoflurane (1.5%, 4 h) results in lasting impairments of oligodendrocyte proliferation and differentiation. These effects lead to defects in myelinations and are associated with cognitive dysfunction. The underlying molecular mechanisms involve the isoflurane-induced activation of the mammalian target of rapamycin pathway and a related decrease in DNA methylation in oligodendrocyte progenitors.

Repeated exposures of adult rats to sevoflurane (2.1%, three times, 3 h on every second day) induce neurobehavioral abnormalities in the exposed males and in male but not female progeny. The neurobehavioral abnormalities in male offspring are accompanied by increased methylation and decreased expression of the potassium ion-chloride ion cotransporter Kcc2 gene that regulates neuronal chloride homeostasis, and, thereby, the functional modalities of γ-aminobutyric acid type A receptor–mediated neurotransmission. Sevoflurane exposure also induces hypermethylation of the Kcc2 gene in both male and female parental germ cells. These observations suggest that epigenetic reprogramming of parental germ cells is involved in transmitting the adverse effects of sevoflurane exposure of adult rats to their male progeny.

Critical Care Medicine

BASIC SCIENCE

In a rat model of hemorrhagic shock, comparing fluid resuscitation with blood, diluted blood, hydroxyethyl starch, or polyethylene-glycolated carboxyhemoglobin, all fluids restored urine output and creatinine clearance, but only blood and diluted blood improved renal P_{O_2}. Postresuscitation histologic renal tubular damage was increased compared with nonresuscitated rats but slightly less with blood, diluted blood, and polyethylene-glycolated carboxyhemoglobin compared with hydroxyethyl starch. Restoration of circulatory hemodynamics and kidney microcirculatory P_{O_2} was comparable with polyethylene-glycolated carboxyhemoglobin and balanced hydroxyethyl starch solution.

Pain Medicine

BASIC SCIENCE

In a female rat model of metastatic breast cancer, expression of vascular endothelial growth factor A and its receptor vascular endothelial growth factor receptor 2 were upregulated in spinal tissue. Blocking vascular endothelial growth factor signaling improved several measures of nociception and function in this model suggesting a role for vascular endothelial growth factor antagonists in reducing cancer-related pain.

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CLINICAL FOCUS REVIEW

Factor Xa inhibitors prevent thrombosis but are associated with severe or life-threatening bleeding. Here, the authors present data on four-factor prothrombin complex concentrates in management of anticoagulation-associated bleeding and restoring hemostasis, including recent results from the UPRATE study.

REVIEW ARTICLE

This narrative review article discusses the anatomy, history, nomenclature, approaches/techniques, pharmacology, indications, potential complications, and alternatives for transversus abdominis plane blocks.

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This Month's ANESTHESIOLOGY Journal-based CME Article

Read the article by Grottke and Schulman entitled "Four-factor Prothrombin Complex Concentrate for the Management of Patients Receiving Direct Oral Activated Factor X Inhibitors" on page 1153.

Learning Objectives

After successfully completing this activity, the learner will be able to recognize the advantages of direct oral anticoagulants administered for the treatment of thromboembolic events, manage the risks associated with direct oral anticoagulants, and effectively manage patients requiring direct oral anticoagulant reversal.

Disclosures

This journal article has been selected for and planned as a journal CME activity, which is designated for *AMA PRA Category 1 Credit*™. The authors disclosed relationships in keeping with ANESTHESIOLOGY's requirements for all journal submissions. All relationships journal authors disclosed to ANESTHESIOLOGY are disclosed to learners, even those relationships that are not relevant financial relationships, per the ACCME's requirements for CME activities.

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Deborah J. Culley, M.D., Editor

Key Papers from the Most Recent Literature Relevant to Anesthesiologists

Effect of flexible family visitation on delirium among patients in the intensive care unit: The ICU Visits randomized clinical trial. JAMA 2019; 322:216–28.

Many hospitals have instituted flexible intensive care unit (ICU) visiting hours but the effect on clinical outcomes remains uncertain. This cluster-crossover randomized trial set out to determine whether a flexible ICU family visitation policy would reduce inpatient delirium. It also looked at whether it effected ICU-acquired infections, family anxiety or depression, and ICU staff burnout. Participants included 1,685 patients, 1,060 family members, and 737 clinicians at 36 adult ICUs. For the study, flexible visitation expanded hours to 12 per day and included in-person family education. Restricted hours meant following usual policy, which was a median of 1.5 h of family visitation daily. The authors found that ICU visitation policies did not affect the incidence of delirium (flexible: 19%, restricted: 20% adjusted difference, −1.7% [95% CI, −6.1% to 2.7%]; $P = 0.44$). Three of nine outcomes showed significant between-group variations based on visitation policy, including ICU-acquired infections (4% *vs.* 5%) and staff burnout (22% *vs.* 25%). With flexible visitation, family members experienced improvements in median anxiety (6 *vs.* 7) and depression scores (4 *vs.* 5). *(Article Selection: Martin J. London. Image: J. P. Rathmell.)*

Take home message: Flexible family visitation policies in an ICU may not reduce the risk of postoperative delirium but may have other positive effects on family and faculty.

Effect of drug disposal bag provision on proper disposal of unused opioids by families of pediatric surgical patients: A randomized clinical trial. JAMA Pediatr 2019 Jun 24 [Epub ahead of print].

Providing patients with opioid disposal products before hospital discharge may help combat opioid abuse and diversion. This study examined whether providing a drug disposal bag increased proper opioid disposal among the families of pediatric patients. Parents of children who had outpatient otolaryngologic or urologic surgery at a major children's hospital and received an opioid prescription participated in this randomized trial. Intervention families received an activated charcoal drug disposal bag plus standard discharge instructions regarding proper opioid storage and disposal. Control families received only the discharge instructions. Participants completed two questionnaires: one at discharge and one 2 to 4 weeks later. Proper opioid disposal was the primary outcome. Among 202 parents, 181 completed follow-up; participation was evenly split between arms. Families who received a disposal bag were more likely to report proper disposal: 66 families (71.7%) *versus* 50 families without a bag (56.2%; 95% CI, 1.7% to 29.3%; $P = 0.03$). Among families who had leftover opioids, a higher percentage of those who received a disposal kit reported proper disposal: 85.7% *versus* 64.9% without a kit (95% CI, 7.6% to 34.0%). *(Article Selection: J. David Clark. Image: The Noun Project.)*

Take home message: This study suggests that providing drug disposal bags to families of children receiving postoperative opioids increased the likelihood of excess opioid disposal.

Association of duration and type of surgical prophylaxis with antimicrobial-associated adverse events. JAMA Surg 2019; 154:590–8.

Antimicrobial prophylaxis offers benefits in the perioperative setting, but the harmful effects associated with continuing prophylaxis after skin closure are not well understood. This study used rates of surgical site infection, acute kidney injury, and Clostridium difficile infection to explore an association between the type of antibiotic prophylaxis and duration on adverse outcomes. This retrospective cohort study included patients from Veterans Affairs who underwent cardiac, joint replacement, colorectal, and vascular procedures during a 5-yr period. The authors analyzed the duration of postoperative antimicrobial prophylaxis in daily increments for up to 3 days in 79,058 patients. After stratifying and adjusting for multiple factors, the authors found that surgical site infection did not correlate with treatment length. However, the odds of acute kidney injury and Clostridium difficile progressively increased with each additional day of antibiotic prophylaxis following both cardiac and noncardiac surgeries such that administration of antibiotics for 72 h after surgery was associated with an increased odds ratio of 1.79 (95% CI, 1.27 to 2.53) for the development of acute kidney injury in noncardiac surgical procedures which is similar to that in cardiac surgery (odds ratio 1.82; 95% CI, 1.54 to 2.16). *(Article Selection: Beatrice Beck-Schimmer. Image: J. P. Rathmell.)*

Take home message: Prolonged antimicrobial prophylaxis was associated with higher odds of postoperative acute kidney injury and Clostridium difficile infection after surgery.
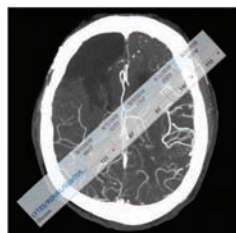
Intensive vs standard treatment of hyperglycemia and functional outcome in patients with acute ischemic stroke: The SHINE randomized clinical trial. JAMA 2019; 322:326–35.

Hyperglycemia in the setting of acute ischemic stroke is common and is associated with adverse outcomes. The SHINE trial examined whether intensive hyperglycemia treatment is effective in this setting. Participants included adults with acute ischemic stroke and glucose concentrations of greater than 110 mg/dl (if diabetic) or 150 mg/dl (if not diabetic) at 63 U.S. hospitals (n = 1,151). Patients were randomized to receive continuous IV insulin with a target blood glucose concentration of 80 to 130 mg/dl (4.4 to 7.2 mmol/l) or subcutaneous insulin on a sliding scale with a target blood glucose concentration of 80 to 179 mg/dl (4.4 to 9.9 mmol/l). In the intensive treatment group, the mean blood glucose level was 118 mg/dl (6.6 mmol/l) and 179 mg/dl (9.9 mmol/l) in the standard treatment group. However, only patients in the experimental group experienced severe hypoglycemia (15/581 [2.6%]; risk difference, 2.58% [95% CI, 1.29% to 3.87%]). There was no statistically significant difference in the percentage of patients who achieved a favorable outcome based on the 90-day modified Rankin Scale score (intensive treatment: 21%; standard treatment: 22%). The adjusted relative risk was 0.97 (95% CI, 0.87 to 1.08, $P = 0.55$; unadjusted risk difference, −0.83% [95% CI, −5.72% to 4.06%]). Accordingly, the trial was stopped for futility. *(Article Selection: Laszlo Vutskits. Image: J. P. Rathmell.)*

Take home message: For patients with acute ischemic stroke, treatment with intensive *versus* standard glucose control for up to 72 h had no effect on functional outcome at 90 days.

Standards for studies of neurological prognostication in comatose survivors of cardiac arrest: A scientific statement from the American Heart Association. Circulation 2019; 140:e517–42.

Patient mortality remains high after cardiac arrest. Massive brain injuries are believed to account for most patient deaths after cardiac arrest. If physicians were better able to predict outcomes for these patients, futile treatments could be avoided and proper care continued for patients who are more likely to recover neurologic function. The American Heart Association Emergency Cardiovascular Care Science Subcommittee convened a writing group composed of adult and pediatric experts in adult and pediatric neurology, cardiology, emergency medicine, intensive care medicine, and nursing to assess the current state of knowledge about prognostication for these patients and when life-sustaining treatment should be withdrawn. The writing group concluded that the existing body of research into neurologic prognostication is of overall low quality, leading to a lack of clinical confidence in predictors and outcomes. Their suggested approach is to develop neurologic function index tests that directly correlate with patient outcomes and quality of life measures. *(Article Selection: Martin J. London. Image: ©gettyimages.)*

Take home message: The ability to identify patients who are likely to have either a good or poor neurologic outcome after cardiac arrest remains low.

Impact of dexmedetomidine on long-term outcomes after noncardiac surgery in elderly: 3-year follow-up of a randomized controlled trial. Ann Surg 2019; 270:356–63.

Postoperative delirium, while common in the elderly, is associated with a variety of both short- and long-term negative outcomes. This randomized clinical trial compared the long-term outcomes of low-dose dexmedetomidine *versus* placebo in 700 patients. The authors previously reported that patients who had stayed in the intensive care unit after noncardiac surgery and were treated with dexmedetomidine were less likely to develop delirium, but the effect on long-term outcomes were unknown. The authors interviewed patients or family members at a 3-yr follow-up to assess survival and cognitive function. Overall, the 3-yr survival rate was comparable between the two groups, with 114 deaths in the intervention group and 122 deaths in the placebo group (hazard ratio 0.87; 95% CI, 0.68 to 1.13; $P = 0.303$). However, patients in the dexmedetomidine group had significantly higher survival rates at 6 months, 1 yr, and 2 yr (rate difference of 5.2%, 5.3%, and 6.7%, respectively; $P < 0.05$). Among 3-yr survivors, patients in the intervention group had significantly better cognitive function and quality of life scores in the physical, psychologic, social relationships, and environment domains when compared to those that received placebo ($P < 0.001$). *(Article Selection: Deborah J. Culley. Image: J. P. Rathmell.)*

Take home message: Among elderly patients admitted to intensive care unit after noncardiac surgery, low-dose dexmedetomidine infusion may not modify 3-yr survival, although it may increase survival for up to 2 yr. Importantly, this study suggests that dexmedetomidine administration in the intensive care unit may be associated with improved cognitive function and quality of life in 3-yr survivors.

Effect of albuterol premedication vs placebo on the occurrence of respiratory adverse events in children undergoing tonsillectomies: The REACT randomized clinical trial. JAMA Pediatr 2019; 173:527–33.

As many as half of the children who undergo tonsillectomy have a surgery-related respiratory adverse event. This study investigated whether pediatric tonsillectomy patients who were treated with inhaled albuterol sulfate (salbutamol sulfate) preoperatively were less likely to experience adverse perioperative respiratory events. The REACT trial was a randomized, triple-blind, placebo-controlled trial conducted in Perth, Australia, among 484 children up to 8 yr of age who underwent tonsillectomy. Patients received either albuterol (two actuations, 200 μg) or placebo preoperatively, and the primary outcome was the development of bronchospasm, laryngospasm, airway obstruction, desaturation, coughing, or stridor before leaving the postanesthesia care unit. The authors found that nearly half of the children in the placebo group (47.9%, n = 114) had an adverse event, while only about one quarter of the intervention group (27.8%, n = 67) did. Even after adjusting for age, airway management, and severity of obstructive sleep apnea, patients in the placebo arm were more likely to experience adverse respiratory events (odds ratio, 2.8; 95% CI, 1.9 to 4.2; $P < 0.001$). (Article Selection: Laszlo Vutskits. Image: ©gettyimages.)

Take home message: Albuterol premedication administered before tonsillectomy in young children may result in fewer adverse perioperative respiratory events when compared to children who did not receive albuterol premedication. Premedication with albuterol should be considered for children undergoing tonsillectomy.

Effect of tanezumab on joint pain, physical function, and patient global assessment of osteoarthritis among patients with osteoarthritis of the hip or knee: A randomized clinical trial. JAMA 2019; 322:37–48.

Many patients with moderate to severe osteoarthritis of the knee or hip do not have adequate pain relief with traditional treatments. This study compared two subcutaneous dosing regimens of the monoclonal antibody tanezumab in patients with osteoarthritis in a randomized, double-blind, multicenter trial. Patients were randomized to receive injections of tanezumab (2.5 mg at day 1 and week 8 or 2.5 mg at day 1 and 5 mg at week 8) or placebo. The primary endpoints were changes at 16 weeks using the Western Ontario and McMasters Universities Osteoarthritis Index Pain and Physical Function and patient global assessment of osteoarthritis indexes. Of the 582 patients who completed the trial, patients in the tanezumab groups experienced statistically significant improvements in joint pain, physical function, and patient global assessment of osteoarthritis over 16 weeks. However, the improvements were limited. Patients in the experimental groups had more total joint replacements, with nearly 7% in the higher-dose group undergoing joint replacement, while less than 2% of the placebo group did. (Article Selection: J. David Clark. Image: J. P. Rathmell.)

Take home message: In patients with moderate to severe osteoarthritis of the knee or hip, tanezumab, compared with placebo, resulted in improvements in scores assessing pain and physical function, but may be associated with an increase in total joint replacements.

A multicenter trial of vena cava filters in severely injured patients. N Engl J Med 2019; 381:328–37.

Venous thromboembolism is a frequent sequela of major trauma and many severely injured patients cannot receive preventive anticoagulation. This multicenter, randomized, controlled trial investigated whether early placement of an inferior vena cava filter reduced the risk of pulmonary embolism or death in severely injured patients. Investigators randomized 240 severely injured patients who were ineligible for anticoagulation to receive an inferior vena cava filter within 72 h or not. The primary endpoint was a composite of symptomatic pulmonary embolism or any-cause death at 90 days. There were no differences in the primary outcome between the group that received inferior vena cava filters and those that did not (filter group: 14%, control group: 14%; hazard ratio 0.99; 95% CI, 0.51 to 1.94; $P = 0.98$). No patients in the filter group (n = 46) who did not receive preventive anticoagulation developed a pulmonary embolism, but five patients in the control group did (n = 34, 15%), including one who died. Trapped thrombi were found in the inferior venal cava filters of six patients. (Article Selection: Laszlo Vutskits. Image: J. P. Rathmell.)

Take home message: Prophylactic placement of a vena cava filter after major trauma may not result in a lower incidence of symptomatic pulmonary embolism or death at 90 days when compared to patients who did not receive a filter.

SCIENCE, MEDICINE, AND THE ANESTHESIOLOGIST

Key Papers from the Most Recent Literature Relevant to Anesthesiologists
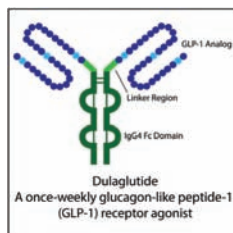
Dulaglutide and cardiovascular outcomes in type 2 diabetes (REWIND): A double-blind, randomised placebo-controlled trial. Lancet 2019; 394:121–30.

Patients with type 2 diabetes and high glycated hemoglobin A1c concentrations are at high risk for cardiovascular events. This randomized, double-blind, placebo-controlled trial performed at 371 sites in 24 countries and included 9,901 patients studied how the glucagon-like peptide-1 receptor agonist dulaglutide affected the risk of major adverse cardiovascular events when added to existing antihyperglycemic regimens. Participants, who had type 2 diabetes with varying degrees of glycemic control, with and without previous cardiovascular disease, were randomized to either weekly injections of dulaglutide 1.5 mg or placebo. The authors used a composite endpoint of nonfatal myocardial infarction, nonfatal stroke, and cardiovascular-related death. During a median follow-up of 5 yr, 12% of patients in the dulaglutide group (n = 594) experienced the primary outcome for an incidence rate of 2 per 100 person-years. This compares with 13% in control patients (n = 663) for an incidence rate of 3 per 100 person-years (hazard ratio 0.88; 95% CI, 0.79 to 0.99; $P = 0.026$). All-cause mortality was comparable between groups: dulaglutide group: 11%, n = 536; placebo group: 12%, n = 592 (hazard ratio 0.90; 95% CI, 0.80 to 1.01; $P = 0.067$). Interestingly, those individuals randomized to the dulaglutide group had a higher risk of gastrointestinal events when compared to the placebo during follow-up. *(Article Selection: Martin J. London. Image: J. P. Rathmell.)*

Take home message: Dulaglutide should be considered for the management of hyperglycemia in middle-aged and older people with type 2 diabetes with a history of cardiovascular disease or cardiovascular risk factors but may increase the risk of gastrointestinal events.

Demographics, care patterns, and outcomes of patients admitted to cardiac intensive care units: The Critical Care Cardiology Trials Network Prospective North American Multicenter Registry of Cardiac Critical Illness. JAMA Cardiol 2019 Jul 24 [Epub ahead of print].

Evolving demographics, care patterns, and patient outcomes in the modern cardiac intensive care unit are not well understood. This study established the Critical Care Cardiology Trials Network, an investigator-initiated multicenter network of 16 advanced cardiac intensive care units in the United States and Canada. Each cardiac intensive care unit sent data for its consecutive admissions over a 2-month period to the central data coordinating center. This study tracked the demographics, diagnoses, management, and clinical outcomes of 3,049 participants. Of 3,310 admissions during a 1-yr period, three quarters (2,557, 77.3%) were for primary cardiac problems. Of the remaining admissions, 337 (10%) were for postprocedural care, 253 (8%) for mixed general and cardiac problems, and 163 (5%) for general intensive care unit overflow. The most common admission diagnoses were acute coronary syndrome 969 (32%) and heart failure 567 (19%). The incidence of coronary syndrome ranged from 15% to 57% depending on the center. Respiratory insufficiency, shock, unstable arrhythmia, and cardiac arrest were the most common entrance diagnoses. The highest mortality rates occurred in patients with cardiac arrest, cardiogenic shock, and/or needed renal replacement therapy. *(Article Selection: Martin J. London. Image: ©gettyimages.)*

Take home message: Cardiac intensive care units are now seeing diverse patient populations ranging from those admitted for monitoring to those with acute life-threatening illnesses, including acute coronary syndrome and congestive heart failure.

To sleep, perchance to dream: Acute and chronic sleep deprivation in acute care surgeons. J Am Coll Surg 2019; 229:166–74.

In-house call has been associated with disrupted sleep. The purpose of this prospective study was to evaluate sleep deprivation in acute care surgeons taking in-house call. Data on age, sex, in-house call schedule, hours and pattern of each sleep stage, and total hours of sleep over a 3-month period of time were gathered. Nights with in-house call were excluded from the study. Sleep was categorized as normal, acute sleep deprivation, or chronic sleep deprivation in 17 acute care surgeons from two level 1 trauma centers. Sixty-five percent of sleep patterns were categorized as either acute or chronic sleep deprivation. Sleep patterns consistent with acute and chronic sleep deprivation were noted on postcall day 1 but peaked on postcall day 2 and were not back to baseline until postcall day 3 ($P < 0.05$), suggesting that a large percentage of surgeons taking call at level 1 trauma centers experience acute and chronic sleep deprivation. *(Article Selection: Deborah J. Culley. Image: ©gettyimages.)*

Take home message: In-house call among surgeons at level 1 trauma centers may be associated with acute and chronic sleep disturbances.

INFOGRAPHICS IN ANESTHESIOLOGY

Complex Information for Anesthesiologists Presented Quickly and Clearly

Infographic created by Jonathan P. Wanderer, Vanderbilt University Medical Center, and James P. Rathmell, Brigham and Women's Health Care/Harvard Medical School. Illustration by Annemarie Johnson, Vivo Visuals. Address correspondence to Dr. Wanderer: jonathan.p.wanderer@vanderbilt.edu.

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Reporting Laboratory and Animal Research in ANESTHESIOLOGY

The Importance of Sex as a Biologic Variable

Laszlo Vutskits, M.D., Ph.D., J. David Clark, M.D., Ph.D., Evan D. Kharasch, M.D., Ph.D.

Biologic differences between the two sexes have been naturally acknowledged from the dawn of humanity. Laboratory, clinical, and epidemiologic data now provide ample evidence for sex-specific differences in both disease and drug responses,[1] and perioperative medicine is not exempt. For example, sexual dimorphism in response to ischemic brain, cardiac, and renal injury has been repeatedly reported.[2–4] The incidence of postoperative cognitive dysfunction is higher in men, and male and female rodents differ in vulnerability to developmental anesthesia neurotoxicity.[5,6] Chronic pain is more frequently reported in women, although the effects of sex on postoperative pain are unclear.[7] Sex-specific differences have been demonstrated in morphine-induced analgesia and opioid-related side effects.[8,9] Postoperative nausea and vomiting are more common in women, and attendant clinical protocols are sex-specific.

Despite this dimorphism, women have long been studied less often in biomedical research. In 1993, the National Institutes of Health Revitalization Act required the inclusion of women in clinical studies, and further amendments of this document advocated for justification on how sex is factored into research design and analysis.[10–12] Full implementation of these recommendations, however, is incomplete. While clinical studies, when applicable, now do systematically include women and men, only a small proportion of them report outcome by sex or include both sexes as a covariate.[13] This limitation can be partially explained by the fact that the Consolidated Standards of Reporting Trials

"**Consideration of sex as a biologic variable in conducting laboratory animal research— and inclusion of both sexes—is strongly encouraged.**"

(CONSORT) statement for randomized controlled trials does not include reporting results by sex.[14] It is notable, however, that sex-specific pathology and pharmacology in perioperative medicine and calls for sex-specific reporting have been known and advocated for more than a decade.[15]

Laboratory and animal research is often a predecessor and driving force behind clinical investigation. It may also ensue from clinical observation. In contrast to the real-life complexity of clinical research, laboratory and animal research is often highly reductionist in nature in an attempt to understand specific mechanisms underlying physiology and disease. One common and traditional approach to presumably reducing data variability in the laboratory is to include only one sex in an experiment or whole investigation. Indeed, most basic science research in mammals is conducted in a single sex, predominantly in males.[16] This is due at least in part to the longstanding belief that females are more variable than males, due to estrous cycles.[17,18] For example, in the field of neuroscience, less than 20% of investigations used both sexes and, disturbingly, a quarter of the investigations did not specify the sex of research animals.[16] Moreover, analysis of results with consideration of sex as a variable remains infrequent.[19] The potentially confounding effects of this overt sex bias on the meaning of single-sex experimental data are, however, rarely considered. This is worrisome because, in light of the evolutionary well conserved sex-specific differences in biology, erroneous conclusions may be drawn when extrapolating

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outcome data from one sex to another.[16,20] Therefore, sex bias in preclinical research can have important translational implications because it may contribute to the frustrating gap between laboratory and clinical advances.

Advocacy, over the past few years, for better attention to sex as a biologic variable in laboratory studies and reformation of single-sex science ignited intense debates among researchers and policymakers.[21–23] Some countered that including both females and males in research would increase variability and consequently necessitate greater numbers of animals with attendant increased costs of laboratory studies.[22] However, the canard of greater variability in female than male animals has been discredited across many species.[17,18] Moreover, when research is specifically preclinical, intending translation to clinical investigation, is it worthwhile to conduct single-sex experiments when results may be explicitly flawed in their applicability to humans? Indeed, the costs of failed clinical trials, and the consequences of therapies inappropriate to women (or men), far outweigh purported cost-saving benefits of ignoring sex as a biologic variable in the laboratory. Studying both animal sexes may be more expensive *per se* yet may enhance translation and be more cost-effective overall. Although it remains unknown whether simply including both sexes in laboratory studies will be salutary, given the many failures in translating laboratory results to human therapeutics and the costs and consequences of failed clinical trials, the axiomatic inclusion of both sexes does appear justified. Additionally, even if translation to clinical investigation is not the immediate goal of animal research, sex differences can inform on the mechanism of underlying biology, pathophysiology, or pharmacology.

In 2015, the National Institutes of Health implemented new expectations on considering sex as a biologic variable in cell and animal investigations, as part of a rigor and transparency initiative.[24] The National Institutes of Health perspective was that (1) sex as a biologic variable is frequently ignored in animal studies, leading to incomplete understanding of potential sex-based differences in basic biologic function, disease processes, and treatment response; (2) overreliance on males in basic and preclinical research may obscure understanding of key sex influences on health processes and outcomes; and (3) accounting for sex as a biologic variable includes research questions, study design, data collection, data analysis, and reporting of findings, because sex may be critical to interpretation, validation, and generalizability. The National Institutes of Health "expects that sex as a biological variable will be factored into research designs, analyses, and reporting in vertebrate animal and human studies. Strong justification from the scientific literature, preliminary data, or other relevant considerations must be provided for applications proposing to study only one sex."[24] Similar statements have been subsequently issued by other international funding agencies, such as the European Commission and the Canadian Institutes of Health Research, and echoed in scientific articles.[25,26] A recent survey noted an important increase, although still incomplete, in appreciation of these requirements by grant reviewers.[27] As with clinical research, however, it remains to be seen how well this will lead to improvement in the conduct of laboratory research.

Changes in ANESTHESIOLOGY

ANESTHESIOLOGY supports the above principles and encourages the consideration of sex as a biologic variable in laboratory as well as clinical research. Adequate consideration of sex by investigators in the formulation of research questions, study design, experimentation, and collection and analysis of data is important and should be considered by sponsors in their funding decisions. Investigators must decide whether to simply include both sexes in research or to deliberately power studies to detect sex differences and make sex-specific conclusions.

The adequate consideration of sex in research *reporting* is also the subject of guidelines[24,28] and is directly in the purview of journals.[29] For example, the Sex and Gender Equity in Research guidelines provide a comprehensive description for reporting of sex in study design, data analysis, and interpretation.[26] Journal editors, editorial policies, and the careful scrutiny of peer reviewers will play a major role in ensuring adequate reporting of sex as a biologic variable, and ANESTHESIOLOGY embraces these principles. Furthermore, consideration of sex as a biologic variable is the purview of journals, including ANESTHESIOLOGY, as it pertains to peer review and the validity of results and conclusions. The reporting of sex as a biologic variable in

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Box 1. Sex and a Biologic Variable in Laboratory Research: ANESTHESIOLOGY Reporting Guidelines

1. Consideration of sex as a biologic variable in conducting laboratory animal research, and inclusion of both sexes, is strongly encouraged.
2. Consideration of sex as a biologic variable in reporting laboratory animal research is required by ANESTHESIOLOGY.
3. The term "sex" rather than "gender" should be used when referring to biologic/genetic differences.
4. A clear indication of the sex(es) of animals included in the research should be included in the Abstract and Methods sections of a manuscript. If only one sex was studied, specification in the title may be appropriate.
5. Data may be reported and analyzed individually by sex, whether showing sex differences or not, where appropriate.
6. The Discussion section should discuss potential implications of sex on results and conclusions, where appropriate.
7. If research or data analysis by sex was not conducted, the Discussion section should provide the rationale and discuss any implications for the interpretation of results.

Note: Although this addresses laboratory and animal research, it continues to apply also to clinical research.

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laboratory/animal research (box 1) has already undergone increased attention and scrutiny in the ANESTHESIOLOGY peer review process over the last 2 yr but has been somewhat informal and variable.

Therefore, after discussion and agreement among the Editorial Board, ANESTHESIOLOGY will attend to sex as a biologic variable more thoroughly and henceforth require greater reporting transparency in submitted and published manuscripts. We will continue to insist on the correct usage of the terms sex (a biologic variable based on chromosomal assignment) and gender (a constellation of sociologic processes that interact with and have the potential to influence human biology).[29] We will evaluate the inclusion of both sexes in research, as relevant to results and conclusions. We will require that if there is only one sex in an animal or human study (excluding studies relevant to only one sex), the Abstract must specify the sex. Inclusion of the single sex in the title is also encouraged, but it should not mistakenly conflate a single-sex study population with results and conclusions that were found specific to only one sex. The Methods section should report the sexes of cells or tissues (if known), animals, and humans studied, justify reasons for any exclusion, and whether/how sex was considered in study design. The Results section should report the sex composition of the final study population. Data may be reported and/or analyzed without disaggregation by sex, individually by sex using descriptive statistics to simply communicate results separately for each sex, or individually by sex where experiments were formally designed to detect effect modification by sex (*i.e.*, an effect × sex interaction) using *a priori* statistical power considerations to properly ground such analyses, as appropriate. Authors are encouraged to report results disaggregated by sex, whether showing sex differences or not, in the main article or supplement, as appropriate. The Discussion section should discuss potential implications of sex on results and conclusions, where appropriate. If research or data analysis by sex was not conducted, the Discussion section should provide the rationale and discuss any implications for the interpretation of results. For convenience, these new reporting standards are summarized in box 1. Although this Editorial and the Guidelines are written to address laboratory and animal research, they have applied and will continue to apply also to clinical research.

It is intended and hoped that these reporting requirements will increase the transparency, rigor, and value of the research published in ANESTHESIOLOGY, strengthen the evidence, and enhance the translation of discovery to practice.

Competing Interests

Dr. Vutskits served as consultant for Primex (Zug, Switzerland) and Regeneron (Tarrytown, New York). Dr. Kharasch is the Editor-in-Chief of ANESTHESIOLOGY, and his institution receives salary support from the American Society of Anesthesiologists (Schaumburg, Illinois) for this position. The other authors declare no competing interests.

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Dexmedetomidine and the Upper Airway

Not as Simple as We Hoped

Denham S. Ward, M.D., Ph.D., Suzanne B. Karan, M.D.

"*Breathing is truly a strange phenomenon of life, caught between the conscious and the unconscious, and peculiarly sensitive to both*"[1]

With the epidemic of obstructive sleep apnea syndrome, concern about airway obstruction during sedation has increased. Dexmedetomidine, used for sedation in the intensive care unit and during procedures, has been thought to have fewer respiratory depressive effects than other sedatives, although airway obstructions and apneas with dexmedetomidine have been noted in several studies.[2,3] This issue of ANESTHESIOLOGY features work by Lodenius *et al.*,[4] who found that dexmedetomidine is not superior to propofol in the propensity for causing airway obstruction at comparable sedation levels.

"[D]exmedetomidine seems to cause no less propensity for airway obstruction than does propofol at a similar level of sedation."

Sedative agents depress ventilation through a variety of actions including direct actions on upper airway muscle tone,[5] on chemosensory pathways, and by removal of the "wakefulness" drive.[6] Loss of the wakefulness input can unmask a profound depression of the chemosensory drive from sedatives and analgesics[7] and reduce the drive to the pharyngeal dilator muscles. Respiratory physiologists routinely discriminate new sedative and opioid agents by their effects on the depression of the hypoxic and hypercapnic chemosensitivity.[8] Moderate depression of the chemoreflexes is well tolerated, particularly when supplemental oxygen is supplied. However, even with supplemental oxygen, upper airway obstruction may result in serious hypoxemia in a matter of minutes. In sleep apnea research, the collapsibility of the upper airway during sleep has been quantified by the estimation of the pharyngeal pressure that is required to close the airway or keep it open.[9,10] This methodology is being used to assess the propensity of medications to increase airway collapsibility.

The subjects in this study had a wider range of age (23 to 66 yr), body mass index (20.3 to 32.4 kg/m²), Mallampati score (1 to 4), neck circumference (31 to 45 cm), and risk for sleep disordered breathing (as indicated by the STOP–BANG questionnaire and apnea hypopnea values) than is usually found in a tightly controlled laboratory study. The subjects were also extensively instrumented, which included an esophageal pressure catheter, Bispectral Index, and three-lead electroencephalogram in addition to the usual laboratory respiratory physiology monitors. The sedation level was assessed by three well established methods. There are two important outcomes from this well designed and executed complex study. The first is that dexmedetomidine seems to cause no less propensity for airway obstruction than does propofol at a similar level of sedation. Interestingly, the three measures of sedation depth did not all show the same dose–response relationship even though the chemoreflex depression as measured by the increase in transcutaneous carbon dioxide (a measure of tissue partial pressure of carbon dioxide that is slightly higher than PaCO₂) showed similar increases for both drugs.

Second, and equally interesting, is the wide variation in the primary outcome of pharyngeal critical pressure. There were several subjects whose airways were resistant to collapse, requiring a subatmospheric pressure to collapse the airway, for both drugs. There are not enough subjects in this study to determine whether there were correlations with any of the subject characteristics. This underscores the need for more studies focused on patient characteristics that may predict airway collapsibility during sedation.

The precise relationship between airway collapsibility measured in a supine subject whose mouth is taped closed and the routine clinical situation during painful stimulation

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Image: S. M. Jarret, M.F.A., C.M.I.

This editorial accompanies the article on p. 962.

is not clear. This study was done in human subjects who were not exposed to painful stimulation, although dexmedetomidine provides analgesia and propofol does not. In clinical situations that require analgesia as well as sedation, the analgesic effect of dexmedetomidine may allow for lighter sedation than with propofol and thus less adverse ventilatory effects.

Nonetheless, to the extent that this laboratory study can be extrapolated to routine clinical situations, it does not appear that light to moderate sedation with dexmedetomidine offers any protection from central ventilatory apneas and airway obstructions over the commonly used sedative, propofol.

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Be Wary of Genes Governing Awareness

Philip G. Morgan, M.D., Max B. Kelz, M.D., Ph.D.

On October 16, 1846 William Morton demonstrated the successful administration of diethyl ether allowing the removal of a neck tumor from a quiescent and pain-free patient. With the advent of muscle relaxants, the significance of immobility as evidence of adequate anesthesia was diminished. However, it was not until 1950 that the first case report of insufficient anesthesia appeared.[1] Recently, a great deal of interest has surfaced in improving our ability to eliminate this catastrophic occurrence.[2]

Aranake *et al.*[3] demonstrated a fivefold increased risk of awareness with recall in patients with a previous history, but whether this increase reflects a genetic predisposition, or is attributable to environmental factors, remains unknown. The study by Sleigh *et al.*[4] in this issue of ANESTHESIOLOGY represents a bold first attempt to determine a potential genetic vulnerability. Assuming that there are a small number of important molecular targets for anesthetics, alleles of those targets may exist which cause an individual to be resistant to general anesthesia. Although we limit our discussion to molecular targets, the reader should realize that similar arguments apply to developmental genes controlling neuronal circuits underlying anesthetic effects; changes at the circuit level could also shift anesthetic sensitivity.

Several molecular targets have been hypothesized to form the site(s) of action of volatile anesthetics although, as yet, no single protein or channel has been identified as the unique anesthetic target to the exclusion of all others. If multiple genes contribute to the behavioral response of anesthesia then the relative importance of each gene product cannot be easily predicted. Regardless of the number of anesthetic targets, the genetic blueprint specifies proteins that determine the nature of all components of a cell; any response to a neuroactive drug must also depend on this genetic information. A natural extension of this logic

> **"[G]enetic data…do not identify a single protein that is uniquely responsible for awareness under anesthesia."**

indicates that specific alterations in appropriate genes will alter specific components of a cell (be they protein, lipid, or other) and in turn the responses to volatile anesthetics. After excluding cases attributable to inadequate drug delivery, awareness under anesthesia should reflect a decreased sensitivity to anesthetics and therefore potentially herald mutations in crucial target genes. Strategies to look for resistance to volatile anesthetics could identify the responsible genes, determine their products, and therefore identify the cellular components responsible for awareness with recall.

Sleigh *et al.*[4] report an important first step in identifying any genetic bases for awareness under general anesthesia. The authors are aided by almost unbelievable progress in genomic sequencing compared with even a decade ago. It is now feasible to identify specific changes in the DNA of patients that correlate to a shared behavioral change. This makes possible the following experimental approach used by the authors.

1. Identify a group of individuals with the phenotype of interest (documented awareness with recall despite appropriate anesthetic exposure)
2. Identify a second group who do not have the phenotype
3. Do a full sequencing of the genetic material that codes for all proteins in each group
4. Determine consistent genetic differences between the two groups
5. Deduce important coding changes that differentially affect the awareness "phenotype"

Although this approach sounds straightforward conceptually, there are a number of complicating factors. First, one must be sure that the test group actually had some type of awareness under adequate anesthesia concentrations with or without recall. Second, one must be sure that all members

Image: ©gettyimages.

This editorial accompanies the article on p. 974.

in the control group would be adequately anesthetized at the same adequate doses. These are incredibly difficult points to ensure, making any study of awareness with (or without) recall open to concern.

Our discussion is limited to the genetic approach and its ensuing caveats. Complications generally arise from the fact that the human genome contains about 30,000 genes and 3 billion base pairs. Therefore, a large number of likely noncontributory differences will be identified, which must somehow be filtered out. One method to address this problem is to take a very large group of normal sequences to use as controls. Because 99.8% of people do not experience awareness with recall, should they exist, important DNA changes must be rare and unlikely to be found in the control population. Any variation that occurs above some cutoff frequency in the general population is automatically assigned as noncontributory. This requires sequencing control genomes of a sufficient quantity to determine the normal distribution. It is difficult to say how many such normal genomes should be sequenced, but typically 5 to 10 times the number of affected genomes is standard.[5] Similarly, known mutations that cause overt disease are eliminated from consideration because there is no known correlation of awareness under anesthesia with known diseases.

One will still be left with a large number of base pair variants in the affected data set, so two further assumptions were made in the present study. The first assumption was that there are probably a small number of such target genes. In that case, one would expect that in a reasonable number of patients experiencing awareness, mutations in the same genes (or at least pathways) should occur multiple times. Consider genetic experiments designed to determine how many genes are in a developmental pathway. One collects mutants until a large percentage of identified genes have been identified by multiple different mutations (although only one per patient, generally); then researchers know that they have sampled the genome adequately so that all genes likely to have a major effect have been identified. In the present study, the authors have only one such duplication. This indicates that complete coverage of the genes controlling sensitivity is unlikely. It is difficult to expect repeated identification when only 12 affected individuals and 12 controls are sequenced, unless the list of possible causative genes is extremely small.

The final assumption was that the variations must be in genes that make sense biologically. However, the targets for volatile anesthetics are not clear, meaning that filtering may eliminate important molecular targets. For example, mitochondrial targets, synaptic proteins, developmental genes, and molecular motors (kinesins, microtubules) are not considered in the screen. If one is going to rule out monogenic causes, one probably needs to look at all possible genes, a monumental task when no consensus exists as to the likely

anesthetic targets. By restricting their genes of interest, the authors may have biased their results. Hence, it seems premature to conclude that awareness is not caused by changes in one or a few genes. In fact, most of the genes identified by Sleigh *et al.* have relatively minor contributors to volatile anesthetic sensitivity. The authors point out this serious limitation, and appropriately state that their study must be extended with a wider net.

Nevertheless, it is interesting to note what genetic data fail to do—they do not identify a single protein that is uniquely responsible for awareness under anesthesia. Unfortunately, the necessarily small study cohort does not carry the power to boldly interpret the sequencing data. Such conclusions will require a much larger group of patients with documented awareness under anesthesia and several-fold more negative controls. Thus, although Sleigh *et al.*'s results are consistent with a lack of monogenetic causes of awareness under anesthesia, they do not yet force that conclusion. Most importantly, the authors have beautifully mapped out an approach to identifying whether a genetic component exists to awareness under general anesthesia. All that remains is to extend the approach to a much larger cohort.

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Mission Impossible or Mission Futile?

Estimating Penetrance for Malignant Hyperthermia

Marie-Anne Shaw, Ph.D., Philip M. Hopkins, M.B.B.S., M.D., F.R.C.A.

In this issue of Anesthesiology, Ibarra Moreno *et al.*[1] report a multicenter evaluation of previous anesthetic history of patients who have a history of malignant hyperthermia (MH) under anesthesia and their family members. This work will have involved considerable effort to collate the data and evaluate the clinical histories. The major and vital clinical message is to reinforce that MH can occur in patients who have previously experienced uneventful anesthesia with MH-triggering anesthetics. The implication is that a negative personal anesthesia history does not obviate the need to take a family history of adverse anesthesia events, nor should it lower the anesthesiologist's index of suspicion concerning the potential for the patient to develop MH. There are several other interesting observations contained within the data generated by Ibarra Moreno *et al.*,[1] one being that the well-known male predominance of MH probands cannot be explained by different levels of exposure to MH triggering anesthesia, but it is one of their key aims—the estimate of penetrance of variants in the *RYR1* gene (the gene encoding the skeletal muscle isoform of the ryanodine receptor, which is the gene principally implicated in MH)—that requires comment.

The concept of penetrance was introduced in the literature almost 100 yr ago as an explanation for patterns of heredity that diverged from the expected patterns of Mendelian inheritance, all of which assumed single-gene traits,[2] and by the 1950s, the term itself was in use.[3] Indeed, the first report of an MH family described incomplete penetrance of the condition because an obligate genetic carrier had received general anesthesia but had not developed

"In [malignant hyperthermia] there are several strands of genetic evidence that [malignant hyperthermia] susceptibility, at least in some families, is associated with two or more genetic abnormalities."

MH.[4] For genetic conditions typically presenting at birth, we can readily estimate penetrance on the basis of its current standard definition as the proportion of individuals who have a disease-causing genotype who express the phenotype. But can such estimates be usefully derived for the penetrance of *RYR1* variants in MH?

Reduced penetrance is often associated with autosomal dominant disorders and is likely due to modifying genetic or environmental factors, or both. It would have been no surprise, therefore, that the inheritance pattern of the first MH family was described as autosomal dominant with incomplete penetrance.[4] However, in a landmark review 20 yr ago, Scriver and Waters[5] illustrated how inheritance patterns that are presumed to represent reduced penetrance in Mendelian heredity could be better explained by non-Mendelian genetic models. Indeed, in MH there are several strands of genetic evidence that MH susceptibility, at least in some families, is associated with two or more genetic abnormalities.[6–8] Furthermore, studies of human MH muscle[9] and *in vivo* and *in vitro* experiments in transgenic *RYR1* knock-in mouse models of MH[10–12] show marked differences in the severity of the MH phenotype caused by different variants in the *RYR1* gene. Collectively, these genetic and functional observations suggest a threshold (non-Mendelian) genetic model for MH susceptibility in which "weaker" *RYR1* variants require coinheritance of other genetic abnormalities in order for their combined effects to be severe enough for a patient to be clinically susceptible.

Even if we assume that at least some *RYR1* variants do operate in an autosomal dominant manner, there are a

number of problems in estimating the penetrance of such rare pharmacogenetic disorders, which require an environmental trigger. The penetrance of MH could be handled similarly to a late-onset disorder such as Huntington disease where the average age by which the condition presents is used to estimate penetrance,[13] but this would not account for the variable number of general anesthetics received by individuals at any specified age. Alternatively, the penetrance of MH could be defined by the presence or absence of a reaction after a certain number of anesthetics, but this ignores observations that not all anesthetic events are equally likely to trigger a reaction in any one susceptible individual.[14,15] With either a time-based or exposure-based cutoff for determining penetrance, identification of the first individual within a family presenting with a clinical reaction, the proband, is a signal to avoid subsequently exposing potentially susceptible family members to triggering anesthesia. Inclusion of these relatives is likely to underestimate penetrance. The rarity of clinical MH reactions means that studies relying on clinical reactions to identify those with a high-risk genotype are likely to lack power, especially when penetrance should relate to individual variants rather than all variants associated with a phenotype. A multicenter approach as used by Ibarra Moreno et al.[1] pools resources to increase power, but inevitably increases the variability of genetic background, which will impact the estimates of penetrance of Mendelian disorders and confound attempts to unravel the genetic bases of complex traits.

Estimation of the likelihood of developing a reaction in probands compared to relatives is also difficult. The MH reaction of the proband is the route to ascertaining such families, yet it is not possible to remove this reaction from the study to correct for ascertainment (sampling) bias, which is standard practice in population genetic studies. Nor is it possible, after diagnosis in the proband, to reliably control for the number of subsequent anesthetic events per individual, resulting in a reaction or otherwise. Furthermore, we do not know if we are selecting for individuals carrying pathogenic variants that lead to a reaction with the first or second exposure to anesthetic triggers, as opposed to those variants that are likely to trigger only after many exposures; again, this fuels ascertainment bias.

The ascertainment bias associated with the selection of families only on the basis of a known MH reaction might artificially inflate any estimate of penetrance. Alternatively, we could approach an estimate of penetrance by comparing the observed incidence of MH reactions to that predicted based on an estimate of the population prevalence of *RYR1* variants predisposing to MH. Based on genomic data from large low-risk (for MH at least) cohorts, an estimate of individuals carrying currently defined pathogenic *RYR1* variants in the general population is approximately 1:1,500. From these data, for a country the size of the United Kingdom with a population of ~60 million, we can project that there are ~40,000 people who carry

pathogenic *RYR1* variants. Contrast this to the fewer than 2,500 MH-susceptible individuals whom we (the United Kingdom national MH referral center in Leeds) have definitively diagnosed during the last 48 yr. Similarly, we would anticipate that 2,000 to 4,000 of those carrying pathogenic *RYR1* variants in the United Kingdom would receive general anesthesia each year, whereas we identify only around 20 new cases of MH per annum. The estimate of penetrance obtained from these data (5 to 10%) is considerably lower than that of Ibarra Moreno et al.,[1] but perhaps more interestingly, seems incompatible with the idea that all of the currently defined pathogenic *RYR1* variants play a major role in determining MH susceptibility.

Our final illustration of the futility of trying to estimate the penetrance of *RYR1* variants in MH goes back to the definition of penetrance. This requires the genotype in any one individual to be described as penetrant or not penetrant, whereas MH-susceptible individuals may express the phenotype during one exposure but not another. There is still much to learn about the genetic factors predisposing to MH. *RYR1* is a large gene, and the 48 variants currently regarded as pathogenic will not be a complete list. Many more rare variants remain to be characterized, and those found to date cause changes in coding sequence only (noncoding sequence has not been studied). If the phenotypic consequences of a pathogenic *RYR1* variant are significantly influenced by other variants, leading to stratification of the combined data, we would be unaware. In the meantime, the work of Ibarra Moreno et al.[1] highlights important clinical messages that need to be understood by every anesthesiologist.

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Preoperative Assessment of Functional Capacity

Looking beyond the Ability to Climb Stairs

Duminda N. Wijeysundera, M.D., Ph.D.

Almost any preoperative evalua-tion—be it by an anesthesiolo-gist, internal medicine physician, or surgeon—involves asking a patient about the ability to climb one to two flights of stairs or walk several blocks on level ground. Patients' responses to these questions provide insights into their usual levels of physical activity and their overall cardiopul-monary fitness, which in turn plau-sibly help with stratifying risk for postoperative morbidity and mortal-ity. In this issue of ANESTHESIOLOGY, Rubin *et al.* present an analysis of the National Health and Nutrition Examination Survey that provides important new data on the validity of patients' self-report as a measure of usual levels of physical activity.[1] Participants in this nationally repre-sentative sample of the United States population responded to questions about their usual physical activities (*e.g.,* walking or climbing stairs) and also wore accelerome-ters to objectively measure their physical activity over a 7-day period. Overall, the authors found that typical interview ques-tions related to physical activity were relatively inaccurate tools for screening out significantly inactive individuals who did not complete at least 2 min of moderate-to-vigorous activity (*i.e.,* walking two blocks at 4 mi/h) over a 7-day period. While the self-reported inability to climb 10 stairs had reasonably good performance (positive likelihood ratio of 3.9) for identifying inactive individuals, the self-reported ability to climb 10 stairs and walk two to three blocks had relatively weak ability to rule out inactivity (negative likelihood ratio of 0.5).

Importantly, the National Health and Nutrition Examination Survey sample analyzed in this study was rela-tively small (*i.e.,* 522 individuals), drawn from a noncontem-porary time period (*i.e.,* 2003 to 2006), and not restricted to surgical patients. The sample therefore differed from typical surgical patients in that participants were sicker yet more physically active. For example, cor-onary artery disease was present in 65% of the National Health and Nutrition Examination Survey sample, compared to 13% of a large, relatively unselected cohort study of patients having major inpatient noncardiac surgery.[2] About 67% of the National Health and Nutrition Examination Survey sample was sedentary based on measurements by older generation uniaxial accel-erometers. By comparison, a recent study of 50 surgical patients in the United Kingdom found that more than 99% were sedentary based on measurements by newer genera-tion triaxial accelerometers over a 3-day period preceding surgery.[3]

This study by Rubin *et al.* adds to a growing body of literature pointing to the important limita-tions of the usual clinical approach of subjectively assessing preoperative functional capacity based on responses to a few simple unstructured questions. The relatively poor perfor-mance of simple questions at screening out unfit patients is consistent with the findings of the Measurement of Exercise Tolerance before Surgery study.[4] In this multicenter pro-spective cohort study, anesthesiologists' subjective rating of poor fitness (defined as being unable to attain four meta-bolic equivalents of activity) had a positive likelihood ratio of 3.8 and negative likelihood ratio of 0.85 for identifying patients with poor performance on objective exercise test-ing. Importantly, in the Measurement of Exercise Tolerance before Surgery study, cardiopulmonary fitness was objec-tively measured by formal exercise testing, while in the National Health and Nutrition Examination Survey sam-ple, usual levels of physical activity were objectively mea-sured by accelerometers. Cardiopulmonary fitness and usual

"[H]ow might clinicians assess preoperative func-tional capacity in a more valid and prognostically accurate manner?"

Image: J. P. Rathmell.

physical activity levels are related, but different, constructs. Indeed, preoperative activity levels measured by accelerometers are only moderately correlated with exercise testing performance (*i.e.,* correlation coefficients ranging from 0.55 to 0.6) in surgical patients.[3] Thus, an individual may conceivably exhibit a higher level of cardiopulmonary fitness on strenuous exercise testing than would be evident during usual daily physical activities. Conversely, usual physical activity levels might be limited by factors other than fitness (*e.g.,* musculoskeletal disease). Cardiopulmonary fitness and usual physical activity levels may also have different prognostic relevance. In the surgical setting, maximal exercise ability on formal objective testing is predictive of moderate or severe postoperative complications, but not cardiac events such as myocardial infarction or myocardial injury.[4] The prognostic relevance of preoperative activity levels remains unclear but could plausibly be superior to objectively measured fitness for some outcomes. Consistent with this possibility, self-reported activity as measured by the standardized 12-item Duke Activity Status Index questionnaire has been shown to predict postoperative cardiovascular complications.[4–6] Thus, future studies should assess the ability of activity levels as objectively measured by accelerometers to predict important postoperative complications.

Moving forward, how might clinicians assess preoperative functional capacity in a more valid and prognostically accurate manner? It is increasingly clear that the current clinical approach of unstructured questions is simply inadequate. There are some promising alternatives, but all require further study before mainstream clinical implementation. Rubin *et al.* have highlighted the potential for the application of accelerometers and other similar wearable healthcare technology. If these devices are to be used to inform preoperative risk stratification, future research must specifically evaluate the prognostic relevance of preoperative accelerometer measurements. Such studies are especially needed since accelerometer measurements have similar correlation to objectively measured exercise capacity as the much simpler Duke Activity Status Index questionnaire.[3] Importantly, there are potential roles for accelerometers and other similar wearable healthcare technology in the perioperative setting beyond estimating preoperative activity levels. Indeed, these devices may be best suited to the postoperative setting, where they can facilitate early identification of patients at risk for poor postsurgical recovery.[7] As with many emerging perioperative care technologies—such as minimally invasive cardiac output monitors, remote postoperative physiological monitors, and wearable technology—the onus lies with anesthesiologists and perioperative physicians to identify the most cost-effective opportunities to apply new technology to improve clinical care and outcomes.

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ANESTHESIOLOGY

Upper Airway Collapsibility during Dexmedetomidine and Propofol Sedation in Healthy Volunteers

A Nonblinded Randomized Crossover Study

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Dexmedetomidine is a relatively new sedative promoted as having minimal effect on ventilatory drive or the propensity to upper airway obstruction

What This Article Tells Us That Is New

- At comparable levels of light to moderate sedation, dexmedetomidine and propofol exhibit similar degrees of pharyngeal collapsibility and reductions in ventilatory drive
- The findings suggest that sedation with dexmedetomidine does not offer inherent protection against upper airway obstruction or ventilatory depression

ABSTRACT

Background: Dexmedetomidine is a sedative promoted as having minimal impact on ventilatory drive or upper airway muscle activity. However, a trial recently demonstrated impaired ventilatory drive and induction of apneas in sedated volunteers. The present study measured upper airway collapsibility during dexmedetomidine sedation and related it to propofol.

Methods: Twelve volunteers (seven female) entered this nonblinded, randomized crossover study. Upper airway collapsibility (pharyngeal critical pressure) was measured during low and moderate infusion rates of propofol or dexmedetomidine. A bolus dose was followed by low (0.5 µg · kg^{-1} · h^{-1} or 42 µg · kg^{-1} · min^{-1}) and moderate (1.5 µg · kg^{-1} · h^{-1} or 83 µg · kg^{-1} · min^{-1}) rates of infusion of dexmedetomidine and propofol, respectively.

Results: Complete data sets were obtained from nine volunteers (median age [range], 46 [23 to 66] yr; body mass index, 25.4 [20.3 to 32.4] kg/m^2). The Bispectral Index score at time of pharyngeal critical pressure measurements was 74 ± 10 and 65 ± 13 (mean difference, 9; 95% CI, 3 to 16; $P = 0.011$) during low infusion rates *versus* 57 ± 16 and 39 ± 12 (mean difference, 18; 95% CI, 8 to 28; $P = 0.003$) during moderate infusion rates of dexmedetomidine and propofol, respectively. A difference in pharyngeal critical pressure during sedation with dexmedetomidine or propofol could not be shown at either the low or moderate infusion rate. Median (interquartile range) pharyngeal critical pressure was −2.0 (less than −15 to 2.3) and 0.9 (less than −15 to 1.5) cm H$_2$O (mean difference, 0.9; 95% CI, −4.7 to 3.1) during low infusion rates ($P = 0.595$) *versus* 0.3 (−9.2 to 1.4) and −0.6 (−7.7 to 1.3) cm H$_2$O (mean difference, 0.0; 95% CI, −2.1 to 2.1; $P = 0.980$) during moderate infusion of dexmedetomidine and propofol, respectively. A strong linear relationship between pharyngeal critical pressure during dexmedetomidine and propofol sedation was evident at low (r = 0.82; $P = 0.007$) and moderate (r = 0.90; $P < 0.001$) infusion rates.

Conclusions: These observations suggest that dexmedetomidine sedation does not inherently protect against upper airway obstruction.

(*ANESTHESIOLOGY* 2019; 131:962–73)

Upper airway obstruction and respiratory depression are common side effects of many sedative and anesthetic agents and may put ventilation and oxygenation at risk.[1–4] A drug that can offer sedation without impairing airway patency and increasing risk of hypoxemia would be ideal in situations where preservation of spontaneous ventilation is desired, such as during sedation in nonintubated patients in intensive care or during diagnostic procedures

or awake tracheal intubation. Dexmedetomidine, a highly selective α2-adrenoceptor agonist,[5,6] has been advocated for use in these patient populations because the drug has been considered to lack respiratory side effects.[7–9]

However, this notion has been challenged by findings from several recent studies. Drug-induced sedation endoscopy studies have shown obstruction of the upper airway at moderate levels of sedation with dexmedetomidine.[10] A reduction in airway dimensions has also been observed during magnetic resonance imaging of the upper airway in children sedated with dexmedetomidine.[11] Further, we recently demonstrated that light to moderate sedation with dexmedetomidine or propofol in adult volunteers caused similar reductions in ventilatory responses to hypoxia and hypercapnia with either drug.[12] Moreover, that study also displayed serious upper airway compromise with almost all participants exhibiting repetitive episodes of snoring and upper airway obstruction during dexmedetomidine sedation, whereas less than half had such events during propofol sedation.[12]

Hence, there is reason to doubt the assertion that use of dexmedetomidine provides sedation without airway compromise. However, no previous study has directly measured upper airway collapsibility during dexmedetomidine sedation in adults or used such measures to compare it with conditions during propofol sedation, a suitable benchmark sedative agent given its well characterized effects on upper airway collapsibility.[2,13] This study addresses this deficiency, using pharyngeal critical pressure, a widely used measure of collapsibility that assesses the intraluminal pressure at which airway closure occurs. Such quantification allows precise assessment of the effects of sedation on airway patency, facilitating comparisons between drug levels and different agents.

The aim of the present study was to establish whether upper airway collapsibility is increased during dexmedetomidine sedation in healthy adult volunteers. We examined the hypothesis that dexmedetomidine sedation would have less effect on upper airway collapsibility than propofol sedation. Participants were to be given dexmedetomidine to achieve light and moderate levels of sedation. This was to be compared to sedation with propofol, a sedative known to increase upper airway collapsibility. The primary endpoint of the study was the effect of dexmedetomidine on upper airway collapsibility, quantified by pharyngeal critical pressure, relative to standard intravenous sedation with propofol. The purpose of undertaking the study was to test the assertion that dexmedetomidine sedation offers protection against obstruction of the unprotected upper airway.

If indeed, dexmedetomidine proved to offer such protection, then it would offer advantages for sedation in nonintubated patients. If not, uninformed use of dexmedetomidine in these situations could compromise patient safety.

Materials and Methods

Ethics

This study was approved by the Sir Charles Gairdner Hospital Human Research Ethics Committee (Perth, Western Australia; approval No. 2009-037). The trial was conducted according to the standard of the Declaration of Helsinki, Good Clinical Practice, and the Consolidated Standards of Reporting Trials guidelines (Supplemental Digital Content, http://links.lww.com/ALN/C52). The study was registered with the Australian New Zealand Clinical Trials Registry (trial ACTRN12616000085471). The Universal Trial Number is U1111-1175-8788. Through oversight, registration was completed after study commencement, but no modification of the original protocol was made from commencement. A more detailed protocol can be provided by request.

The study was performed from August 2015 to September 2016 at the research facilities at the West Australian Sleep Disorders Research Institute, Department of Pulmonary Physiology and Sleep Medicine, Sir Charles Gairdner Hospital, Nedlands, Western Australia. Analyses of plasma propofol concentrations were made at the Department of Physiology and Pharmacology, Section for Anesthesiology and Intensive Care, Karolinska Institutet, Stockholm, Sweden. Plasma dexmedetomidine concentrations were analyzed at the Clinical Research Services Turku, University of Turku, Finland.

Study Subjects. Eligible participants in this randomized, crossover study were nonsmoking adults (age 18 to 65 yr), with an American Society of Anesthesiologists (ASA) physical status score of I or II and a body mass index of less than 37 kg/m². Predisposition to obstructive sleep apnea was assessed with the STOP-BANG questionnaire. Exclusion criteria were allergy to the study drugs, upper airway pathology, severe obstructive sleep apnea, uncontrolled cardiovascular or respiratory disease, or other systemic disorder. Participants were recruited through advertising at an affiliated university campus or sleep research institute or from individuals previously known by the study team. They were randomized and assigned for intervention by the study team after oral and written informed consent.

Randomization was undertaken to establish the order of sedation with dexmedetomidine or propofol in a first block of five volunteers, followed by a second block of seven volunteers, using an online resource.[14] Concealment regarding randomization was not made. A crossover design was used, each volunteer acting as his/her own control with a minimum interval of 48 h between administration of each drug protocol (fig. 1). Blinding of the intervention was considered difficult because of the different colors of the study drug infusions and their palpably different pharmacokinetics and was therefore not done. The entire study team was involved in data collection, and therefore the study drug was not concealed during data analysis.

Participant Preparation and Monitoring. No premedication was administered. Standard perioperative monitoring was applied. An intravenous cannula was placed in each arm, one used for drug and compound sodium lactate administration (Viaflex;

Fig. 1. Consolidated Standards of Reporting Trials flow diagram showing inclusion, randomization, and exclusion of participants.
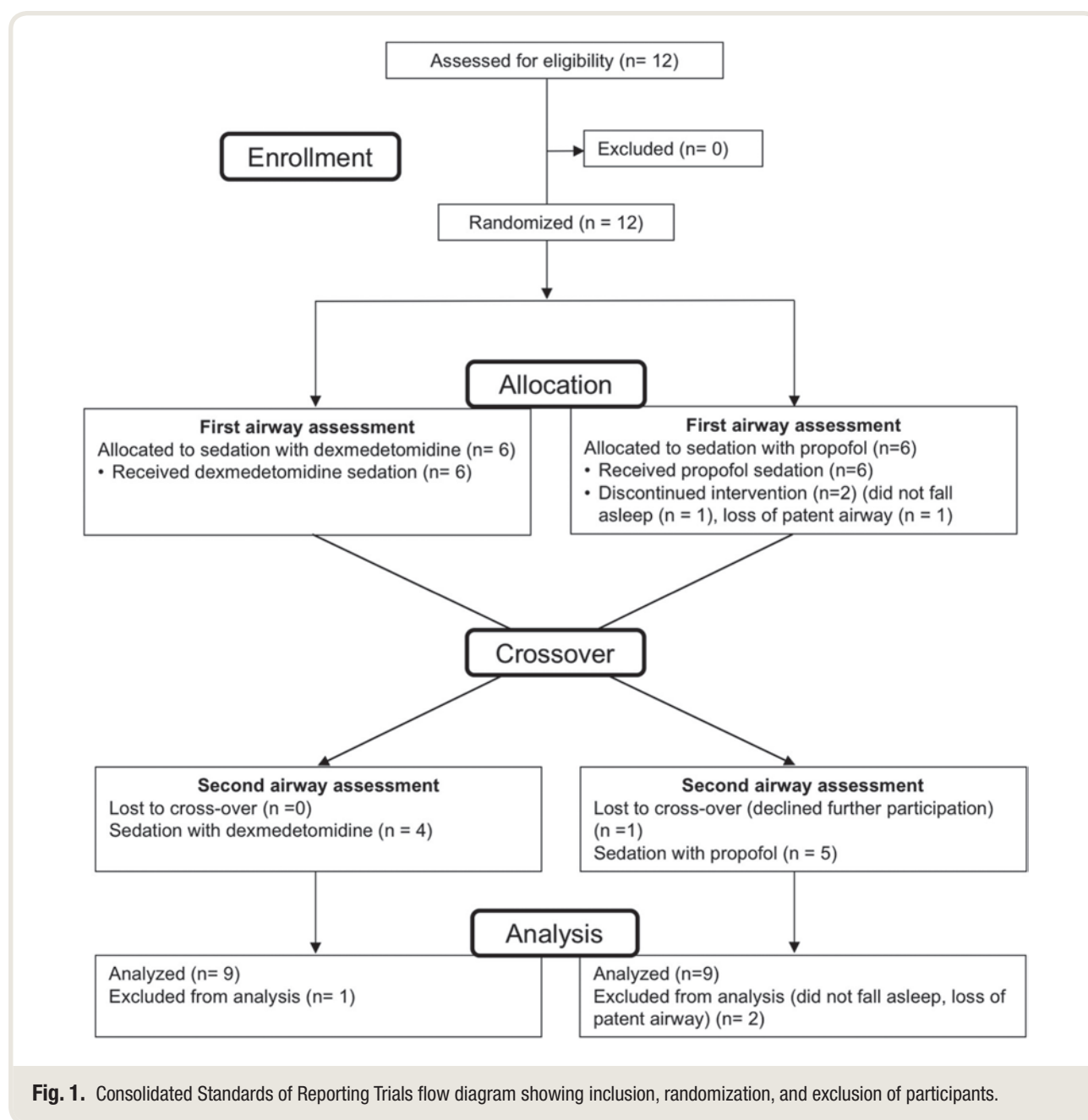
Baxter, Australia) and the other used for venous blood sampling. Transcutaneous carbon dioxide (TCM 4 series; Radiometer Medical ApS, Denmark) and Bispectral Index score (A-2000 Bispectral Index score monitor) were continuously recorded along with a three-lead electroencephalogram (O1, C3, F3).

The participants were supine with the head maintained in the neutral position (Frankfort plane perpendicular to the bed surface) using a modified Shea headrest. A volume-calibrated pneumotachograph (Korr Medical Technologies, USA), an expiratory port, and a custom-built pressure source (ResMed, Australia) capable of delivering positive and negative pressure (+20 to −20 cm H$_2$O) were connected in series to a well sealed nasal mask. Nasal mask pressure was continuously

measured from a sample port in the mask connected to a pressure transducer (model 143 PC, Micro Switch; Honeywell, USA). Maintenance nasal pressure was the pressure at which inspiratory flow limitation was abolished and was applied at all times other than when measures of airway collapsibility were being performed.[15,16] An air–oxygen mix (FIO$_2$ ~0.5) was delivered *via* a Bain circuit attached to the pressure source and the nasal mask, at a minimum flow of 10 l/min. The mouth was sealed with occlusive tape, and a chin strap was fitted.

A calibrated four-sensor pressure transducer catheter (CTO-4; Gael Tec, United Kingdom) was passed into the esophagus, as previously described,[17] to measure respiratory effort by pharyngeal and esophageal pressures during

flow-limited breathing, as well as retropalatal and hypopharyngeal pressures. In addition, thoracic and abdominal respiratory inductance plethysmography (Respitrace; Ambulatory Monitoring, USA) was applied. All signals were recorded continuously at 1,000 Hz on a Power Lab data acquisition and analysis system (model 16s; AD Instruments, Australia).

Sedation during Study Protocol. Sedation was initiated using clinically accepted set dosages targeting light and deep sedation. A standard syringe pump (Alaris PK; Cardinal Health, Switzerland) was used to deliver a bolus infusion over 10 min of either dexmedetomidine (Precedex; Hospira Inc., USA) 0.6 µg/kg or propofol (Diprivan; Astra Zeneca, Australia) 750 µg/kg. This was followed by continuous infusion of dexmedetomidine $0.5\ \mu g \cdot kg^{-1} \cdot h^{-1}$ or propofol $42\ \mu g \cdot kg^{-1} \cdot min^{-1}$, respectively, aiming for light sedation. The first set of measurements of airway collapsibility was done at least 20 min after the start of the maintenance dose to allow for a steady state to occur.

Immediately after these measurements, the infusion rate was increased to dexmedetomidine $1.5\ \mu g \cdot kg^{-1} \cdot h^{-1}$ or propofol $83\ \mu g \cdot kg^{-1} \cdot min^{-1}$, aiming for deep sedation. The second set of airway collapsibility measurements was performed 20 min after initiation of this higher infusion rate (fig. 2).

Sedation level was monitored with continuous electroencephalogram, Bispectral Index score recordings, and two clinical sedation scales scored at discrete points of time (fig. 2). The modified five-point composite Observer's Assessment of Alertness/Sedation scale[18] and the Richmond Agitation–Sedation Scale[19] were used on two occasions for each infusion rate: 10 min after the start of each maintenance dose and immediately after airway measurements were completed. These times were selected for sedation checks to avoid provoking arousal and possible interference with airway measurements. Blood samples were collected for analysis of plasma

concentrations of dexmedetomidine or propofol before sedation (baseline value) and after completion of airway measurements at each infusion rate. After the second airway measurement, the infusion was stopped, and the protocol was ended.

Upper Airway Collapsibility Measurement: Pharyngeal Critical Pressure. Upper airway collapsibility was assessed using pharyngeal critical pressure as previously described at each infusion rate (fig. 3).[1,2,13,15–17,20] Briefly, stable breathing was established at a maintenance nasal pressure level ("maintenance pressure") sufficient to abolish inspiratory flow limitation (the presence of which was recognized by appearance of a plateau in the inspiratory flow profile or a failure of inspiratory flow to increase despite a decrease in esophageal pressure of at least 1 cm H_2O). The mask pressure was abruptly reduced from maintenance pressure to a range of positive and, if necessary, negative pressures to induce variable degrees of inspiratory flow limitation over a five-breath sequence before a return to maintenance pressure. End-expiratory pressure and mid-inspiratory flow for each of breaths 3–5 of the pressure drop were measured, and a mean was calculated. A minimum of three pressure drops to levels associated with flow limitation was obtained. Pharyngeal critical pressure was derived from linear regression of the mask pressure–plateau flow rate relationship during these pressure drops to calculate the pressure at which zero flow occurs (fig. 3). If brief arousal (less than 45 s of increase in electroencephalogram frequency followed by return to the previous state) occurred during a pressure drop or a pressure-drop sequence, any affected pressure levels were excluded from the pharyngeal critical pressure analysis and repeated. However, if the participant aroused for a more lengthy period, the entire pressure-drop sequence was abandoned, and a new
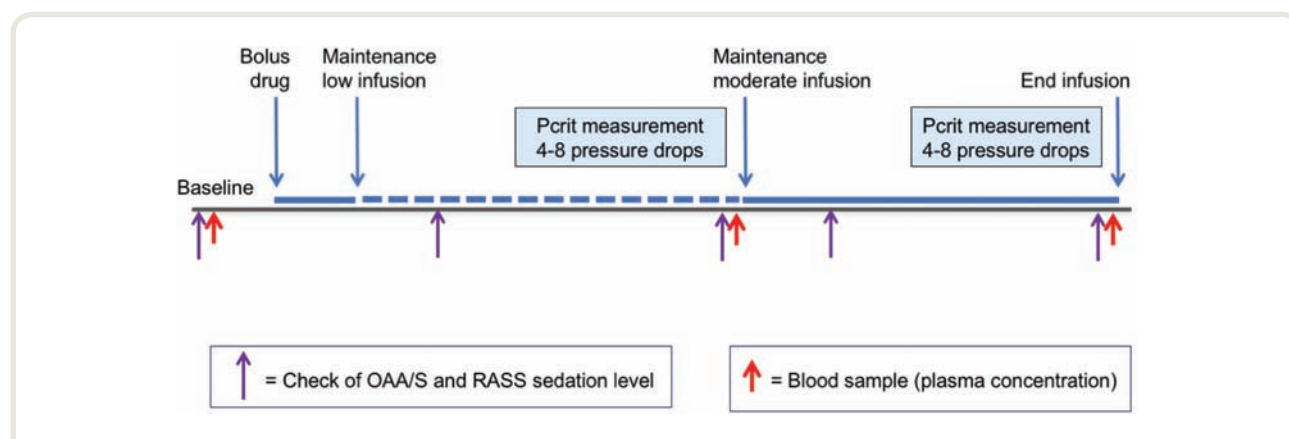
Fig. 2. Protocol used for sedation and acquisition of upper airway collapsibility data during dexmedetomidine or propofol sedation. Sedation was assessed with the modified five-point composite Observer's Assessment of Alertness/Sedation (OAA/S) scale and the Richmond Agitation–Sedation Scale (RASS). The sedation scale scores were as follows: (1) OAA/S scores 1: deep sedation, 2 to 4: light to moderate sedation, and 5: alert state; and (2) RASS score 0: alert and calm, −1: sustained awakening more than 10 s to voice, −2: briefly awakens to voice less than 10 s, −3: movement to voice but no eye contact, −4: movement to physical stimulation, and −5: unarousable. Pcrit, pharyngeal critical pressure.
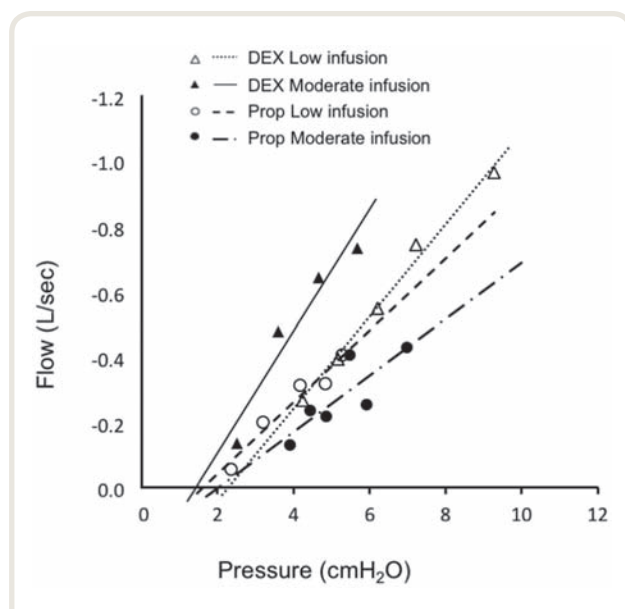
Fig. 3. A representative graph showing linear regression of airway flow and airway pressure in flow limited breaths used for pharyngeal critical pressure calculation during sedation with low and moderate infusion rates of dexmedetomidine and propofol in one individual (No. 7). *Open triangles* with *dotted line* indicate dexmedetomidine low infusion. *Closed triangles* with *solid line* indicate dexmedetomidine moderate infusion. *Open circles* with *dashed line* indicate propofol low infusion. *Closed circles* with *dashed and dotted line* indicate propofol moderate infusion. DEX, dexmedetomidine; Prop, propofol.

sequence was initiated after return to a stable level of sedation and breathing. The pressure drops were limited to a minimum of $-10\,cm\,H_2O$ (a level indicative of an airway highly resistant to collapse) as a precaution against untoward reflex responses, arousal, or gastroesophageal reflux. If a pharyngeal critical pressure was unable to be generated with this minimum pressure and flow limitation was not present to allow extrapolation of the flow–pressure relationship to zero flow (so a pharyngeal critical pressure value could be derived at a pressure greater than $-15\,cm\,H_2O$),[1] then a pharyngeal critical pressure value of less than $-15\,cm\,H_2O$ was recorded.

Analysis of Plasma Concentrations of Dexmedetomidine and Propofol. Blood was collected for measurement of drug concentrations in plasma before sedation and after completion of airway measurements at each infusion rate. Five ml of venous blood was drawn from the arm contralateral to drug infusion and placed into EDTA-containing tubes that were stored in wet ice and centrifuged at $+4°C$ for 10 min at $1,500g$ within 3 h. The plasma was then immediately transferred to precooled polypropylene tubes and frozen at $-20°C$ or colder. Concentrations of dexmedetomidine and propofol in plasma were determined as described previously.[12]

Statistical Analysis. Because data relating to upper airway collapsibility and dexmedetomidine sedation was unavailable, a prospective sample size estimate was generated partly based on earlier studies by the research group in Perth with a difference in pharyngeal critical pressure of 3 cm H_2O, considered to be of clinical significance, and a variability of 3.4.[21] An interim analysis was performed after completion of n = 12 participants, with complete data available from n = 9. No adjustments were made to the P values for this interim analysis. Power analysis at that time indicated insufficient power to detect a difference in pharyngeal critical pressure between dexmedetomidine and propofol sedation or confirm equivalence, based on the sample size and data available. A significantly larger sample would be required to do this. Given the need for a sample size widely exceeding the one originally calculated, the demanding nature of the study, and the consequent difficulty with subject recruitment, the study was ended.

Continuous data are presented as means $\pm$ SD or median (interquartile range). Categorical data are expressed as medians (interquartile range) or numbers. Normal distributions of data were tested using histograms, Q–Q plots, and the Shapiro–Wilk's test.

The primary outcome variable, pharyngeal critical pressure, was compared between drugs, dexmedetomidine and propofol, on two different occasions: "low infusion rate" and "moderate infusion rate," using a generalized equation estimation model to account for the repeated measures and a small sample size. If there was no sign of airway collapse, we used the Winsorizing method to reduce bias by the effect of extreme outliers, replacing the pharyngeal critical pressure value with a value of $-15\,cm\,H_2O$. These values were also used in Pearson correlation analyses, performed to identify the strength of the relationship between pharyngeal critical pressure during sedation with dexmedetomidine and pharyngeal critical pressure during propofol, at both infusion rates.

Bispectral Index score, mean arterial blood pressure, heart rate, and transcutaneous carbon dioxide were compared between drugs at seven different occasions using two-tailed testing with repeated-measures ANOVA with two within factors: time and drug. If differences were revealed by the repeated-measures ANOVA, preplanned pairwise comparison between drugs was made using a Bonferroni *post hoc* test. Sedation levels according to sedation scales Observer's Assessment of Alertness/Sedation and Richmond Agitation–Sedation Scale were compared with a sign test. Bispectral Index score and transcutaneous carbon dioxide were averaged over predefined time periods and are presented as means $\pm$ SD. A P value of less than 0.05 was considered statistically significant. All statistical analyses were undertaken using IBM SPSS Statistics version 24 software (IBM, USA). Graphs were made using Prism 7.0 (GraphPad, USA).
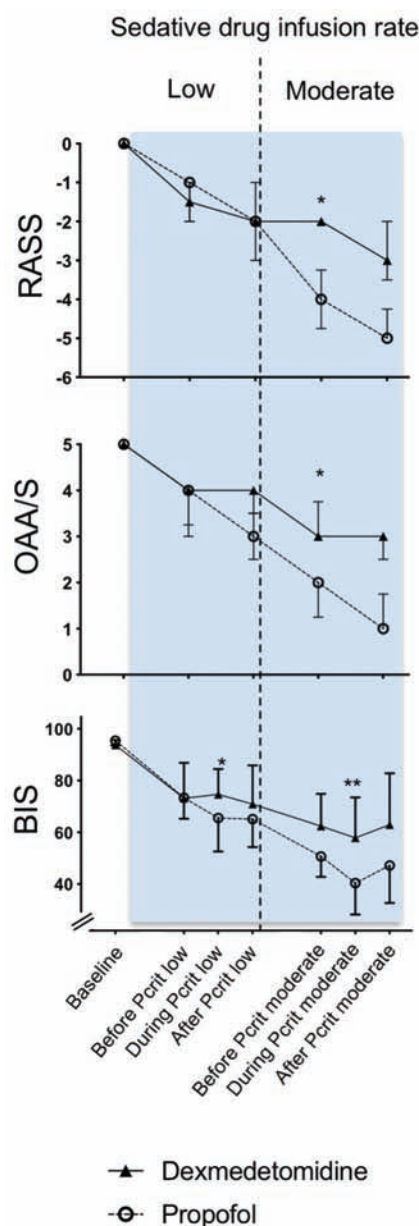
Fig. 4. Richmond Agitation Sedation Scale (RASS), modified 5-point composite Observer's Assessment of Alertness/Sedation (OAA/S), and Bispectral Index score (BIS) before and during sedation with dexmedetomidine (*closed triangles* with *solid line*) or propofol (*open circles* with *dotted line*). The *shaded area* shows the time for sedation with drug infusion at low and moderate rates. Sedation checks, OAA/S, and RASS, were conducted at baseline and before and immediately after airway measurements. BIS was recorded before, during, and after airway measurements. The *vertical broken line* indicates the time point when the infusion rate was changed from low to moderate. Data are presented as means ± SD for BIS and as median (interquartile range) for OAA/S and RASS. Please note that *error bars* of SD are unidirectional for clarity. If no *error bars* are visible they are smaller than the *symbols*. *P < 0.05; **P < 0.01.

Results

Twelve subjects, seven female, were included in this study. They had a median (range) age of 46 (23 to 66) years, body mass index of 25.4 (20.3 to 32.4) kg/m^2, and ASA physical status score of 1.5 (I to II). The six subjects with ASA class II comprised two subjects with treated hypothyroidism, two with hyperlipidemia and/or non–insulin-dependent diabetes mellitus, and one with treated hypertension. Their modified Mallampati score was 1 (1 to 4), thyromental distance was 10 (8 to 12) cm, and neck circumference was 35 (31 to 45) cm. Data regarding thyromental distance were missing for two participants. The STOP-BANG score was assessed and found to be 1 (0 to 5). The apnea hypopnea index had been determined in two subjects (No. 4 and No. 6) and was 25 and 7, respectively. Nine of twelve participants completed the full study protocol (fig. 1). Of the three other participants, one chose not to participate on the second occasion (propofol) because of time commitments, in one a patent airway could not be satisfactorily maintained without manual intervention, and one subject remained awake at the prescribed infusion rates. These three individuals lacked complete data for pharyngeal critical pressure measurements and were therefore excluded from analysis. Consequently, nine data sets evaluating upper airway behavior during both dexmedetomidine and propofol sedation were analyzed. No adverse events were experienced during the conduct of the study.

Sedation

The total time of drug infusion was 101 ± 11 min for dexmedetomidine and 100 ± 17 min for propofol. Assessment of airway collapsibility took 15 ± 6 *versus* 12 ± 7 min during low and 13 ± 8 *versus* 12 ± 10 min during moderate infusion rates for dexmedetomidine and propofol, respectively. Results from measurements of the Bispectral Index score, the modified Observer's Assessment of Alertness/Sedation, and the Richmond Agitation–Sedation Scale sedation scales are presented in figure 4.

Mean Bispectral Index score levels at the time of airway measurements were higher during dexmedetomidine infusion than propofol infusion during both low infusion rates 74 ± 10 and 65 ± 13, with a mean difference of 9 (95% CI, 3 to 16), respectively (P = 0.011) and moderate infusion rates of 57 ±16 and 39 ± 12, mean difference of 18 (95% CI, 8 to 28), respectively (P = 0.003), but were similar between dexmedetomidine at moderate infusion rates and propofol at low infusion rates of 57 ± 16 *versus* 65 ± 13, mean difference of 8 (95% CI, 0 to 16; P = 0.048; fig. 4). A statistical difference in sedation scale levels was not observed when dexmedetomidine and propofol were compared at low infusion rates but was observed during moderate infusion rates before pharyngeal critical pressure measurement for both Observer's Assessment of Alertness/Sedation (3.0 [3.0 to 4.0] *vs.* 2.0 [1.0 to 2.0], respectively; P = 0.016) and

Richmond Agitation–Sedation Scale (−2.0 [−2.0 to −2.0] *vs.* −4.0 [−3.25 to −4.75] respectively; $P = 0.016$; fig. 4).

Measurement of drug concentrations in plasma verified drug exposure. Specifically, the observed mean concentrations were 0.6 ± 0.1 and 1.6 ± 0.3 ng/ml at low and moderate infusion rates of dexmedetomidine and 0.9 ± 0.2 and 1.4 ± 0.2 µg/ml during low and moderate infusion rates of propofol.

Primary Outcome: Upper Airway Collapsibility, Pharyngeal Critical Pressure, during Sedation with Dexmedetomidine or Propofol

The maintenance airway pressure required to prevent inspiratory flow limitation varied between 4 (the minimum maintenance pressure used) and 15 cm H_2O. The primary outcome, pharyngeal critical pressure, was −2.0 (less than −15 to 2.3) and 0.9 (less than −15 to 1.5) cm H_2O (mean difference, 0.9; 95% CI, −4.7 to 3.1) during low infusion rates *versus* 0.3 (−9.2 to 1.4) and −0.6 (−7.7 to 1.3) cm H_2O (mean difference, 0.0; 95% CI, −2.1 to 2.1) during moderate infusion of dexmedetomidine and propofol, respectively. In five participants, pharyngeal critical pressure was more than 0 cm H_2O (indicating upper airway obstruction at atmospheric pressure), and in two other participants, pharyngeal critical pressure was less than −15 cm H_2O (beyond the limit of our testing [Materials and Methods] and indicating high resistance to upper airway collapse) during sedation both with dexmedetomidine and with propofol at low and/or moderate infusion rates. In the remaining two participants, pharyngeal critical pressure was consistently less than 0 cm H_2O with both sedatives, although it was less than −15 cm H_2O with only one or the other sedative. Consistent with these concordant observations, a statistically significant difference in pharyngeal critical pressure could not be shown during sedation with dexmedetomidine or propofol at either low the infusion rate ($P = 0.595$) or the moderate infusion rate ($P = 0.980$). Furthermore, a strong relationship between pharyngeal critical pressure during dexmedetomidine and propofol sedation was evident at the low and moderate infusion rates (fig. 5).

Effect of Sedation with Dexmedetomidine or Propofol on Respiration and Circulation

Three of the participants had periods of central apnea starting during the 10-min bolus dose administration of dexmedetomidine with durations up to 70 s: a representative polygraph example is shown in figure 6. These resolved at the latest within 3 min after completion of the bolus dose infusion. Apneic episodes were also seen in these same three individuals during propofol bolus dose administration, although the episodes were less frequent and of shorter duration than during dexmedetomidine infusion. In participant No. 3, seven apnea periods up to 60 s long during the dexmedetomidine bolus dose *versus* four apnea episodes with the longest duration of 50 s during the propofol bolus were recorded. Participant No. 5 had four apnea episodes, up to 30 s long, during bolus doses of both drugs. In participant No. 6, four apneas up to 75 s long were recorded in the last 4 min of the dexmedetomidine bolus dose infusion, and irregular breathing persisted for a further 5 min after completion of the bolus dose. This participant also exhibited three apnea episodes up to 50 s long during the propofol bolus infusion, beginning within 2 min from start of the bolus dose infusion. Apnea episodes resolved spontaneously with continued infusion in each case.
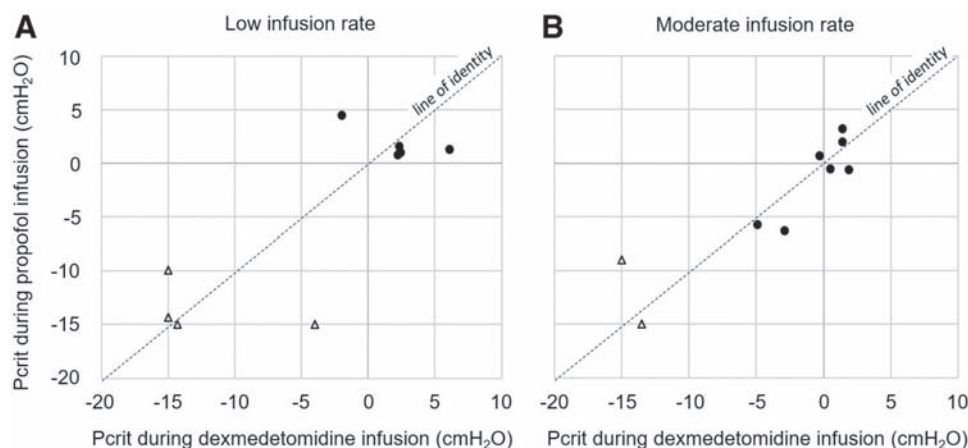
Fig. 5. Relationship between pharyngeal critical pressure (Pcrit) during dexmedetomidine and Pcrit propofol sedation during low (*A*) and moderate (*B*) infusion rates. *Open triangles* represent participants with an airway highly resistant to collapse (Pcrit was less than or equal to −15 cm H_2O). The *dotted line* indicates line of identity. Note the concordance between Pcrit values during sedation at low (r = 0.82; *P* = 0.007) and moderate (r = 0.90; *P* < 0.001) infusion rates with both drugs (n = 9).
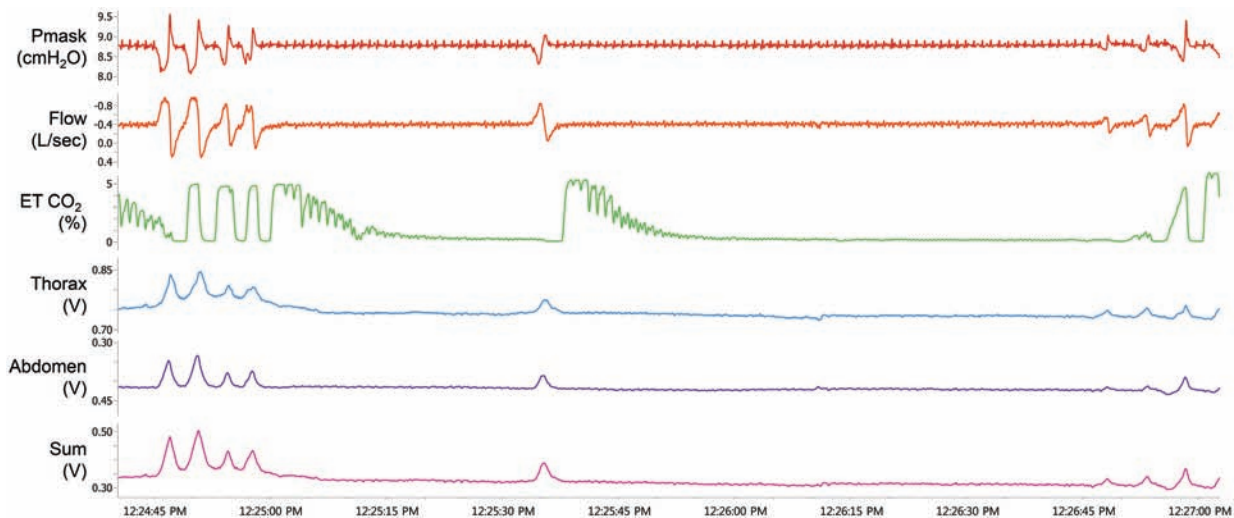
Fig. 6. Original data recording of apnea episodes in one subject (No. 3) appearing 7 min after induction of dexmedetomidine sedation with a bolus dose of 0.6 µg/kg administered during 10 min. Rows 1 to 3 from the top display mask pressure (Pmask), airway flow (Flow) and end-tidal carbon dioxide (ET CO$_2$). In rows 4 to 6, inductance plethysmography of the thorax and abdomen show an absence of respiratory effort indicative of centrally evoked apnea.

Transcutaneous carbon dioxide at the end of bolus dose infusion did not differ between individuals with (n = 3) and without apnea during either dexmedetomidine (42 ± 4 *vs.* 48 ± 2 mmHg; $P = 0.570$) or propofol (46 ± 7 *vs.* 47 ± 4 mmHg; $P = 0.857$) infusion. Transcutaneous carbon dioxide increased to a similar extent from baseline to the end

Fig. 7. Transcutaneous carbon dioxide (TcCO$_2$) before and during low and moderate infusion rates of dexmedetomidine (*closed triangles*) or propofol (*open circles*). The data are shown as means ± SD. If no *error bars* are visible, they are smaller than the *symbols*. Pcrit, pharyngeal critical pressure.

of the sedation protocol with dexmedetomidine (from 41 ± 5 to 47 ± 4 mmHg) and propofol sedation (from 42 ± 5 to 48 ± 6 mmHg) with no statistically significant difference between drugs ($P = 0.281$; fig. 7).

Peripheral oxygen saturation did not decrease below 96% at any time in any participant. Mean heart rate was lower during dexmedetomidine compared with propofol sedation ($P = 0.001$), but mean arterial blood pressure was similar during infusion of both agents ($P = 0.297$; fig. 8).

Discussion

A difference in upper airway collapsibility during dexmedetomidine and propofol sedation was not observed in this study, regardless of level of sedation. Importantly, we observed pharyngeal critical pressure values indicative of total obstruction at atmospheric pressure (*i.e.*, pharyngeal critical pressure values of at least 0 cm H$_2$O) in five of the nine subjects during dexmedetomidine infusion at low and/or moderate infusion rates, which is contrary to previous assertions that this drug protects against such obstruction.[7–9] Furthermore, the same five subjects were the only subjects to exceed this 0 cm H$_2$O threshold during propofol sedation at low and/or moderate infusion rates. Correlation analysis confirmed a strong relationship in pharyngeal critical pressure between both drugs and at both infusion rates. Moreover, induction of sedation was associated with prolonged apneas in three of the nine subjects with either drug, suggesting similar effects of the two drugs on ventilatory drive.
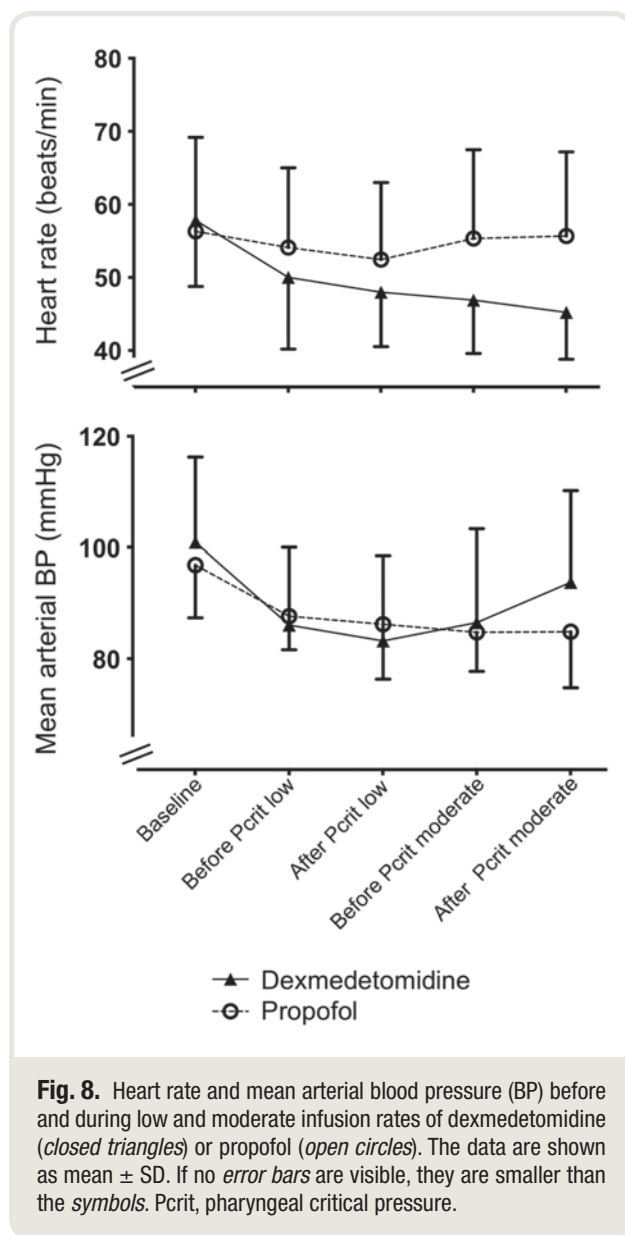
Fig. 8. Heart rate and mean arterial blood pressure (BP) before and during low and moderate infusion rates of dexmedetomidine (*closed triangles*) or propofol (*open circles*). The data are shown as mean ± SD. If no *error bars* are visible, they are smaller than the *symbols*. Pcrit, pharyngeal critical pressure.

Dexmedetomidine is increasingly used for sedation in both children and adults, with and without obstructive sleep apnea, in the belief that both upper airway patency and ventilatory drive are less compromised than with other sedative agents.[22] With these purported benefits, its use for drug-induced sedation endoscopy as a diagnostic tool for identifying the level of airway obstruction during sleep is being increasingly advocated.[23] However, systematic evaluation of drug-induced sedation endoscopy during propofol *versus* dexmedetomidine sedation has identified no differences in airway obstruction in terms of anatomical location (velum, oropharyngeal lateral walls, tongue base, and epiglottis) and severity (none, partial, or complete) between the sedative agents during both light (Bispectral Index score of 65 to 75) and deep (Bispectral Index score of less than

60) sedation.[10,24,25] These findings and those of the present study seriously question the assumption that dexmedetomidine, when used for sedation, has minimal adverse effect on airway stability.

Consistent with the findings of the present study and one we previously conducted,[12] magnetic resonance imaging demonstrates that dexmedetomidine sedation is associated with reductions in upper airway dimensions in healthy children,[11] although clinically significant airway obstruction was not observed. However, in two other studies comparing upper airway behavior during dexmedetomidine and propofol sedation for magnetic resonance imaging in children with obstructive sleep apnea, there was a need for an artificial airway or airway maneuvers in 14% and 11% of children sedated with dexmedetomidine *versus* 40% and 23%, respectively, when propofol was used.[26,27]

The range of upper airway collapsibility seen in our participants reflects that seen in the general community.[20,28] We observed similarities in airway behavior between dexmedetomidine and propofol sedation across this range of individual collapsibility (fig. 5). However, we did not study patients with difficult airways specifically. Although it is possible that the airway behavior of individuals with difficult airways could be different, there is no indication of this from the data of those with more collapsible airways in our study (fig. 5).

Contrary to previous studies of pharyngeal critical pressure during propofol sedation, the pharyngeal critical pressure level did not consistently increase with a deeper level of sedation with either drug,[2,13] although we did not explore as deep levels of sedation in the present study. The relatively stable pharyngeal critical pressure levels observed here may reflect the relatively profound muscle deactivation that accompanies the transition from wakefulness to unconsciousness, leaving little room for further change.[2] Indeed, although previous studies have demonstrated a dose effect with propofol, the increase in pharyngeal critical pressure values with increased effect site concentrations was relatively small.[2,13]

Although both drugs have powerful sedative effects, dexmedetomidine, a noradrenergic drug, and propofol, a γ-aminobutyric acid–mediated drug, have different molecular targets within the brain. In producing sedation, dexmedetomidine inhibits stimulatory pathways, whereas propofol activates inhibitory ones, each molecular target having different structural correlates within the brain.[5,6,29] It may be that these different actions result in differences in other characteristics that could influence upper airway behavior and protection from upper airway collapse, such as their effects on arousal thresholds with, perhaps, a readier tendency to arouse from obstructive events with dexmedetomidine than with propofol. In this regard, it is of interest to note the different relationships between infusion rates and depth of sedation between the two drugs evident in figure 4. With propofol, there is a progressive increase in depth of sedation

assessed both objectively (Bispectral Index score) and subjectively (sedation scales), whereas with dexmedetomidine, there appears to be a plateauing of effect with less change in sedation levels between low and moderate infusion rates than between the wake baseline and low infusion rate. This suggests a wider therapeutic index with dexmedetomidine sedation than with propofol sedation. It should be noted that in our assessment of arousal thresholds, we were careful to minimize environmental disturbance, which included limiting the number of arousals inducing sedation checks; hence, the relative stability of sedation with each drug was not formally assessed.

It was notable that, in addition to their effects on airway collapsibility, other aspects of breathing behavior were similarly affected by the two drugs. The three subjects who experienced persistent central apneic events (prolonged pauses in respiratory effort) during induction of sedation did so with both drugs, although the effect appeared to be a little more amplified with dexmedetomidine. Although the reason for these pauses is not entirely clear, our observations are in line with previous findings of irregular breathing and apnea that have been reported during administration of both dexmedetomidine[7,12] and another α2-adrenoceptor agonist, clonidine.[30–32] α2-Adrenoceptors are widely distributed in the central nervous system including brainstem sites associated with respiratory control. α2-Adrenergic agonists have been shown to reduce the firing rate of neurons and cause disturbances in respiratory pattern not dependent on the carotid body, suggesting an effect on central α2-adrenoceptors that affects the rhythm of breathing.[33,34] It is also possible that these apneic periods may represent perturbations in ventilation that accompany changes in the apneic threshold for hypercapnic ventilatory drive that accompanies loss of consciousness with sleep or sedation, which can vary between individuals.[35] Regardless of the mechanism, transcutaneous carbon dioxide increased to a similar degree with sedation with both drugs, suggesting a similar overall ventilatory depressant effect.

There are several limitations to this study. First, there was a small number of participants, reflecting the commitment required of them and the complexity of the study. Given the low numbers involved, although we failed to demonstrate a difference in airway collapsibility between the drug regimens, this does not equate to confirmation of equivalence. However, there were sufficient subjects to demonstrate that dexmedetomidine sedation is associated with substantial airway collapsibility in some of them and that the degree of collapsibility observed is related to that seen with propofol at equivalent infusion rates. These findings are contrary to the widely held belief that the upper airway is less vulnerable to obstruction during dexmedetomidine sedation than during sedation with propofol. Second, the variability in collapsibility between subjects potentially complicated interpretation given the small number involved. A difference in upper airway collapsibility between the two drugs

may exist, but to detect this, a study with much greater sample sizes would be necessary. However, the observed variability can also be regarded as strength because it reflects the variability found in the general population. Third, our use of a set infusion rate for administration of the sedative agent, which was done to standardize conditions as much as possible, resulted in different sedation levels, as reflected by Bispectral Index scores and sedation scales, between the agents and across the subjects. Adding to this, the Bispectral Index score is not validated for dexmedetomidine, and earlier studies have shown that the Bispectral Index score at equal clinical levels of sedation tends to be lower for dexmedetomidine than for propofol.[36] However, comparison of collapsibility at both these prescribed infusion rates and at nearly equivalent levels of sedation demonstrated a similar effect on upper airway collapsibility. Nonetheless, a more pronounced effect on airway collapsibility during dexmedetomidine sedation at an infusion rate increased to produce an equivalent sedation level to that induced by moderate propofol infusion cannot be ruled out. Last, the study was not conducted in a blinded fashion and was therefore susceptible to bias during data collection and analysis. Blinding during data collection would have been possible although challenging because of the different appearance of the study drugs. Blinding during analysis would have also been possible with a larger research team.

Strengths of the study are a careful experimental setup designed to evaluate upper airway collapsibility directly that is well validated in several previous studies[1,2,13,17,21] and the use of propofol, a sedative drug that is well characterized regarding upper airway collapsibility, as a direct comparator in a crossover design, *i.e.* the same individuals were tested with both drugs. In addition, drug concentrations in plasma were quantified as an objective indirect measure of sedation, and the concentration results indicated that light to moderate levels of sedation were achieved.[6,8,36,37]

Conclusions

Upper airway collapsibility is similar during dexmedetomidine and propofol sedation, questioning the belief that dexmedetomidine offers protection against upper airway obstruction relative to sedation with this other commonly used anesthetic agent. Episodes of clinically significant apnea can occur during induction of sedation with both drugs.

Acknowledgments

Competing Interests

Dr. Eriksson received lecture fees from Merck Inc. Sweden (Stockholm, Sweden) and is a medical advisor to Saniona A/S (Copenhagen, Denmark). Dr. Scheinin has contract research relationships with Orion Corporation (Espoo, Finland); AstraZeneca (Espoo, Finland); Hoffmann–La Roche (Espoo, Finland); Novartis (Espoo, Finland); and AC Immune SA (Lausanne, Switzerland). Dr. Scheinin has also received speaker's fees and research support from Orion Corporation and speaker's fees from Pharma Industry Finland (Helsinki, Finland). Drs. Maddison, Eastwood, Hillman, and Walsh have contract research relationships with Zelda Therapeutics Pty Ltd. (Perth, Australia); Nyxoah S.A. (Mont-Saint-Guibert, Belgium); and Oventus (Indooroopilly, Australia). The other authors declare no competing interests.

Reproducible Science

Full protocol available at: Ase.Lodenius@remeoclinic.se. Raw data available at: Ase.Lodenius@remeoclinic.se.

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ANESTHESIOLOGY

Genetic Analysis of Patients Who Experienced Awareness with Recall while under General Anesthesia

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- The incidence of explicit recall of intraoperative events, or awareness with recall, is less than 0.2%
- Anesthetic dosing is apparently adequate in 10 to 25% of awareness with recall patients
- The awareness with recall phenotype only reveals itself when patients are exposed to anesthesia

What This Article Tells Us That Is New

- A preliminary study sought to determine whether there is evidence that awareness with recall is caused by a few rare variants with high penetrance in 12 patients who had experienced awareness with recall in the presence of apparently adequate anesthesia
- Whole exome sequencing was conducted and identified variants were filtered and prioritized to identify a candidate list that might be suitable for further investigation of causes of awareness with recall
- No candidate gene(s) suggestive of a monogenic etiology were identified, possibly because of the application of a filtering strategy, the small sample size, or use of exome sequencing, which does not interrogate potentially important regulatory noncoding sequences

ABSTRACT

Background: Intraoperative awareness with recall while under apparently adequate general anesthesia is a rare, unexplained, and often very distressing phenomenon. It is possible that a relatively small number of genetic variants might underlie the failure of general anesthetic drugs to adequately suppress explicit memory formation and recall in the presence of apparently adequate anesthesia concentrations.

Methods: The authors recruited 12 adult patients who had experienced an episode of intraoperative awareness with recall (compared with 12 controls), performed whole exome sequencing, and applied filtering to obtain a set of genetic variants that might be associated with intraoperative awareness with recall. The criteria were that the variant (1) had a minor allele frequency less than 0.1% in population databases, (2) was within exonic or splicing regions, (3) caused a nonsynonymous change, (4) was predicted to be functionally damaging, (5) was expressed in the top 50% of genes expressed in the brain, and (6) was within genes in Kyoto Encyclopedia of Genes and Genomes pathways associated with general anesthesia, drug metabolism, arousal, and memory.

Results: The authors identified 29 rare genetic variants in 27 genes that were absent in controls and could plausibly be associated with this disorder. One variant in *CACNA1A* was identified in two patients and two different variants were identified in both *CACNA1A* and *CACNA1S.* Of interest was the relative overrepresentation of variants in genes encoding calcium channels and purinergic receptors.

Conclusions: Within the constraints of the filtering process used, the authors did not find any single gene variant or gene that was strongly associated with intraoperative awareness with recall. The authors report 27 candidate genes and associated pathways identified in this pilot project as targets of interest for future larger biologic and epidemiologic studies.

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Explicit recall of intraoperative events is a rare, but often distressing, phenomenon. We will use the nomenclature *awareness with recall* for these episodes. For 90% of the population, the amnesic effects of general anesthetic drugs occur at concentrations well below those required for unconsciousness (typically 0.1 to 0.3 minimum alveolar concentration [MAC]).[1] However, in approximately 10 to 25% of awareness with recall patients anesthesia dosing is apparently adequate (greater than 0.5 MAC),[2,3] indicating that the anesthetic drug has failed to

disrupt conscious perception and the memory consolidation process. A genetic predisposition for awareness with recall may exist and could explain resistance to levels of anesthesia that are normally considered adequate for the majority of patients. However, we are not aware of any study that has previously investigated this hypothesis.

Many neurobiologic events must be fulfilled to lay down a properly consolidated memory.[4–6] It is noteworthy that the awareness with recall phenotype only reveals itself when patients are exposed to anesthesia; typically, awareness with recall patients display no other identified phenotypic disturbance in day-to-day life, and awareness with recall has no clear demographic or disease associations. Also, the incidence of awareness with recall is low (less than 0.2%).[7] If there is a genetic component to awareness with recall, these observations would suggest one of two parsimonious hypotheses for potential genetic causes of awareness with recall: (1) awareness with recall could be a polygenic trait with common variants in many weakly penetrant genes—and having interactions with other genes or the environment—each contributing a small amount to the risk; or that (2) awareness with recall could be caused by a few rare variants, each with a high penetrance. Identifying common variants of low impact is difficult, and typically involves undertaking a genome-wide association study, usually requiring hundreds if not thousands of affected individuals. Without access to these resources, we undertook a preliminary study examining whether there is evidence for the rare-variant model. To do this we conducted whole exome sequencing of a set of carefully chosen patients who have experienced awareness with recall in the presence of apparently adequate anesthesia concentrations. The identified variants were filtered and prioritized to identify a candidate list of variants/genes that might form a reasonable basis for further investigation of the causes of awareness with recall.

Materials and Methods

This study was conducted according to the Strengthening the Reporting of Observational Studies in Epidemiology guideline and Strengthening the Reporting of Genetic Association Studies extension.[8] Prospective approval was obtained from the ethics committees of Royal Melbourne Hospital, Australia (June 9, 2011; approval number 2011.008) and Waikato Hospital, New Zealand (June 29, 2011, Northern Y Regional Ethics Committee approval reference, NTY/11/03/027), as investigators at these sites recruited and interviewed the patients. The study was not registered because it was not a clinical trial.

Patient Cohort

Patients were eligible for this study if they were fluent in English, were at least 18 yr of age at the time of enrollment and at least 13 yr of age at the time of the awareness episode, experienced an awareness episode during the last 10 yr (on

December 14, 2013 amended to the last 30 yr, *i.e.*, since the widespread use of end tidal volatile anesthetic agent monitoring), and reported an awareness episode that included the following features:

1. General anesthesia was intended for the case
2. Somatic sensations, pain, sounds, conversations, or emotions were experienced while the patient was supposed to be unconscious
3. These feelings were experienced during the procedure (*i.e.*, conversations confirmed to have occurred during surgery, sounds that could only have been heard during surgery)

Patients were recruited *via* advertisements in local newspapers, in the investigators' hospitals, on hospital websites and the website of the Australian and New Zealand College of Anaesthetists, and through free media opportunities (such as features in popular magazines and television programs). Patients who answered the advertisements were screened over the phone. Those who met the eligibility criteria were mailed the patient information and consent form. Patients were given an opportunity to ask questions of the investigators or obtain independent advice. After receipt of written informed consent, trained research nurses at each institution conducted a phone or in-person interview with the patient. The nurses or investigators provided general advice to patients, or referral to their own physician or hospital, if requested or required. The investigators did not seek access to medical records related to the reported awareness episodes.

The following information was obtained at interview:

1. Date of birth
2. Sex
3. Date of index surgery
4. Name of index surgery
5. Family history of awareness
6. Description of awareness episode
7. Presence of somatic sensations, pain, paralysis or weakness, sounds or conversations audible, visual perceptions, tried or able to move, emotions experienced, feelings of helplessness, anxiety, panic, or impending death, other (free text)
8. Description of any consequences of awareness episode
9. Presence of sleep disturbance, nightmares, daytime anxiety, depression, fear of future anesthetics, late psychologic problems, posttraumatic stress disorder
10. Description of treatment
11. Presence of consultation with health professional, counseling, medication, or other treatment for awareness episode
12. Details of the explanation provided to the patient by their anesthetist (especially in relation to the adequacy of anesthesia during the episode)
13. Any written material regarding incident in possession of patient and which patient is willing to share

Interview reports were adjudicated by three of the investigators (J.W.S., K.L., A.J.D.). The adjudicators independently classified the cases as "awareness," "possible awareness," and "no awareness," as per our previous studies.[9,10] Only cases that were universally coded as "awareness" were included. Furthermore, the adjudication panel decided by consensus whether a patient was aware despite apparently adequate general anesthesia.

DNA Sample Collection, Storage, and Analysis

A saliva sample was obtained from all included patients and genomic DNA was isolated using the Oragene kit as per the manufacturer's instructions (DNA Genotek, Canada). DNA quality and concentration was determined using a NanoDrop 2000c (ThermoFisher Scientific, USA) and samples were stored at −20°C until analyzed.

Whole Exome Sequencing and Variant Calling

Because the whole exome sequence of each individual can vary considerably, it is necessary to have a process to maximize the reliability of identification of the variants; which is done by comparing the sequence of participants with a reference genome databases. The technical description of the process we used is as follows. Whole exome sequencing was performed at the Australian Genome Research Facility, Melbourne, Australia, using the Agilent SureSelect Human All Exon V5+UTR capture platform.[11] The raw whole exome sequencing data from the 12 awareness with recall cases were analyzed using an in-house bioinformatics pipeline. In summary, the raw sequencing reads were aligned to the hg19 version of the reference human genome assembly with Novoalign (www.novocraft.com; accessed January 11, 2016) and polymerase chain reaction duplicates were removed using Picard MarkDuplicates.[12] It is necessary to determine the existence (likelihood) of the gene variants from the raw nucleotide sequence. To identify sequence variants in cases compared with the reference, the open source program (GATK HaplotypeCaller[13]) was used. The consequence and potential significance of identified variants was then determined by comparison with previously identified and reported variants using the bioinformatics tool ANNOVAR.[14,15] Standard quality control checks were performed during all stages of the analysis pipeline. We used our in-house database to select 12 other whole exome sequencing datasets as controls for filtering, in addition to the Genome Aggregation Database Exome Aggregation Consortium[16] (gnomAD/ExAC) and 1,000 Genomes variant frequency databases.[17] These are databases that catalogue the genetic variation in tens of thousands of unrelated individuals that can be used to establish the accuracy and incidence of any variants, and are critical in eliminating false positives, which can arise as a result of differences in the data analysis processing steps involved in variant calling. The controls were selected to match the samples with respect to sex, age, and ethnicity; they also underwent the same sequencing process at the same facility.

Filtering Strategies in Candidate Genes

Approximately 5.5 million variants were identified in the whole exome sequencing data generated for the 12 participants (fig. 1); therefore, a filtering strategy needed to be used. Filtering of variants was performed based on the assumption that the causal variants are rare and likely to affect either expression of the transcript or amino acid sequence of the protein. Application of these criteria reduced the candidate list to 8,706 variants. Initially, we searched this candidate list for the presence of variants in the Mendeliome, a subset of ~4000 genes with reported disease associations in the Online Mendelian Inheritance in Man.[18,19] We did not detect any variants fulfilling the criteria of either (1) previously reported as pathogenic or likely pathogenic or (2) novel/very rare high impact variants, that could plausibly explain either a dominant or recessive genetic model.

To further reduce the candidate variant list, we next focused on a subset of genes that had a higher *a priori* chance of being involved in awareness with recall. The R statistical software package KEGGgraph (http://bioconductor.org/packages/KEGGgraph/; accessed January 11, 2016) was used to extract the genes involved in Kyoto Encyclopedia of Genes and Genomes pathways to assemble the anesthetic awareness candidate gene list. Kyoto Encyclopedia of Genes and Genomes is a database of gene clusters associated with various known cellular functions. General literature searches involving likely targets of general anesthesia, drug metabolism, arousal, and memory were used to assist in finding the likely Kyoto Encyclopedia of Genes and Genomes pathways. The pathways and their member genes are listed in the Supplemental Digital Content (http://links.lww.com/ALN/C5).

To be included in the final dataset, all variants needed to pass the following filtration criteria: have a minor allele frequency less than 0.1% in the ExAC database; be within exonic or splicing regions; cause a nonsynonymous change; predicted to be functionally damaging by one of SIFT[20] and PolyPhen-2[21] (these databases predict the potential for a given variant to be detrimental to protein function based on sequence homology and physical properties of the amino acids involved); be expressed in the top 50% of genes expressed in the brain based on the genotype tissue expression (GTEx) dataset; be within genes in Kyoto Encyclopedia of Genes and Genomes pathways associated with general anesthesia, drug metabolism, arousal, and memory; and be detected in at least one of the 12 cases, but fewer than three controls. Genes were further annotated with residual variation intolerance scores,[22] genic intolerance scores[17] and Online Mendelian Inheritance in Man.

Validation of Variants

Prioritized variants were validated by standard polymerase chain reaction amplification and Sanger sequence analysis. This analysis was performed fee for service by Genewiz Corporation (USA).

Sample Size and Statistics

Descriptive statistics were used to describe the included patients and the quality of the sequencing data. Continuous data were summarized using median (range), and categorical data were summarized using number (percent). We did not perform formal inferential statistical testing because there was insufficient statistical power to achieve significance after multiple testing because of the small number of rare variants identified and the small sample size.

Results

Patients were screened between September 2011 and January 2014. Of 102 screened patients, 52 met the eligibility criteria and were interviewed. Among these, seven were determined to have had the awareness episode too long ago and one did not return the DNA sample. Twelve of these patients, with most phenotypic consistency, were selected for sequence analysis (fig. 2).

The characteristics of the 12 included patients are reported in table 1. The median age was 44.5 (range 23 to 88) years at the time of surgery. Eleven of the 12 patients (92%) were female. The interval between the surgery and the interview was 6.5 (range 0 to 36) yr. The single patient who had an episode of awareness 36 yr ago was included because this patient had a very clearly defined episode and also reported a family history of awareness

with recall. Patients had the following experiences during their awareness episode: somatic sensations (83%), pain (42%), weakness (33%), sounds (75%), voices (67%), visual perceptions (17%), attempts to move (33%), able to move (17%), emotions (75%), and feelings of helplessness (75%). Psychologic consequences were experienced by 42% of patients.

Whole Exome Sequencing and Variant Calling

The sequencing data generated were of high quality, the median depth of coverage ranged from 31 to 38 reads with a mean of 35. The percentage of targeted bases covered by at least 10 reads ranged from 92.4 to 94.9%, with a mean of 94.0%.

Filtering and Validation

The pathway search led to nine pathways containing a total of 658 genes that were chosen as the candidate gene set for variant analysis. Because we preselected pathways to examine, we were unable to perform pathway enrichment analyses.

The variant filtering strategy is summarized in figure 1. This reduced the number of variants from an initial 5,465,705 variants to a final list of 29 variants in 27 genes in the 12 individuals with awareness with recall (table 2). None of the final 29 variants was observed in any of the
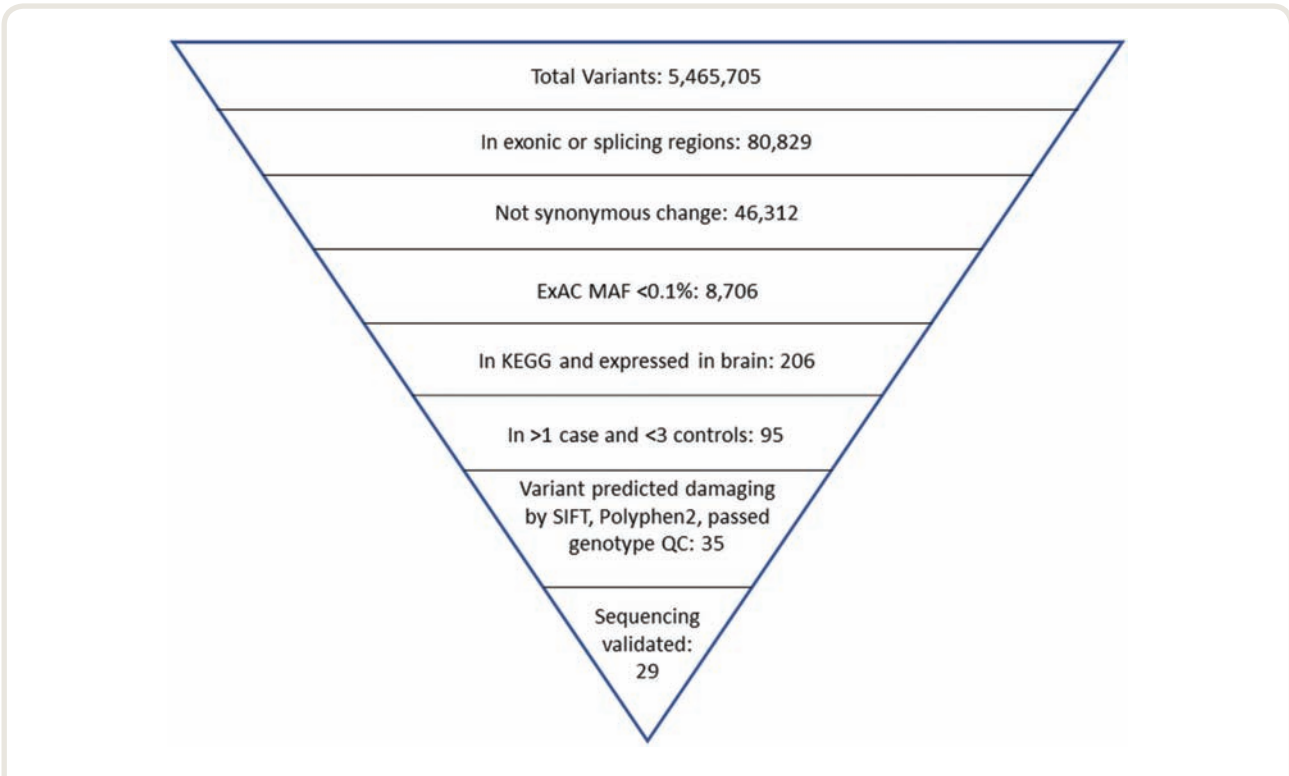
Fig. 1. Variant filtering strategy. ExAC, Exome Aggregation Consortium; KEGG, Kyoto Encyclopedia of Genes and Genomes; SIFT, sorting intolerant from tolerant database; polyphen2, polymorphism phenotyping v2.

Table 1. Characteristics of Included Patients

Patient	Sex	Type of Surgery	Age at Surgery (yr)	Years since Surgery (yr)	Family History	Somatic	Pain	Weakness	Sounds	Words	Visual	Move Attempt	Move	Emotions	Helpless	Consequences
1	f	gynae	36	28	–	–	–	–	–	+	–	–	+	+	–	+
2	f	gynae	36	0	–	+	+	–	–	–	–	+	+	–	–	+
3	f	spine	37	9	–	+	+	+	+	+	+	+	–	+	+	+
4	f	gynae	23	36	+	+	–	+	+	+	–	+	–	+	+	–
5	f	gynae	41	4	+	–	–	+	+	+	+	+	–	–	+	–
6	f	general	50	0	–	+	+	+	+	+	–	+	–	+	+	+
7	f	general	53	6	–	+	–	+	+	+	–	+	–	+	+	+
8	f	many	48	11	–	+	–	+	+	+	–	+	–	+	+	–
9	f	ortho	50	7	–	+	–	+	+	+	–	+	–	+	+	–
10	f	ortho	36	9	–	+	+	+	+	+	–	+	+	–	+	–
11	f	ortho	76	4	–	+	–	–	–	–	–	–	+	–	–	–
12	m	cardiac	88	0	–	+	+	–	+	–	–	+	–	+	+	–

Plus signs indicate present; *minus signs* indicate not present; f, female; general, general surgery; gynae, gynecologic surgery; m, male; and ortho, orthopedic surgery.

12 control whole exome sequencing datasets, and all were observed in only a single patient, except for the variant in *CACNA1A* (NM_001127222.1:c.6658_6659insACC, p.[His2219dup]), which was observed in two patients. Two different variants in the gene encoding CACNA1S were identified (NM_000069.2:c.3322C>G; p.[Gln1108Glu] and NM_000069.2:c.1819G>A; p.([Val607Ile]).

Possible Associated Disorders

Awareness with recall has no known associations with other diseases. Twenty-five of the variants caused nonsynonymous coding sequence alterations, two resulted in frameshift insertion deletion (*P2RX1* and *CREB3L3 indels*), one resulted in a nonframeshift duplication of a histidine (*CACNA1A*), and one resulted in an intronic deletion of five base pairs 16 base pairs upstream of a splice site (*RPS6KA6*). Of the 27 genes of interest that we identified, a few had some reported association with various clinical disorders (such as malignant hyperthermia, long QT syndrome, epilepsy, spinocerebellar ataxia, myopathy, and leukemia [table 2]). However, the variants we identified in the awareness with recall patients are probably not clinically significant for these disorders, because they are described as either not present or of conflicting interpretation in ClinVar, which is the main publicly available database for linking gene variants with phenotypes.[23]

Likely Candidate Genes for Awareness with Recall

There was no single gene variant, or multiple different variants in a single gene, present in a high proportion of the awareness with recall patients that would indicate a monogenic or paucigenic mechanism in our cohort. We did identify a cluster of variants for nine CACN genes in 10 of the patients (table 2), and most of the other variants are linked to calcium signaling pathways in the Genecards database.[24] In addition, there were six variant identified in genes that encode proteins involved in purinergic receptor function and metabolic signaling. These observations are suggestive that a polygenic mechanism may be associated with awareness with recall in this cohort, although general conclusions regarding underlying genetic mechanisms will require analysis of larger cohorts.

Discussion

The purpose of this observational study is to report some possible genetic methodologies and explanations regarding the clinical issue of awareness with recall. We do not make any definitive causal claims, but present these as hypothesis–generating data. This study also highlights some of the methodologic difficulties in understanding the implications of genetic studies.

In the cohort analyzed we did not identify any candidate gene(s) suggestive of a monogenic cause. This may be attributable to one or more factors, including the
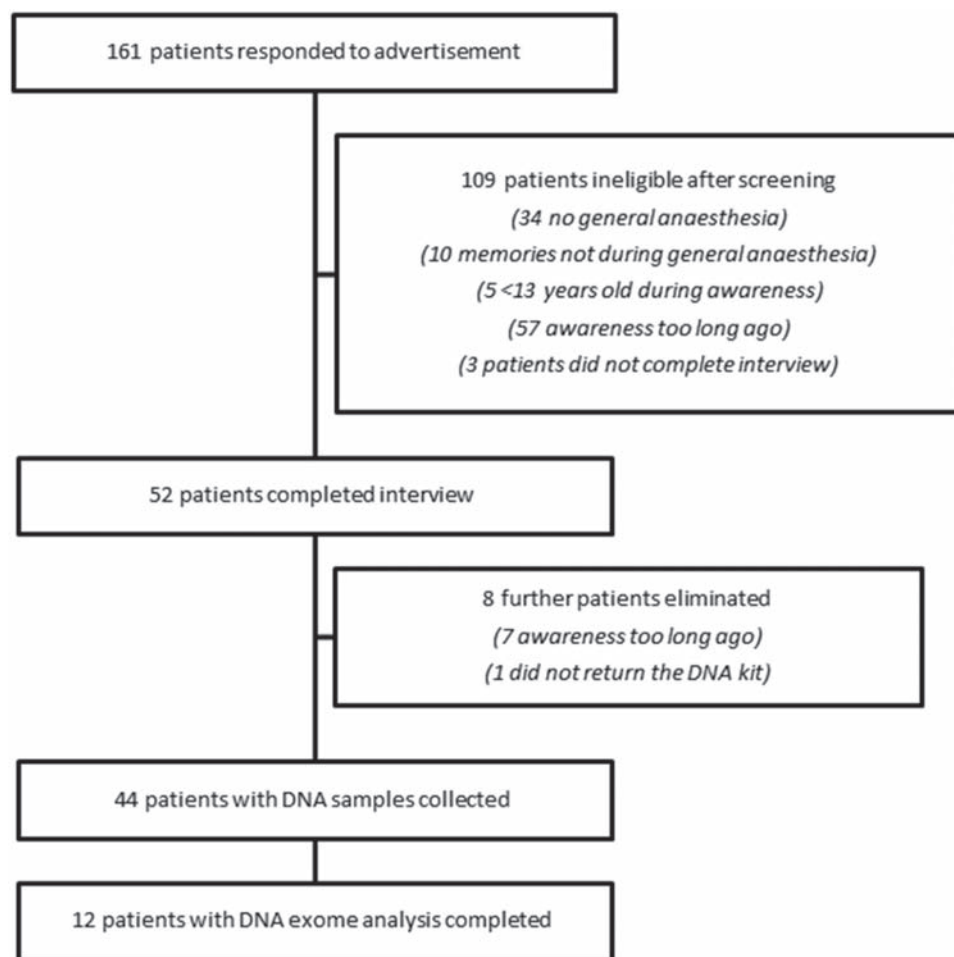
Fig. 2. Study flowchart.

application of our filtering strategy, the relatively small sample size, or the use of exome sequencing, which does not interrogate potentially important regulatory noncoding sequences. We did identify variants in multiple genes that function in the calcium signaling, purinergic receptor, and metabolic signaling pathways. These observations are consistent with the hypothesis that awareness with recall is a polygenic trait. Other large next-generation studies in neurogenetic disorders—which seek to identify rare causal variants—commonly identify multiple genes with different variants showing elevated risk.[25] The variants that we found merit further definitive investigation through a replication study in an independent cohort of awareness with recall patients to increase statistical power, and with experimental functional studies—such as mouse models with these variants inserted into the genome artificially by genome editing methodologies such as CRISPR/Cas9.

The role of calcium signaling pathways and their relationship to purinergic pathways is worth exploration. Clearly they are pivotal in memory formation[26] and arousal. In animal models mutations of these genes have been associated with hyperfunction diseases, such as epilepsy and migraine. As yet there is limited evidence to associate these mutations with anesthetic sensitivity, although Tatsuki *et al.*[27] suggested that impaired CACNA-1H /Cav2.3 decreases slow wave sleep and is a critical regulator of N-methyl-D-Aspartate receptor function—and hence memory. As regards general anesthesia, Takei *et al.* somewhat confusingly found that CACNA-1B mutants were resistant to propofol sleep time but more sensitive to halothane.[28]

Measures of likely harmfulness—such as residual variation intolerance score and the loss of function intolerant scores—do not appear to be a useful guide for determining potential pathogenicity in this setting as a broad range of often conflicting scores, including very high (tolerant to mutations) for the known malignant hyperthermia gene *CACNA1S,* and yet very low for *RYR*1, were observed. The interpretation of the possible role of the gene variations

Table 2. Final List after Filtering for 29 Variants in 27 Genes in the 12 Individuals with Awareness with Recall

Chr	Start	Ref	Alt	Gene	Gene Number	Protein	dbSNP	ExAC/ gnomAD	RVIS	R	Is Variant in ClinVar?	OMIM
1	18167498	G	A	CACNA1E	NM_001205293.1:c.6467G>A	p.(Arg2156His)	rs2480373	0.000465	3.34	6.61 (pLI=1)	No	NA
1	201029878	G	C	CACNA1S	NM_000069.2:c.3322C>G	p.(Gln1108Glu)	rs765685405	0.00002436	61.36	-0.97 (pLI=0)	Uncertain	MALIGNANT HYPERTHERMIA, SUSCEPTIBILITY TO, 5
1	201046056	C	T	CACNA1S	NM_000069.2:c.1819G>A	p.(Val607Ile)	rs377461013	0.0000528	61.36	-0.97 (pLI=0)	No	MALIGNANT HYPERTHERMIA, SUSCEPTIBILITY TO, 5
2	128083346	G	A	MAP3K2	NM_006609.4:c.635C>T	p.(Ser212Phe)	NA	0.0000041	19.17	0.93 (pLI=1)	No	NA
4	26483637	T	G	CCKAR	NM_000730.2:c.910A>C	p.(Asn304His)	rs201121639	0.000364	74.38	0.12 (pLI=0.02)	No	NA
4	104640318	C	G	TACR3	NM_001059.2:c.515G>C	p.(Ser172Thr)	rs201237591	0.00000416	31.78	1.06 (pLI=0)	No	HYPOGONADOTROPIC HYPOGONADISM
4	14624641	T	C	MGST2	NM_002413.4:c.262T>C	p.(Tyr88His)	NA	NA	63.43	-0.27 (pLI=0.06)	No	NA
5	159399049	C	CCGCCGCCGA	ADRA1B	NM_000679.3:c.1113_1114insCGCCGCCGA	p.(Arg378_Arg380dup)	rs764801657	0.000948	31.87	3.79 (pLI=0.18)	No	NA
7	31864488	G	A	PDE1C	NM_005020.2:c.1399C>T	p.(Arg467Cys)	rs765961590	0.000008132	49.05	1.14 (pLI=0)	No	NA
10	18827163	C	T	CACNB2	NM_201596.2:c.1357C>T	p.(Leu453Phe)	rs145638628	0.000209	73.81	0.36 (pLI=0)	Uncertain	Not specified
11	1580181	C	T	DUSP8	NM_004420.2:c.475G>A	p.(Val159Met)	rs747581475	0.0000244	N	4.06 (pLI=0.68)	No	NA
11	67432827	C	T	ALDH3B2	NM_000695.3:c.635G>A	p.(Arg212Gln)	rs61736819	0.0005779	99.55	0.23 (pLI=0)	No	NA
11	113813713	C	T	HTR3B	NM_006028.4:c.706C>T	p.(Arg236Cys)	rs150117061	0.0000434	91.61	-0.51 (pLI=0)	No	NA
12	2797785	G	A	CACNA1C	NM_001129827.1:c.6101G>A	p.(Ser2034Asn)	rs755280013	0.0000325	3.51	6.41 (pLI=1)	Uncertain	Long QT syndrome
12	49221405	C	G	CACNB3	NM_000725.3:c.1178C>G	NM_000725.3:p. (Ser393Cys)	rs749648436	0.0000181	23.44	1.71 (pLI=0.05)	No	NA
12	121603187	C	G	P2RX7	NM_002562.5:c.561C>G	p.(Asn187Lys)	rs112631221	0.0000541	95.98	0.34 (pLI=0)	No	LEUKEMIA, CHRONIC LYMPHOCYTIC
14	70634202	C	T	SLC8A3	NM_033262.4:c.938G>A	p.(Arg313His)	rs149478505	0.0000397	7.02	0 (pLI=0.62)	No	NA
15	42442027	G	A	PLA2G4F	NM_213600.3:c.943C>G	p.(Arg315Gly)	rs144191503	0.0001	92.74	-1.71 (pLI=0)	No	NA
16	1252303	C	T	CACNA1H	NM_021098.2:c.1853C>T	p.(Pro618Leu)	rs60734921	0.000541	25.99	-2.59 (pLI=0.76)	Uncertain	EPILEPSY, CHILDHOOD ABSENCE, SUSCEPTIBILITY TO, 6
16	4770329	C	G	PHKB	NM_000293.2:c.2593C>G	p.(Pro865Ala)	rs142281844	0.000195	35.71	-0.55 (pLI=0)	No	NA
17	3593422	C	T	P2RX5	NM_002561.3:c.556G>A	p.(Glu186Lys)	rs147009070	0.00052	78.48	-0.22 (pLI=0)	No	NA
17	3806566	-	G	P2RX1	NM_002558.3:c.676_677insC	p.(Leu226Profs*23)	rs758752485	0.0000108	41.88	1.06 (pLI=0)	No	NA
17	3844399	G	A	ATP2A3	NM_005173.3:c.1966C>T	p.(Arg656Cys)	rs140404080	0.000663	5.77	3.13 (pLI=0.06)	No	NA
19	4171445	C	T	CREB3L3	NM_001271997.1:c.934C>G	p.(Arg312*)	rs143545033	0.000743	83.25	-0.55 (pLI=0)	No	NA
19	13318222	A	G	CACNA1A	NM_001127222.1:c.7426T>C	p.(Tyr2476His)	rs779631503	0.0000795	1.68	7.23 (pLI=1)	No	SPINOCEREBELLAR ATAXIA 6
19	13319691	-	GGT	CACNA1A	NM_001127222.1:c.6658_6659insACC	p.(His2219dup)	rs768950814	0.00828	1.68	7.23 (pLI=1)	Uncertain	SPINOCEREBELLAR ATAXIA 6
19	38995965	C	T	RYR1	NM_000540.2:c.8327C>T	p.(Ser2776Phe)	rs147707463	0.000712	0.03	4.44 (pLI=0)	Uncertain	MINICORE MYOPATHY WITH EXTERNAL OPHTHALMOPLEGIA
20	9401989	G	A	PLCB4	NM_000933.3:c.2164C>A	p.(Val722Ile)	rs772944610	0.00000406	25.88	2.78 (pLI=0.27)	No	NA
X	83372466	AAACA	-	RPS6KA6	NM_014496.4:c.790-16_790-12delTGTTT	NA	rs778822899	0.000181	16.12	1.7 (pLI=0.74)	No	NA

Alt, alternative nucleotide in the variant; chr, chromosome; dbSNP, reference number of the variant; ExAC.gnomAD, minor allele frequency in ExAC (Exome Aggregation Consortium) database; OMIM, Online Mendelian Inheritance in Man; ref, reference nucleotide; R, ExAC missense constraint; RVIS, Residual Variation Intolerance Score. 0, no missense constraint; –ve is more variation than expected; +ve is less variation than expected; pLI near 1, LoF constraint.

beyond those already implicated through clinical databases, such as ClinVar, is difficult.

Limitations

This study is of a preliminary nature, primarily to explore methodologic issues and generate hypotheses. There are two important limitations to acknowledge. The first is the question of whether our filtering processes were over restrictive. Because we filtered the genes that were analyzed according to the gene variant frequency and known association with likely anesthesia mechanisms, we have therefore precluded discovering unexpected genes, whose function lies outside our present understanding of brain function. Our approach was primarily driven by the fact that there is a growing recognition that unfiltered genome-wide analyses result in too many false positives—especially with such small sample sizes. We refer readers to an excellent review paper on the problems of rare variant association studies by Bomba *et al.*[29] They highlight the fact that false positives arise both because of incorrect statistical assumptions and bias, as well as heterogeneity in allelic estimation.

Successful implementation of genetic analysis is clearly a balance between overrestrictive filtering *versus* excessive false positive results. A minimum plausible filter would be that the gene variant: was expressed in the brain; caused a change in the protein (non-synonymous, or copy-number variants); was exonic; and was not very common (perhaps less than 1% if the variant had poor penetrance). The analysis of the resultant set of hundreds, or thousands, of genetic variants would probably produce a false positive result; even for a paucigenic Mendelian pattern with our small data set. If we had the resources for analyzing a large data set, this reduced filtering approach might accurately detect a paucigenic pattern. However, our study suggests that awareness with recall may represent a disorder with a substantial polygenic contribution, as observed in late onset diabetes. So there will be substantial difficulties in teasing out the complex web of gene-expression-protein interactions, even with a large dataset.

The other limitation of this study is the accuracy of determining the phenotype from a retrospective study design. awareness with recall may occur as a result of delivering low concentrations of anesthesia either intentionally if the patient cannot tolerate higher doses, or unintentionally as a result of error. In our selection we chose cases where it was thought highly likely that anesthesia should have been adequate, however without prospectively collecting detailed data this assumption can never be completely verified.

In conclusion, we hypothesize that a number of genetic variants found in our sample of awareness with recall patients—in particular those related to calcium signaling and purinergic pathways—could be associated with awareness with recall and should be considered as putative targets in future prospective studies into awareness with recall. To this end a collaborative international anesthesiology database would be a useful tool to start collecting rare variants of interest for anesthesia.

Competing Interests

The authors declare no competing interests.

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ANESTHESIOLOGY

An Assessment of Penetrance and Clinical Expression of Malignant Hyperthermia in Individuals Carrying Diagnostic Ryanodine Receptor 1 Gene Mutations

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Malignant hyperthermia is a rare life-threatening disorder triggered in genetically predisposed individuals by exposure to certain anesthetics
- The ryanodine receptor 1 (*RYR1*) gene, which encodes the Ca2+ release channel of skeletal muscle sarcoplasmic reticulum, is the major malignant hyperthermia-associated locus
- Malignant hyperthermia diagnostic mutations are more prevalent than the reported incidence of clinical malignant hyperthermia episodes because many mutation carriers are never exposed to anesthetic triggers and some may have several uneventful anesthetics before developing malignant hyperthermia reaction

What This Article Tells Us That Is New

- In a multicenter case-control study of 229 genotype-positive subjects with previous recorded exposure to trigger anesthetics, there were 93 malignant hyperthermia cases, for an overall penetrance for the analyzed *RYR1* mutations of 40.6%
- The probability of developing malignant hyperthermia on exposure to triggers was 0.25 among all *RYR1* mutation carriers and 0.76 in survivors of malignant hyperthermia reactions (95% CI of the difference 0.41 to 0.59)
- Young age, male sex, and the use of succinylcholine were major nongenetic risk factors influencing expression of the *RYR1* mutations conferring malignant hyperthermia susceptibility

ABSTRACT

Background: Malignant hyperthermia (MH) is a potentially lethal disorder triggered by certain anesthetics. Mutations in the ryanodine receptor 1 (*RYR1*) gene account for about half of MH cases. Discordance between the low incidence of MH and a high prevalence of mutations has been attributed to incomplete penetrance, which has not been quantified yet. The authors aimed to examine penetrance of MH-diagnostic *RYR1* mutations and the likelihood of mutation carriers to develop MH, and to identify factors affecting severity of MH clinical expression.

Methods: In this multicenter case–control study, data from 125 MH pedigrees between 1994 and 2017 were collected from four European registries and one Canadian registry. Probands (survivors of MH reaction) and their relatives with at least one exposure to anesthetic triggers, carrying one diagnostic *RYR1* mutation, were included. Penetrance (percentage of probands among all genotype-positive) and the probability of a mutation carrier to develop MH were obtained. MH onset time and Clinical Grading Scale score were used to assess MH reaction severity.

Results: The overall penetrance of nine *RYR1* diagnostic mutations was 40.6% (93 of 229), without statistical differences among mutations. Likelihood to develop MH on exposure to triggers was 0.25 among all *RYR1* mutation carriers, and 0.76 in probands (95% CI of the difference 0.41 to 0.59). Penetrance in males was significantly higher than in females (50% [62 of 124] *vs.* 29.7% [30 of 101]; *P* = 0.002). Males had increased odds of developing MH (odds ratio, 2.37; 95% CI, 1.36 to 4.12) despite similar levels of exposure to trigger anesthetics. Proband's median age was 12 yr (interquartile range 6 to 32.5).

Conclusions: Nine MH-diagnostic *RYR1* mutations have sex-dependent incomplete penetrance, whereas MH clinical expression is influenced by patient's age and the type of anesthetic. Our quantitative evaluation of MH penetrance reinforces the notion that a previous uneventful anesthetic does not preclude the possibility of developing MH.

(*ANESTHESIOLOGY* 2019; 131:983–91)

Malignant hyperthermia (MH) is a rare life-threatening disorder caused by dysregulation of intracellular calcium homeostasis in skeletal muscle and triggered by exposure to certain anesthetics in genetically predisposed individuals.[1] A progressively better understanding of the pathomechanism of MH, advances in anesthesia monitoring, and the introduction of dantrolene have been crucial in reducing MH mortality, which remains around 10%.[2]

Variants in ryanodine receptor 1 (*RYR1*),[3] calcium voltage-gated channel subunit alpha1 S (*CACNA1S*),[4–6] and in SH3 and cysteine rich domain 3 (*STAC3*) genes[7] are associated with MH. The *RYR1* gene—encoding the Ca²⁺ release channel of skeletal muscle sarcoplasmic reticulum (RyR1)—is the major MH-associated locus, involved in more than half of MH cases, whereas variants in *CACNA1S* and *STAC3* account for less than 1%. At present, 48 *RYR1* and 2 *CACNA1S* variants are

recognized as MH-causative mutations.[8] Recent availability of data on thousands of human exomes[9] has allowed to determine the true combined prevalence of all known MH-causative mutations as 1:2,750,[10] which is close to earlier estimates.[11–13] The prevalence of MH diagnostic mutations is considerably greater than the reported incidence of clinical MH episodes (1:35,000 to 1:68,000 surgical discharges).[2] This striking discrepancy can be attributable to the fact that many mutation carriers may never be exposed to anesthetic triggers.[14] The discrepancy may also reflect a reduced—or incomplete—penetrance of the MH trait.[10,11,15] Indeed, not all subjects carrying a causative mutation develop MH on first exposure to anesthesia, and some may have several uneventful anesthetics before developing MH in the operating room.[16,17] In addition, the onset, progression, and severity of MH reaction are variable. The time of onset of MH seems to be influenced by the type and dose of volatile anesthetic, whereas severity is also dependent on the duration of exposure.[18–20]

Although our knowledge of factors influencing MH penetrance is limited, it is known that MH penetrance may depend on the additive effect of more than one genetic factor,[21] and allele-specific differences in RyR1 mRNA expression levels may explain the observed reduced penetrance and variations in MH phenotype among individuals.[22] Several studies[23–26] indicate higher incidence of MH in younger males, but the reason for that remains unclear.

Challenged by a paucity of clinically affected individuals with variable phenotype and known genotype, quantification of the MH trait penetrance remains an elusive subject, albeit being essential for an optimal risk assessment at the time of genetic counseling. In this study, we analyzed the available clinical and genetic data on 125 European and North American MH families collected at MH centers in Europe and Canada. We hypothesized that the penetrance of diagnostic RYR1 mutations is incomplete, and that reduced penetrance and MH clinical expression are genotype-specific. Therefore, our objectives were to examine the penetrance of RYR1 diagnostic mutations and the likelihood of RYR1 mutation carriers to develop MH, and to identify factors that may affect MH clinical expression.

Materials and Methods

After research ethics board approval and institutional authorization, for this multicenter case–control study, we collected clinical and genetic data on MH susceptible individuals from families with a positive history of MH reaction from the registries of three German, one Belgian, and one Canadian MH diagnostic centers between January 1, 1994 and December 31, 2017. Because of the retrospective nature of the study, consent was waived by the ethics board of all the participating centers.

Selection Process and Data Collection

Probands (survivors of MH reactions) and their relatives (family members who carried a familial RYR1 mutation and had one or more uneventful exposures to anesthetic triggers) were included in the study provided that: (1) they carried only a single MH diagnostic RYR1 mutation and had no additional potentially pathogenic RYR1 variants; (2) data on two or more probands sharing a RYR1 mutation were available; (3) three or more relatives (excluding the probands) shared a RYR1 mutation and each had a documented history of at least one uneventful exposure to MH triggers (fig. 1).

RYR1 mutation refers to nonsynonymous variants that have been functionally validated and included in the MH diagnostic mutations list of the European Malignant Hyperthermia Group.[8] When referring to specific RYR1 mutations we describe the inferred amino acid change at the protein level using the Human Genome Variation Society recommendations for the description of sequence variants.[27]

Data collected on probands included RYR1 genotype, number of exposures (i.e., number of surgeries under general anesthesia with inhalational agents or succinylcholine, including the one during which the MH crisis occurred), trigger agent(s) used, sex, age at the time of the MH reaction, Clinical Grading Scale[28] scores, and onset time defined as the period from the start of the trigger anesthetic to first sign of MH on record. Data were extracted from the anesthetic records, where available, or otherwise from the MH center records.

Available data on relatives of the probands included RYR1 genotype, sex, and number of exposures (i.e., uneventful anesthetics with trigger agents). The relatives' ages at the time of triggered anesthetics were not extracted because they were not available for all.

Penetrance[29] of an MH causative RYR1 mutation was defined as the percentage of probands among all carriers of the mutation who had been exposed to general anesthesia:

$$Penetrance = \frac{n_{probands}}{\left(n_{relatives} + n_{probands}\right)} \times 100$$

$n_{probands}$ and $n_{relatives}$ refer to the number of probands and of relatives, respectively. Because of the rarity of MH, penetrance
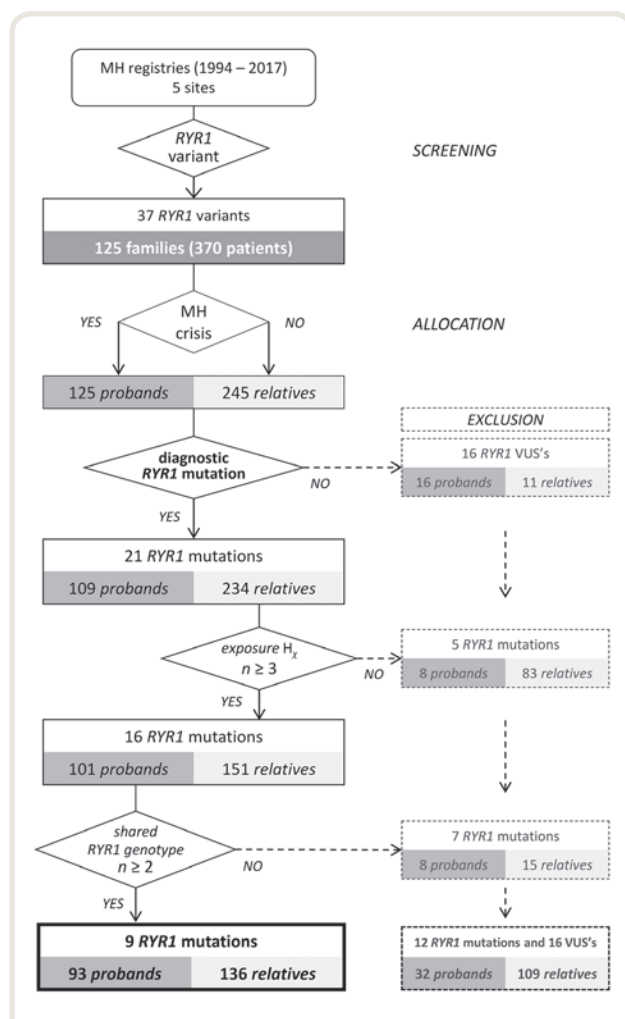
Fig. 1. The five diamonds on the flowchart contain the criteria used in this research for screening, allocation and inclusion/exclusion according to: (1) variant pathogenicity as per the list of diagnostic malignant hyperthermia (MH) mutations from the European Malignant Hyperthermia Group; (2) history (H$_x$) of uneventful exposure to trigger anesthetics in relatives bearing a given mutation; (3) available data from at least two probands sharing a ryanodine receptor 1 (*RYR1*) mutation. VUSs, variants of unknown significance.

in this study is a byproduct of pooled pedigrees with a shared *RYR1* mutation and therefore it is subject to the influence of different familial genetic backgrounds.

MH susceptible individuals may have several uneventful exposures to triggering anesthetics before developing an MH reaction. We assessed the probability to develop MH on exposure to triggers in carriers of a *RYR1* mutation both in probands ($P_{\text{MH probands}}$) and in the entire cohort ($P_{\text{MH all}}$), respectively, as follows:

$$P_{\text{MH probands}} = \frac{n_{probands}}{Exp_{probands}}$$

$$P_{\text{MH all}} = \frac{n_{probands}}{\left(Exp_{probands} + Exp_{relatives}\right)}$$

Here, $n_{probands}$ is the number of probands, which in this study is equal to the number of MH reactions, and $Exp_{(x)}$ is the number of exposures to triggers in probands or in relatives, as specified.[30,31]

Clinical Grading Scale scores and MH onset time were used as indices of MH phenotype severity for comparison of the different *RYR1* genotypes.

Statistical Analysis

No statistical power calculation was conducted before the study, so the sample size was based on the available data. Hypothesis testing was two-tailed. Normality of the different variables was graphically assessed by histograms or by the Kolmogorov–Smirnov test for normality. Mean and SD were used to describe normally distributed data, whereas median and interquartile range were used for skewed variables. Chi-square test was used to look for associations between the phenotype groups (*probands* or *relatives*) and the *RYR1* genotype, the number of exposures to anesthetic triggers, and sex. Pairwise comparisons of penetrance and P_{MH} across the *RYR1* mutations were performed using *Z*-test with Bonferroni's correction to decrease the likelihood of committing type 1 error ($P < [\alpha / C_2^9] \rightarrow [0.05/36] = 0.0014$). Nonparametric between-subjects one-way ANOVA was used to assess differences in MH phenotype severity. Spearman's coefficient was used to explore the correlation between proband's age and each indicator of clinical MH severity (*i.e.*, Clinical Grading Scale score and MH onset time). Only this latter analysis was data-driven (*post hoc*), whereas all the former were done in accordance to our original statistical plan. $P < 0.05$ was considered significant, unless otherwise specified. The software used for statistical analysis was SAS Studio (Enterprise Edition, version 3.7; SAS Institute Inc., USA).

Results

An initial screening of the four European and one Canadian MH registries revealed 125 unrelated MH pedigrees with 370 individuals carrying 37 different potentially pathogenic *RYR1* variants. After applying the inclusion criteria (see Materials and Methods), 229 subjects from 93 MH pedigrees (93 probands and 136 relatives) carrying nine MH diagnostic mutations were included in this study (fig. 1). Among those excluded, there were eight families with more than one potentially pathogenic *RYR1* variant. Data were extracted from anesthetic records in 76 probands and from the referring anesthesiologists' reports in the rest of the study sample.

The selected *RYR1* mutation carriers had in total 365 exposures to anesthesia with MH triggers, of which 93

Table 1. Penetrance and Likelihood to Develop MH, by *RYR1* Genotype

RYR1 Mutation	Relatives		Probands		Penetrance (%)	MH Probability (P_{MH})	
	n	*#Exp*	*n*	*#Exp*		$P_{MH\ all}$	$P_{MH\ probands}$
p.Gly341Arg	18	25	11	17	37.9	0.26	0.65
p.Arg614Cys	33	64	30	39	47.6	0.29	0.77
p.Arg614Leu	3	5	5	6	**62.5**	**0.45**	0.83
p.Thr2206Met	36	57	18	19	33.3	0.24	**0.95**
p.Arg2336His	5	12	3	5	37.5	0.18	**0.60**
p.Ala2350Thr	4	9	3	4	42.9	0.23	0.75
p.Gly2375Ala	7	11	4	5	36.4	0.25	0.80
p.Gly2434Arg	9	17	12	18	57.1	0.34	0.67
p.Arg2454His	21	43	7	9	**25**	**0.13**	0.78
Overall	136	243	93	122	40.6 ± 0.12	0.25 ± 0.09*	0.76 ± 0.11
Exposures	2 [1–2]*		1 [1-1]				

Overall penetrance and P_{MH} expressed as mean ± SD; exposures expressed as median [interquartile range]. Penetrance = $n_{probands}$ / ($n_{relatives}$ + $n_{probands}$) × 100; $P_{MH\ probands}$ = $n_{probands}$ / $\#Exp_{probands}$; $P_{MH\ all}$ = $n_{probands}$ / ($\#Exp_{probands}$ + $\#Exp_{relatives}$). #Exp, number of exposures (*i.e.*, general anesthetics received with trigger agents); MH, malignant hyperthermia; *RYR1*, ryanodine receptor 1. Range (highest and lowest) values are showed in boldface, *P* = 0.047, 0.054 and 0.038 for penetrance, $P_{MH\ all}$ and $P_{MH\ probands}$ range values, respectively.

*P < 0.001.

resulted in MH reactions (table 1). The median exposure to MH triggers was higher in relatives than in probands (2 *vs.* 1 exposures per subject, *P* < 0.0001; table 1). Among probands, 79.6% (74 of 93) developed an MH reaction during the first anesthetic, 17.2% (16 of 93) during the second, and 3.2% (3 of 93) after more than two exposures to MH triggers (fig. 2).
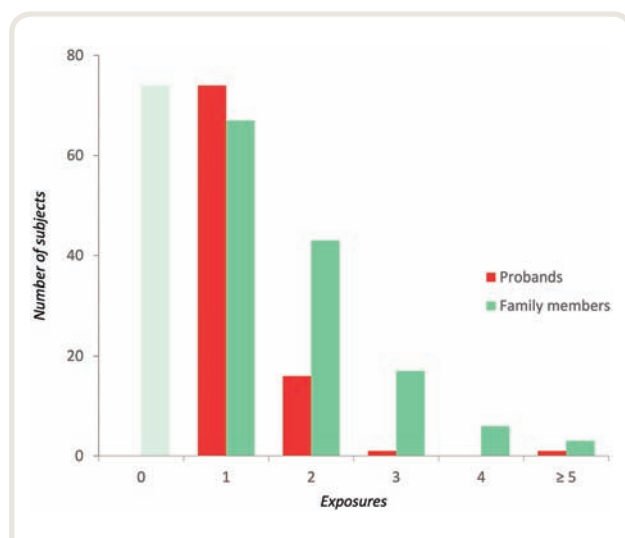
Fig. 2. Exposures to general anesthetics with trigger agents in carriers of ryanodine receptor 1 mutations. On the vertical and horizontal axes are represented the number of subjects exposed to trigger anesthetics and the total number of exposures, respectively. Exposures in probands (*red*) include the general anesthetic during which the actual malignant hyperthermia crisis occurred, whereas in family members (*green*) they comprise the total number of uneventful anesthetics with trigger agents; family members with no anesthetic history (*shaded green bar*) were excluded from analysis.

Penetrance

RYR1 Genotype. The MH-diagnostic *RYR1* mutations harbored by the study participants included three amino-terminal mutations (p.Gly341Arg, p.Arg614Cys, and p.Arg614Leu) and six mutations within the central RyR1 region (p.Thr2206Met, p.Arg2336His, p.Ala2350Thr, p.Gly2375Ala, p.Gly2434Arg, and p.Arg2454His).

There were no missing data for genotype, neither in probands nor in relatives. There were 93 MH cases among 229 genotype-positive subjects with previous recorded exposure to trigger anesthetics; that yields an overall penetrance for the analyzed *RYR1* mutations of 40.6% (95% CI, 34.3 to 47.3%). Notably, levels of penetrance were not significantly different (*P* = 0.303) among the analyzed *RYR1* mutations (table 1). Even the difference between mutations with the highest and lowest penetrance did not reach statistical significance (62.5% [5 of 8] for p.Arg614Leu, and 25% [7 of 28] for p.Arg2454His, respectively; *P* = 0.047, whereas *P* < 0.0014 was required after Bonferroni's correction for 36 pairwise comparisons).

The overall probability that a carrier of any of the nine *RYR1* diagnostic mutations will develop MH on exposure to triggers, $P_{MH\ all}$, was 0.25 (95% CI, 0.21 to 0.30). $P_{MH\ all}$ ranged from 0.45 to 0.13 for the same aforementioned pair of mutations (*P* = 0.054). However, if only probands were considered, the probability of developing MH ($P_{MH\ probands}$) increased to 0.76 (95% CI, 0.67 to 0.83; table 1).

Demographic Factors and MH Triggers. There was a significant association between sex and phenotype: 67.4% (62 of 92) probands were males, whereas among relatives males comprised 46.6% (62 of 133; *P* = 0.002; table 2). One proband and three relatives did not have data for sex and were excluded from the analysis.

Table 2. Sex Distribution by Phenotype Group and *RYR1* Genotype (Upper Section) and Penetrance, Likelihood to Develop MH, and Exposure Rate by Sex (Lower Section)

RYR1 mutation	Males		Females	
	Proband	Relative	Proband	Relative
p.Gly341Arg	8	6	3	11
p.Arg614Cys	18	15	12	18
p.Arg614Leu	3	2	2	1
p.Thr2206Met	11	19	6	17
p.Arg2336His	2	2	1	3
p.Ala2350Thr	3	2	0	2
p.Gly2375Ala	4	3	0	4
p.Gly2434Arg	9	4	3	5
p.Arg2454His	4	9	3	10
N	*62*	*62*	*30*	*71*
# Exposures	87	110	34	126
Penetrance	50 ± 0.11*		29.7 ± 0.18	
Exposures	1 [1-1]		1 [1-1]	
$P_{MH\text{-}all}$	0.31 ± 0.09		0.19 ± 0.12	
$P_{MH\text{-}probands}$	0.71 ± 0.16		0.88 ± 0.14	

Sex data were missing in one proband and three relatives. Penetrance and P_{MH} are expressed as mean ± SD; *Exposures* as median [interquartile range]. MH, malignant hyperthermia; *RYR1*, ryanodine receptor 1.

*$P < 0.01$.

The overall penetrance of the MH trait was significantly higher in males compared with females (50% [62 of 124] *vs.* 29.7% [30 of 101]; 95% CI of the difference 7 to 32%, $P = 0.002$]. Moreover, males had increased odds of developing MH compared with females (odds ratio, 2.37; 95% CI, 1.36 to 4.12) despite similar levels of exposure to trigger anesthetics for both sexes, with an overall rate of 1.6 exposures per subject (table 2).

Age distribution in probands was positively skewed (fig. 3), with a median age of 12 yr and an overwhelming
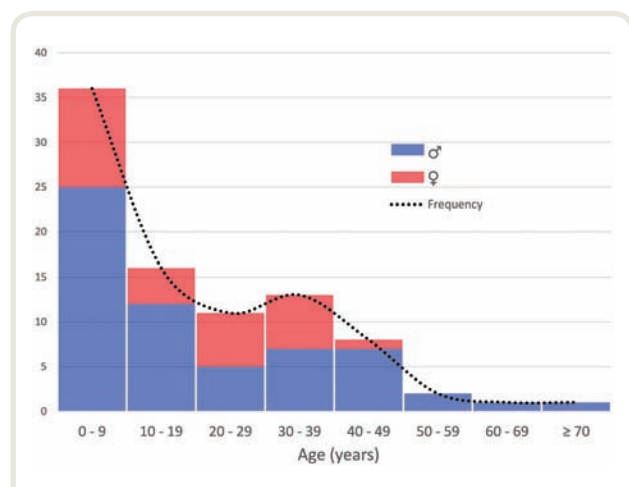
Fig. 3. Age distribution at the time of malignant hyperthermia (MH) crisis by sex, in MH probands carrying ryanodine receptor 1 mutations.

majority being younger than 33 yr old (interquartile range 6 to 32.5; table 3). Age was missing for five probands, who were excluded from the MH phenotype severity analysis.

There were no missing data regarding the anesthetic triggers used in probands. Succinylcholine was used in 76.3% (71 of 93) MH cases, either in combination with volatiles (71%, 66 of 93) or alone (5.4%, 5 of 93), whereas volatile agents were administered without succinylcholine in 23.7% (22 of 93) cases. Succinylcholine use did not differ significantly in male (77.4%, 48 of 62) *versus* female probands (73.3%, 22 of 30; $P = 0.667$).

MH Phenotype Severity

We used Clinical Grading Scale scores and MH onset time as quantitative indicators of MH phenotype severity. The median time to MH onset was 10 min (interquartile range 5 to 60) after exposure to trigger anesthetics, whereas the mean Clinical Grading Scale score was 47.9 (SD: 17.1). There was no significant association between the *RYR1* genotypes and either indicator of MH phenotype severity (table 3).

Both Clinical Grading Scale score and MH onset time positively correlated with age (Spearman's correlation coefficient 0.31, $P = 0.003$; and 0.39, $P = 0.001$, respectively), which implies that MH reactions in older patients were identified later and were more severe as per Clinical Grading Scale score (fig. 4).

Table 3. Proband's Age at MH Crisis and Clinical MH Indices as per *RYR1* Mutation

RYR1 Mutation	Age (yr)	Onset t (min)	CGS
p.Gly341Arg	23 [14–34]	25 [8–45]	55 ± 9
p.Arg614Cys	8 [6–32]	5 [2–5]	47 ± 21
p.Arg614Leu	12 [6–23]	2 [2–11]	46 ± 10
p.Thr2206Met	11 [5–25]	10 [5–79]	48 ± 16
p.Arg2336His	9.5 [7.75–11.25]	10 —	46 ± 11
p.Ala2350Thr	31.5 [23.75–39.25]	60 —	33
p.Gly2375Ala	41.5 [28.5–49.5]	33 [19–46]	50 ± 15
p.Gly2434Arg	6 [5.5–28.5]	12 [10–80]	46 ± 20
p.Arg2454His	13 [8.5–26]	98 [56–161]	45 ± 20
Overall	12 [6–32.5]	10 [5–60]	50 ± 17

Age and MH onset time are shown as median [interquartile range]; Clinical Grading Scale (CGS) score is expressed as mean ± SD. MH, malignant hyperthermia; *RYR1*, ryanodine receptor 1.

Fig. 4. Scatter plot showing a positive correlation between age and (*A*) the malignant hyperthermia (MH) Clinical Grading Score, or (*B*) the MH crisis onset time.
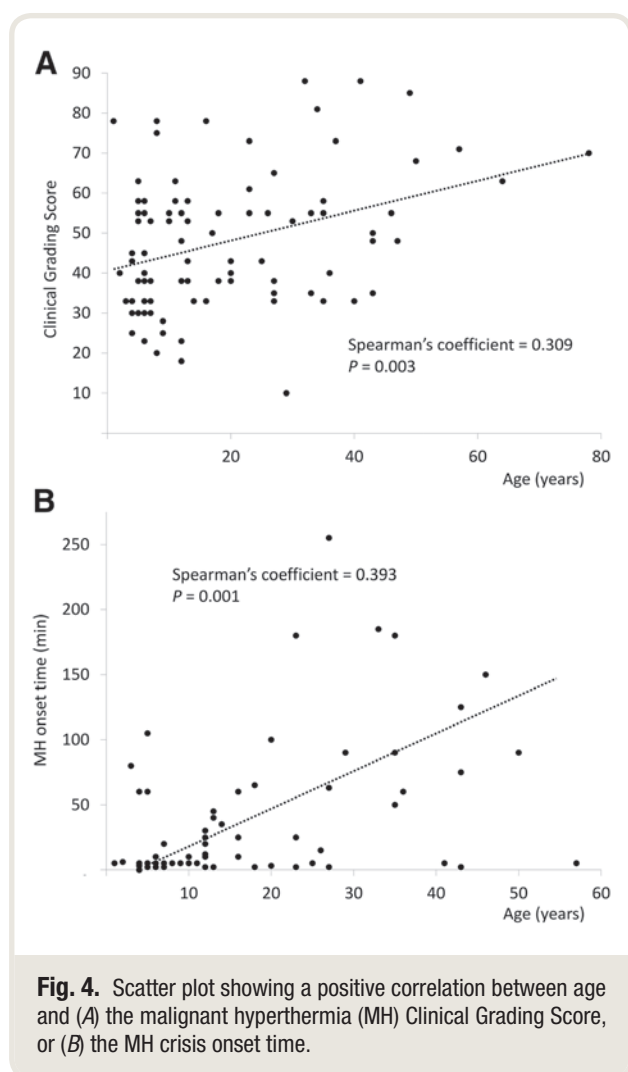
Discussion

This multicenter case–control study aimed to assess the penetrance of MH diagnostic *RYR1* mutations and examine factors that influence their expression. MH is a rare genetic syndrome with an incidence of 1:35,000 to 1:68,000 MH crises per all surgical discharges,[2,32] which makes it challenging for a single MH research center to amass sufficient data for a comprehensive penetrance study. Therefore, we combined clinical and genetic data on 125 European and Canadian MH families.

We quantified the penetrance of nine MH diagnostic mutations to be around 40%. We found that probands' likelihood to develop MH on exposure to triggers is higher compared with other *RYR1* mutation carriers. In fact, most probands in our sample developed MH during their first exposure to general anesthesia. We also found higher penetrance in males, despite similar exposures to triggers in both sexes.

Reduced penetrance is a phenomenon that blurs the distinction between genetically complex disorders and monogenic conditions with Mendelian inheritance, resulting from the interaction of multiple genetic and nongenetic factors that hamper establishing straightforward causation from known genotypes to specific phenotypes.[33] Factors influencing the penetrance of a genetic trait include the degree of dysfunction caused by a specific mutation(s), the modulating influence of additional variants on allelic expression, interindividual variations in gene expression, allele dosage causing homozygotes or compound-heterozygotes to have more severe phenotype, age- and sex-specific epigenetic changes such as genomic imprinting leading to the mutually exclusive expression of either the maternal or the paternal allele, and environmental influences such as the diet, alcohol intake, drugs, body habitus, and comorbid disease states. Any of these factors may either ameliorate or exacerbate the impact of the underlying genetic predisposition.[29]

Concerning MH, although a possible role of allele silencing in relation to other MH loci has not been explored yet, monoallelic silencing does not seem to affect the penetrance of MH–associated *RYR1* mutations,[34] and the role of allele-specific differences in expression levels of RyR1 transcript remains to be elucidated.[22]

Our study may lack the necessary power to achieve statistical significance regarding the variation in penetrance among the analyzed *RYR1* mutations. However this should not be interpreted as lack of differences in severity of phenotypes. Comparison of *in vivo* and *in vitro* in knock-in mouse models of MH demonstrate differences among the p.G2435R,[35] p.R163C,[36] and p.T4826I variants.[37]

In a large retrospective study by Carpenter *et al.*,[38] different *RYR1* genotypes in a group of MH probands were associated with magnitude of contracture in the *in vitro* contracture test and serum creatine kinase concentration, and both parameters were also associated with the clinical phenotype severity defined as MH onset time. The functional data from animal models alongside the human data from Carpenter *et al.* may suggest that the observed differences in severity of phenotype and penetrance among different variants may actually be real.

The Clinical Grading Scale scoring system was conceived to estimate the likelihood that an observed adverse anesthetic outcome was attributable to MH.[28] However, because of its *post hoc* nature, Clinical Grading Scale reliability depends on the availability of clinical data. It may underestimate the likelihood of MH in cases where the crisis is promptly recognized and treated. Despite these, we deemed it reasonable to use Clinical Grading Scale as quantitative indicator of MH phenotype severity because it rates the importance of clinical variables that appear during an MH event by assigning them a score. The positive correlation of both Clinical Grading Scale scores and time of MH onset with the patient's age observed in this study may suggest that diagnosis tends to be more delayed with increasing age.

We also explored the influence of nongenetic factors, such as sex and age, on MH penetrance. In our pool of probands, there were at least two males for every female, but there was a slightly greater proportion of women among relatives (71 of 133, 53.3%). A bias from sex imbalance would not arise if our study groups were matched according to sex, but then no effect estimate for sex could be derived.

Sex differences pervade the literature on MH, from the earliest epidemiologic reports[23,24] to the most recent survey demonstrating that the prevalence of MH in male patients doubles that of females.[2] It was also shown that more males than females test positive on the diagnostic contracture test for MH.[26] Sex-dependent susceptibility to MH is also present in mouse models.[37] Recently, male sex and body build subjectively assessed as muscular have been reported as independent predictors of MH susceptibility.[39] However, whether a larger muscle mass is associated with MH sex discrepancy warrants objective assessment. On the whole, the pathomechanism leading to sex differences in the penetrance of MH is still unknown, but epigenetic *RYR1* allele silencing has been ruled out as a cause of reduced penetrance of MH susceptibility in females.[34]

Age distribution in MH probands is known to be positively-skewed with younger people being most affected.[1,40] Several studies found that children aged 15 or younger comprised more than 50% of all reactions.[1,41,42] Although the majority of cases described here involved also children younger than 15 yr old, our probands' median age of 12 yr is lower than previously reported.[40] This possibly reflects the difference in composition of the investigated cohorts, because the former study included mostly adult patients whose MH status were confirmed by muscle biopsy and contracture testing.

The contrast between the low penetrance of some *RYR1* mutations and the high likelihood of probands to develop MH ($P_{\text{MH probands}}$) strengthens the notion of a multifactorial origin of MH, where genetic predisposition is necessary but not sufficient to unleash the MH syndrome. Although co-inheritance of other genetic factors should be taken into account, nongenetic influences, such as the anesthetic technique, the type of surgery, and patient's comorbidities, may play a pivotal role. In fact, we observed that in our series succinylcholine was used in 76% of MH cases (71% along with volatile anesthetics), which is far above the 10% average use in a typical North American hospital nowadays.[43] Because a number of our cases date back to the 1990s, this may reflect the bygone routine use of succinylcholine. Despite lacking relevant data about the use of succinylcholine in the relatives of our probands, the observed bias in the drug use in probands could be explained by the higher occurrence of MH during emergency surgery where succinylcholine is commonly used. Although ear, nose, and throat procedures seem to be the most common reason for surgery in the majority of MH cases in children,[2,44] orthopedic surgery

and other emergent procedures are preponderant among adults.[29,41,45] Anecdotal reports of MH occurring during or shortly after emergent surgery for acute appendicitis are not rare in the medical literature.[46–54] It seems thus reasonable to suggest that a 10- to 20-fold increased risk of MH secondary to succinylcholine use[42,43] might not be solely attributable to the effects of the drug on the intracellular calcium dynamics of genetically predisposed patients, but also to a threshold shift induced by fever or a concomitant inflammatory process like sepsis or trauma, priming the onset of MH. We deem our observation worthy of further investigation.

There are limitations inherent to our study design. Data collection was retrospective and based on prevalent cases, carrying the risk of recall bias. Although the sample of patients was selected based on all available data, its composition may differ from that of the whole MH population because of participation and ascertainment bias (*e.g.*, identification of a causal mutation in a family preventing further exposures to MH triggers on its members, probands with weaker or abortive reactions remaining unrecognized, lower likelihood of asymptomatic relatives to be tested). We excluded patients with missing data and those with *RYR1* variants of unknown significance, which may have affected the overall power of the study. Because data from first- and second-degree relatives only were available for this study, penetrance in higher-order relatives remains an important investigation for the future.

In conclusion, the penetrance of nine MH-diagnostic *RYR1* mutations is incomplete and sex-dependent. Among genotype-positive subjects, probands have the highest risk of developing MH on exposure to trigger anesthetics. Young age, male sex, and the use of succinylcholine seem to be major nongenetic risk factors influencing expression of the *RYR1* genotypes conferring MH susceptibility in our cohort. Our quantitative evaluation of MH penetrance reinforces the notion that a previous uneventful anesthetic does not preclude the possibility of developing MH.

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ANESTHESIOLOGY

Accuracy of Physical Function Questions to Predict Moderate-Vigorous Physical Activity as Measured by Hip Accelerometry

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Functional capacity is thought to be an important part of preoperative assessment, but it is hard to assess without formal testing
- Standardized physical function questions might identify patients with adequate capacity (greater than or equal to 4 metabolic equivalents)

What This Article Tells Us That Is New

- Results from standardized physical function questions and hip accelerometers were compared in 522 participants
- Physical function questions were sensitive but nonspecific
- Other approaches to assessing physical functional status should be considered

ABSTRACT

Background: Functional capacity assessment is a core component of current perioperative cardiovascular evaluation and management guidelines for noncardiac surgery. The authors investigated the ability of standardized physical function questions to predict whether participants engaged in moderate physical activity as measured by hip accelerometers.

Methods: Participant responses to physical functioning questions and whether they engaged in moderate physical activity were extracted from the National Health and Nutrition Examination Survey (2003 to 2004 and 2005 to 2006). Physical activity intensity was measured using hip accelerometers. Adult participants with at least one Revised Cardiac Risk Index condition were included in the analysis. Standardized physical function questions were evaluated using a classification and regression tree analysis. Training and test datasets were randomly generated to create and test the analysis.

Results: Five hundred and twenty-two participants were asked the physical functioning questions and 378 of 522 (72.4%) had a bout of moderate-vigorous activity. Classification and regression tree analysis identified a "no difficulty" response to walking up 10 stairs and the ability to walk two to three blocks as the most sensitive questions to predict the presence of a 2-min bout of moderate activity. Participants with positive responses to both questions had a positive likelihood ratio of 3.7 and a posttest probability greater than 90% of a 2-min bout of moderate-vigorous activity. The sensitivity and specificity of positive responses to physical functioning questions in the pruned tree were 0.97 (95% CI, 0.94 to 0.98) and 0.16 (95% CI, 0.10 to 0.23) for training data, and 0.88 (95% CI, 0.75 to 0.96) and 0.10 (95% CI, 0.00 to 0.45) for the test data. Participants with at least one 2-min bout of moderate activity had a greater percentage of overall daily active time (35.4 ± 0.5 *vs.* 26.7 ± 1.2; $P = 0.001$) than those without.

Conclusions: Standardized physical function questions are highly sensitive but poorly specific to identify patients who achieve moderate physical activity. Additional strategies to evaluate functional capacity should be considered.

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Activity Status Index. The two physical function questions recommended by textbooks are the ability to walk up a flight of stairs or walk one to two blocks without symptomatic limitations.[4] Patients unable to achieve an activity level of 4 metabolic equivalents are at increased risk of adverse cardiac events.[5–13] Subjective functional capacity assessment thus impacts perioperative risk stratification and dictates whether patients should be considered for further cardiac testing before noncardiac surgery.

However, the ability of standardized individual physical activity questions to accurately assess functional capacity in patients with an increased risk of major adverse cardiac

Functional capacity as used in the preoperative assessment may be defined as the ability to perform submaximal physical activities during daily life, and it plays a core role in the current practice guidelines on perioperative cardiovascular evaluation and management of patients undergoing noncardiac surgery.[1–3] Functional capacity is subjectively assessed by asking patients their ability to perform activities requiring 4 or more metabolic equivalents or using a formalized questionnaire, such as the Duke

events is unclear. Previous work in nonoperative settings has observed that individuals frequently under- or overestimate their physical activity in response to physical function questions when compared to accelerometer-based physical activity measurements.[14] Self-reported physical activity tools are particularly poor among individuals with low routine activity levels.[15] Additionally, a 2018 multicenter trial found that subjective functional capacity assessment does not accurately predict adverse perioperative outcomes.[16]

Accelerometers offer a novel approach to measuring the intensity and duration of physical activity and validating responses to questions regarding physical function. Accelerometers can detect the presence of moderate-vigorous physical activity, which includes the 4 metabolic equivalents threshold.[14] The National Health and Nutrition Examination Survey is a nationally representative sample of the U.S. population that measured physical activity using a hip-worn accelerometer for 7 days during the 2003 to 2004 and 2005 to 2006 time period, and in addition, asked participants about their physical function.

Our study had two main aims. The first was to use the National Health and Nutrition Examination Survey to establish the accuracy of physical function questions to predict the presence of moderate-vigorous physical activity as assessed using accelerometers during 1 week of wear time. The second was to evaluate for differences in overall physical activity between participants who achieve moderate-vigorous physical activity and those who do not. We hypothesized that any single physical function question would not adequately distinguish between participants who routinely participate in moderate-vigorous physical activity and those who do not. Furthermore, we sought to determine if the presence of at least one bout of moderate-vigorous physical activity would identify significant differences in overall activity between these two groups.

Materials and Methods

National Health and Nutrition Examination Survey

National Health and Nutrition Examination Survey is a complex, multistage probability survey conducted by the National Center for Health Statistics designed to assess the health and nutritional status of the civilian noninstitutionalized U.S. population. The survey design has been detailed elsewhere.[17] Briefly, the survey includes participants of all ages and oversamples several under-studied groups including adults older than 70 yr of age, low-income white persons, and non-Hispanic black and Mexican-American persons. Trained interviewers administered a household interview and performed an on-site examination. Interview data included demographic, dietary, and health-related questions and the examination included medical and physiologic measurements. The National Health and Nutrition Examination Survey cycle for the 2003 to 2004 and 2005 to 2006 included a substudy that measured the daily physical

activity of the participants using hip-worn accelerometers. Only data from these two cycles were analyzed for the current study. The University of Chicago institutional review board (Chicago, Illinois) deemed this study exempt since it used a publicly accessible data source (IRB No. 18–1268).

Participants

Participants were included in the analysis if they had at least one Revised Cardiac Risk Index condition. Such participants are considered at increased risk (greater than or equal to 1%) of major adverse cardiac events if they have at least one positive Revised Cardiac Risk Index condition during intrathoracic, intraabdominal, or suprainguinal vascular surgery (high-risk surgery) according to current guidelines.[3] Conditions were identified using questionnaire and laboratory data from the National Health and Nutrition Examination Survey and included diabetes requiring insulin (Diabetes Questionnaire 050: "[Are you] Taking insulin now?"), congestive heart failure (Medical Condition Questionnaire 160B "[Have you] Ever [been] told you have congestive heart failure?"), coronary artery disease (Medical Condition Questionnaire 160C "[Have you] Ever [been] told you have coronary artery disease?"; Medical Condition Questionnaire 160D "[Have you] Ever [been] told you had angina/angina pectoris?"; Medical Condition Questionnaire 160E "[Have you] Ever [been] told you had a heart attack?"), cerebrovascular disease (Medical Condition Questionnaire 160F "[Have you] Ever [been] told you had a stroke?"), and chronic kidney disease (serum creatinine greater than 2.0 mg/dl or Kidney Conditions Question 025 "[Have you] Received dialysis in [the] past 12 months?").[18] The serum creatinine from the 2005 to 2006 National Health and Nutrition Examination Survey cycle was recalibrated according to the analytic notes provided to ensure comparability with standard creatinine values.[19]

Functional Capacity

Questions from the physical functioning questionnaire were used to evaluate self-reported functional capacity. The physical functioning questionnaire (PFQ) was not presented to all participants in the National Health and Nutrition Examination Survey study and these participants were excluded from the current study. Participants under the age of 59 who did not require any special equipment to walk (*e.g.,* walker or cane), had no limitations keeping them from working, or experienced no confusion or memory problems were assumed to have no functional capacity limitations. Physical functioning questions were included in the analysis based on the likelihood that they would be able to discriminate participants based on activity levels they could achieve.

The questions included were: (1) "[Do you have] difficulty walking for a quarter mile/2 to 3 blocks?" (PFQ061B); (2) "[Do you have difficulty] walking up 10 stairs?" (PFQ061C); (3) "[Do you have] lifting or carrying difficulty?" (PFQ061E);

(4) "[Do you have] house chore difficulty?" (PFQ061F); (5) "[Do you have] preparing meals difficulty?" (PFQ061G); and (6) "[Do you have difficulty] walking between rooms on [the] same floor?" (PFQ061H). Responses to the physical function questions were standardized and included: "No difficulty," "Some difficulty," "Much difficulty," "Unable to do," "Do not do this activity," "Refused," "Don't know," or "Missing." In the sample used for the current study, no responses included "Refused" or "Don't know" and all physical function question data were present.[18]

Accelerometry Measures

The National Health and Nutrition Examination Survey used a uniaxial accelerometer (ActiGraph AM-7164; ActiGraph, USA) to measure physical activity among participants older than the age of 6.[20,21] Participants randomly assigned to this substudy were told to wear the monitor at home for 7 consecutive days. Participants who used wheelchairs or had other impairments that prevented walking or wearing the physical activity monitor device were excluded from the substudy. The monitor was placed on an elasticized fabric belt that was worn on the right hip. The device was not water resistant and was removed during bathing/water activities, preventing capture of these activities. Additionally, subjects were instructed to remove the monitor at bedtime and thus, this time period was not captured.

The monitors were programed to start recording at 12:01 AM the day after the participant's health examination. Data from the National Health and Nutrition Examination Survey was obtained using the nhanesdata package and processed using the nhanesaccel package.[22] Activity was summarized into unitless counts per 1-min epoch and processed using the R package nhanesaccel. Non–wear time was defined as any interval 60 min or longer in which all count values were 0. Consistent with previous studies, monitoring days with more than 600 min (more than 10 h) of wear time were considered valid for analysis.[23] At least 4 valid days of wear time were required to be considered a representative characterization of the participant's activity.[23]

The primary outcome measure was the presence of at least one 2-min bout of moderate-vigorous physical activity during the 7-day accelerometer wear time. A 2-min bout was selected because a patient walking at a steady 4-mph pace should take 2 min to travel two blocks. Further, the activity intensity of moderate-vigorous physical activity includes the 4 metabolic equivalent cutoff recommended by the current perioperative practice guidelines.[3,14] Thus, a 2-min bout was deemed the lowest threshold of sustained moderate-vigorous physical activity to meet the guidelines requirements for adequate functional capacity.[3] Activity intensity was identified using predefined cut-points that have been previously validated in the National Health and Nutrition Examination Survey data.[14] Activity intensities were defined as moderate-vigorous (greater than 2,020 counts per min), lifestyle (760 to 2019 counts per min),

light (100 to 759 counts per min), and sedentary (less than 100 counts per min).[14] Lifestyle activities include household chores, gardening, and golf and is performed at a lower intensity than moderate-vigorous physical activity.[24]

Secondary outcome measures included average percent of the day spent in sedentary, light, lifestyle, and moderate-vigorous activity which were calculated by summing the minute spent in each activity intensity and dividing by the total number of valid minute for each valid day. We also calculated the average total daily activity counts for each valid day by summing the total activity counts for each day and dividing by the number of wear days. We also calculated mean activity counts during the total wear time. Average daily steps were unavailable for the 2003 to 2004 cycle and so were not included in our analysis.[23] All models were adjusted for total wear time and the number of weekend days worn (0, 1, 2).

Demographic data collected from the participants included age (yr), sex (male or female), race (white, black, Mexican-Hispanic, other Hispanic, other), highest education achieved (less than ninth grade, ninth to eleventh grade, high school diploma/General Education Development Degree, some college or associate's degree, college or above), height (cm), weight (kg), and body mass index (kg/m²).[25]

Statistical Analysis

The National Health and Nutrition Examination Survey uses a complex, multistage, probability survey design and requires appropriate weights be applied for accurate national population and standard error estimates. For the combined analysis of 2003 to 2004 and 2005 to 2006, we created 4-yr sample weights to account for the different reference populations.[26] Estimates created from this study are thus representative of the U.S. population at the mid-point of the combined survey period (January 1, 2005). Continuous variables are reported as mean ± SD, and categorical variables are reported as frequency. Continuous variables were compared using an adjusted Wald test of the means and categorical variables were compared using Pearson chi-square test. Survey weights were applied to all estimates and statistical tests to ensure accurate standard errors. No *a priori* power calculation was performed and the analysis was based on the available data from the National Health and Nutrition Examination Survey.

Model Development

Classification and regression tree analysis produces a binary decision tree through recursive partitioning of the data to identify variables with the most explanatory power to predict a chosen response variable. Classification and regression tree analysis considers every value of a predictor variable as a potential split point, and the optimal split is chosen such that the resulting two subgroups are more homogenous with respect to predicting the response variable.

Classification trees select the first node through identifying the variable with the most explanatory power, thus producing two intermediate nodes. Intermediate nodes can be further bifurcated until the nodes reach their minimum size or until no significant improvements can be made in the decision tree through splitting. Nodes were prevented from further splitting if they contained less than 10% of the sample or the complexity parameter threshold (10^{-6}) was reached. The complexity parameter represents the tradeoff of how well the tree explains the data and the overall complexity of the tree (number of nodes), thus creating a tree that maximizes prediction and minimizes complexity and overfitting of the training data. The accuracy of the pruned tree was compared to that of a full tree constructed without any limitation on node sizes to ensure no significant loss of predictive ability when using the pruned tree.

The unweighted sample was 522 of 15,915 (3.3%) of the entire National Health and Nutrition Examination Survey sample and was randomly split into a training (90%, n = 469) and validation data set (10%, n = 53). The training sample consisted of 90% of the observations to maximize the accuracy of the classification tree. To offset the risk of overfitting the model we used stringent pruning criteria to avoid deep node splits in the data which would have a greater tendency to overfit our training data. Classification and regression tree analysis was performed to predict the presence of a 2-min bout of moderate-vigorous physical activity using physical function questions, as implemented using the R packages rpart and randomForest.[27,28] Nodes in the classification tree were restricted from further splitting if the complexity parameter threshold was reached and the depth of the tree was limited to two steps. The random forest model is an ensemble classification method that involves the construction of multiple bootstrapped classification trees. Plots from a random forest generated from the data were used to identify the most important predictors using the Gini impurity criterion. Weights were not applied in the classification and regression tree analysis as the analysis focused on classification of the data rather than statistical inference.

Sensitivity Analysis

A *post hoc* sensitivity analysis was performed upon an expanded sample size. Patients with a diagnosis of diabetes (Diabetes Questionnaire 010: "[Has a] Doctor told you [that you] have diabetes?") were included in the sensitivity analysis even if they reported they were not currently taking insulin as validation studies of the Revised Cardiac Risk Index have demonstrated the inclusion of only diabetic patients receiving insulin does not improve the model.[19] The unweighted sample was 695 of 15,915 (4.4%) of the entire National Health and Nutrition Examination Survey sample and was randomly split into a training (89%, n = 625) and validation data set (11%, n = 70). The tables and figures for the sensitivity analysis can be seen in the online supplemental index.

We performed additional classification and regression tree analyses on longer bouts of moderate-vigorous physical activity of 4 min and 6 min in length; however, we do not report these results. The percentage of participants who engaged in moderate-vigorous activity of 4 min (195 of 522, 37.4%) and 6 min (142 of 522, 27.2%) in the original sample were low enough that the analysis could not improve node classification through the use of physical function questions.

Statistical analyses were carried out using STATA-MP V14 (Statacorp, USA) and R V3.5.1 (http://www.r-project. org). All statistical analysis, except for the classification and regression tree analysis, accounted for the complex survey design of the National Health and Nutrition Examination Survey, and survey weights were adjusted for the two cycles of data. Survey analysis was done using the svy and subpop commands of STATA.

Results

A total of 852 (age 20 yr or older) unweighted participants in the survey had at least one Revised Cardiac Risk Index condition. Five hundred and twenty-two were asked the physical functioning questions, all of whom had at least 4 valid days of accelerometer wear time. This cohort represents an estimated 10,174,803 persons in the United States. The mean age for all participants who were asked the physical functioning questions was 69 ± 11 yr and 56% were male. The Revised Cardiac Risk Index condition with the greatest prevalence was coronary artery disease (66.8%), followed by cerebrovascular disease (23.0%), congestive heart failure (24.6%), diabetes requiring insulin (15.8%), and chronic kidney disease (2.7%; table 1). Participants with two or more Revised Cardiac Risk Index conditions totaled 26.8%.

At least one 2-min bout of moderate-vigorous physical activity was present in 72.4% of participants during accelerometer wear time (table 1). Participants with one 2-min bout of moderate-vigorous physical activity were younger and more likely to be male. Participants without a bout of 2 min of moderate-vigorous physical activity were more likely to have a diagnosis of congestive heart failure and cerebrovascular disease. The two groups did not differ in regard to race, education, or body mass index.

The accelerometer parameters measured throughout the week of physical activity monitoring are listed in table 2. Participants with at least one 2-min bout of moderate-vigorous physical activity had more valid days, more overall minutes of valid wear time and a higher average daily wear time of the accelerometer. Additionally, participants with one 2-min bout of moderate-vigorous physical activity spent a higher proportion of time in light and lifestyle activity than those who did not have a 2-min bout of moderate-vigorous physical activity when controlling for number of valid days and wear-time minutes. Participants without a 2-min bout of moderate-vigorous physical activity had a higher proportion of sedentary time.

Table 1. Participant Characteristics Stratified by a 2-Min Bout of Moderate-Vigorous Physical Activity during the Week of Physical Activity Monitoring

Participant Characteristics	2-Min Bout MVPA, 72.4% (n = 378)	No Bout of MVPA, 27.6% (n = 144)	P Value
Age, mean ± SD (yr)	68 ± 11	74 ± 10	0.001
Sex (%)			0.001
Male	62	39	–
Female	38	61	–
Race, (%)			0.582
White	81.8	85.6	–
Black	7.5	8.2	–
Mexican Hispanic	3.8	2.4	–
Other Hispanic	1.4	1.1	–
Other race	5.5	2.7	–
Interview language (%)			0.035
English	98.2	99.8	–
Spanish	1.8	0.2	–
Education, estimate (%)			0.346
< 9th Grade	11.6	8.8	–
9th–11th grade	15.9	17.6	–
High school diploma/GED	27.3	34.2	–
Some college or associate degree	27.3	28.2	–
College or above	17.9	10.8	–
Refused	0	0.5	–
Body Measures, mean ± SD			
Weight (kg)	82.1 ± 16.3	81.2 ± 17.8	0.601
Height (cm)	168.6 ± 9.4	164.5 ± 9.8	0.001
BMI (kg/m²)	28.8 ± 5.0	29.9 ± 5.5	0.110
RCRI conditions (%)			
Diabetes requiring insulin	14.4	19.4	0.263
Chronic kidney disease	2.5	3.4	0.585
Congestive heart failure	19.9	33.1	0.045
Coronary artery disease	67.2	65.9	0.781
Cerebrovascular disease	21.4	33.0	0.050

Sample size (n) is unweighted. Mean and prevalence are weighted to account for the survey design.

BMI, body mass index; GED, General Education Development degree; MVPA, moderate-vigorous physical activity; RCRI, Revised Cardiac Risk Index.

The responses to the physical function questions stratified by 2-min of moderate-vigorous physical activity are listed in table 3. Participants with a 2-min bout of moderate-vigorous physical activity were more likely to report "no difficulty" walking a quarter mile (two to three blocks), walking up 10 stairs, and performing house chores. The groups did not differ with respect to questions about difficulty lifting or carrying 10 pounds, preparing meals, or walking between rooms on the same floor.

Figure 1 presents the results of the classification and regression tree analysis. The classification tree identified self-reported difficulty walking up 10 stairs and walking two to three blocks as predictive of the presence of a 2-min bout of moderate-vigorous physical activity. The stair walking question with a response of "No difficulty" was the first split in the tree, indicating that this question was the strongest predictor of an adequate functional capacity. Among participants responding that they had "no difficulty" walking up 10 stairs, 78.9% achieved a bout of moderate-vigorous physical activity. The split on the ability to walk two to three blocks was dependent on being able to walk up

10 stairs with "No difficulty." Among participants who reported "No difficulty" walking up stairs and responded they were able to walk two to three blocks, 80.5% achieved a bout of moderate-vigorous physical activity. Participants who responded "unable to do" to walking two to three blocks were classified as unlikely to have a 2-min bout of moderate-vigorous physical activity. Of the participants who responded they had "some" or "much difficulty" walking up 10 stairs, 54.6% participated in a bout of moderate-vigorous physical activity. Of the participants who responded they were "unable" to walk upstairs, 35.0% participated in a bout of moderate-vigorous physical activity. Other physical function questions were not included in the classification tree as they did not improve the performance of the decision tree without adding additional complexity.

Model parameters from the classification and regression tree analysis for the training and test samples are presented in table 4. The sensitivity and specificity of the pruned tree on the training data were 0.97 (95% CI, 0.94 to 0.98) and 0.16 (95% CI, 0.10 to 0.23), respectively. The sensitivity and specificity of the pruned tree on the test data were 0.88

Table 2. Physical Function Questions Stratified by Presence or Absence of a 2-Min Bout of Moderate-Vigorous Physical Activity

Physical Function Question	2-Min Bout MVPA (n = 378)	No Bout of MVPA (n = 144)	P Value
Walking for a quarter mile difficulty (%)			0.001
No difficulty	69.8 (264)	45.8 (65)	–
Some difficulty	21.0 (74)	24.5 (38)	–
Much difficulty	4.9 (23)	13.9 (18)	–
Unable to do	2.4 (8)	12.2 (16)	–
Do not do this activity	2.0 (9)	3.6 (7)	–
Walking up 10 steps difficulty (%)			0.007
No difficulty	78.9 (302)	56.4 (80)	–
Some difficulty	13.6 (45)	27.0 (38)	–
Much difficulty	4.7 (20)	8.6 (12)	–
Unable to do	1.5 (6)	5.0 (9)	–
Do not do this activity	1.3 (5)	3.1 (5)	–
Lifting or carrying difficulty (%)			0.085
No difficulty	83.0 (304)	70.6 (103)	–
Some difficulty	9.3 (43)	16.5 (25)	–
Much difficulty	2.9 (11)	37.2 (4)	–
Unable to do	3.2 (17)	7.5 (9)	–
Do not do this activity	0.9 (2)	1.6 (3)	–
Don't know	0.7 (1)	0 (0)	–
House chore difficulty (%)			0.003
No difficulty	75.9 (284)	55.5 (85)	–
Some difficulty	14.7 (57)	29.7 (42)	–
Much difficulty	2.8 (11)	3.7 (4)	–
Unable to do	1.5 (5)	5.5 (5)	–
Do not do this activity	5.0 (21)	5.7 (8)	–
Preparing meals difficulty (%)			0.366
No difficulty	88.8 (329)	82.7 (115)	–
Some difficulty	5.4 (21)	8.4 (13)	–
Much difficulty	0.6 (3)	0.7 (2)	–
Unable to do	0.03 (1)	0.5 (1)	–
Do not do this activity	5.1 (24)	7.7 (13)	–
Walking between rooms on the same floor (%)			0.101
No difficulty	96.2 (362)	93.2 (134)	–
Some difficulty	3.7 (15)	6.7 (9)	–
Much difficulty	0.08 (1)	0.1 (1)	–
Unable to do	0 (0)	0 (0)	–
Do not do this activity	0 (0)	0 (0)	–

MVPA, moderate-vigorous physical activity.

Table 3. Accelerometer Measurements during the Week of Physical Activity Monitoring Stratified by a 2-Min Bout of Moderate-Vigorous Physical Activity

Accelerometer Parameter (Mean ± SD)	2-Min Bout MVPA (n = 378)	No 2-Min Bout (n = 144)	P Value
Valid days	6.3 ± 0.9	6.1 ± 1.0	0.018
Valid min of wear time	5401.3 ± 985.3	4972.5 ± 1085.3	0.006
Average daily wear time	848.0 ± 77.1	818.2 ± 86.7	0.002
Average counts per minute	218.7 ± 97.4	110.6 ± 49.4	0.001
Percent time sedentary	64.6 ± 9.9	73.3 ± 10.0	0.001
Percent time active	35.4 ± 9.9	26.7 ± 10.0	0.001
Percent time light activity	27.0 ± 7.0	24.0 ± 8.5	0.013
Percent time lifestyle activity	6.9 ± 4.0	2.6 ± 1.9	0.001
Percent time MVPA	1.4 ± 1.6	0.1 ± 0.1	0.001

Sample size (n) is unweighted. Mean and prevalence are weighted to account for the survey design. Comparisons were adjusted to control for differences in total wear time and number of weekend days worn (0,1,2). A valid day consisted of at least 10 h of activity. Accelerometer cut points to classify activity intensity were: MVPA (more than 2020 counts per min), lifestyle (760–2019 counts per min), light (100–759 counts per min), and sedentary (less than 100 counts per min).
MVPA, moderate-vigorous physical activity.
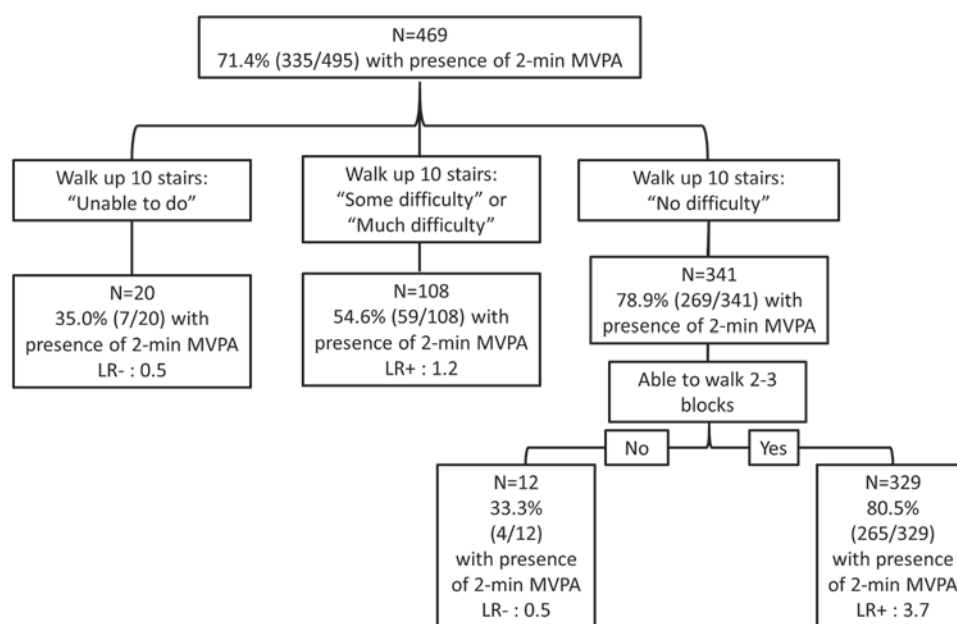
Fig. 1. Classification tree of National Health and Nutrition Examination Survey physical function questions using the presence of a 2-min bout of moderate-vigorous physical activity as the outcome variable. The first split is for participants who responded they had no difficulty walking up 10 steps, of which 78.9% had a 2-min bout of moderate-vigorous physical activity. The sensitivity of detecting a 2-min bout of activity increased further when participants answered they had no difficulty walking up 10 stairs and were able to walk two to three blocks. The classification tree was unable to further improve prediction of the outcome variable with additional questions for participants who answered they had some or much difficulty or unable to walk upstairs. Questions: (Stairs) "By yourself and without using any special equipment, how much difficulty do you have walking up 10 steps without resting?" (Walk two to three blocks) "By yourself and without using any special equipment, how much difficulty do you have walking for a quarter mile [that is about 2 to 3 blocks]?" Possible responses to the physical function questions: (1) No difficulty; (2) Some difficulty; (3) Much difficulty; (4) Unable to do; or (5) Do not do this activity. LR+, positive likelihood ratio; LR−, negative likelihood ratio; MVPA, moderate-vigorous physical activity.

Table 4. Model Parameters from Classification and Regression Tree Analysis of Physical Functioning Questions

Model Parameter	Training Data (n = 469)	Test Data (n = 53)
Prevalence of 2-min bout of MVPA	0.71	0.81
Sensitivity	0.97 (0.94–0.98)	0.88 (0.75–0.96)
Specificity	0.16 (0.10–0.23)	0.10 (0.00–0.45)
Positive likelihood ratio	1.15 (1.06–1.24)	0.98 (0.78–1.24)
Negative likelihood ratio	0.21 (0.10–0.42)	1.16 (0.15–8.89)
Accuracy	0.74 (0.69–0.78)	0.74 (0.60–0.85)

Sample size (n) is unweighted. Weights were not applied to the classification and regression tree analysis as the analysis focused on classification of the data rather than statistical inference. The training and test data were randomly partitioned from the full dataset.

MVPA, moderate-vigorous physical activity.

(95% CI, 0.75 to 0.96) and 0.10 (95% CI, 0.00 to 0.45), respectively. Random forest analyses results are presented in figure 2 and support the classification and regression tree analysis that walking up 10 stairs and walking two to three blocks are the strongest predictors of a 2-min bout of moderate-vigorous physical activity as assessed by the mean decrease in accuracy and mean decrease in Gini impurity if they were removed from the classification tree. None of the other physical function questions or Revised Cardiac Risk Index conditions were reliable predictors of the outcome.

The results of the sensitivity analysis can be seen in the Supplemental Digital Content, table S1 (http://links.lww.com/ALN/C36), which contains the participant characteristics and Supplemental Digital Content, figure S1 (http://links.lww.com/ALN/C39), which contains the classification tree. The accelerometer parameters measured throughout the week of physical activity monitoring are listed in Supplemental Digital Content, table S2 (http://links.lww.com/ALN/C37), and demonstrate increased physical activity in participants who engage in a 2-min bout of moderate-vigorous physical activity. Similar to the primary analysis, stair walking is the optimal question identified by the classification and regression tree analysis to improve the prediction of a bout of moderate-vigorous physical activity with splits identified at "no difficulty," "some/much difficulty," and "unable to do." The likelihood ratio for participants who responded, "no difficulty," is 4.2, which is
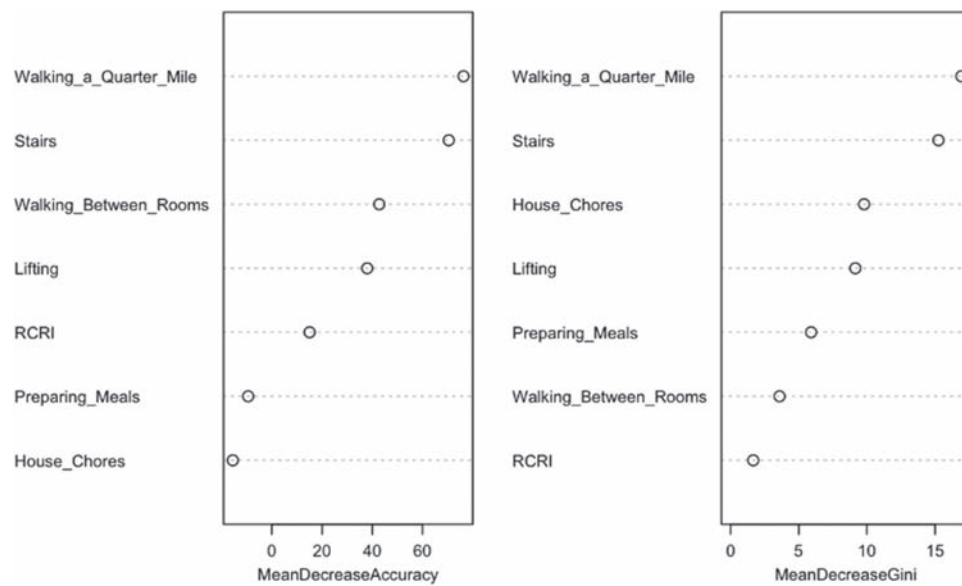
Fig. 2. Results of random forest analyses illustrating the importance of each physical function question in the classification and regression tree analysis. Stair climbing and walking two to three blocks are the most important questions for predicting the presence of a 2-min bout of moderate-vigorous physical activity. (*Left*) Indicates the mean decrease in accuracy of the tree as the result of removing a physical functioning question. (*Right*) The Gini coefficient is a measurement of the likelihood of an incorrect classification of the outcome. Physical function questions that decrease the mean Gini coefficient improve the prediction of that outcome. In each figure, the greater the decrease in accuracy and the greater the mean decrease in Gini impurity is associated with increased variable importance to the final classification and regression tree analysis. RCRI, Revised Cardiac Risk Index.

equivalent to a posttest probability of greater than 90%.[28] The full model parameters are similar to the primary analysis and can be seen in Supplemental Digital Content, table S3 (http://links.lww.com/ALN/C38), and Supplemental Digital Content, figure S2 (http://links.lww.com/ALN/C40), which contains the random forest analyses.

Discussion

In our analysis of the National Health and Nutrition Examination Survey database, self-reported ability to walk up 10 stairs without difficulty best predicted the presence of a 2-min bout of moderate-vigorous physical activity during a week of accelerometer wear among adult participants with at least one Revised Cardiac Risk Index condition. The prediction was further improved when participants responded that they were able to walk two to three blocks, with a positive likelihood ratio of 3.7 and a posttest probability greater than 90% that the participant engaged in a 2-min bout of moderate-vigorous physical activity.[29] Additionally, the sensitivity analysis participants who responded "No difficulty" to walking up 10 stairs also had a posttest probability greater than 90% of a 2-min bout. However, the overall specificity was low in both the training and test data sets, thus calling into question the utility of subjective functional capacity assessment in patients at increased risk of major adverse cardiac events before noncardiac surgery. With respect to our

second aim, participants who engaged in a 2-min bout of moderate-vigorous physical activity had a greater percentage of overall active time and a lower percentage of sedentary time as compared to participants who did not engage in a 2-min bout of moderate-vigorous physical activity.

Our data suggest that individual physical activity questions are insufficiently specific to identify patients who do not engage in moderate activity. Functional capacity as assessed by cardiopulmonary exercise testing has been used for decades for perioperative risk stratification and guidelines on the use of cardiopulmonary exercise testing before surgery were published in 2018.[6–9,13,30] Older *et al.* identified an oxygen consumption at ventilatory threshold of less than $11.0\,\mathrm{ml}\cdot\mathrm{kg}^{-1}\cdot\mathrm{min}^{-1}$ as a critical value identifying patients at risk for developing severe morbidity and mortality during major abdominal surgery.[6] In 2016, the American Heart Association emphasized the role of formal cardiopulmonary testing to identify patients at high perioperative risk who are scheduled for an elevated risk surgical procedure, and in whom functional capacity is unknown.[31] However, cardiopulmonary exercise testing is constrained by time, personnel, and cost. In contrast, subjective assessment is easy to perform, and the questions used are surprisingly consistent over time. In a 1999 single center study, Reilly *et al.* used patient self-reported exercise tolerance to predict perioperative complications.[5] Exercise tolerance was

evaluated by asking patients two physical activity questions: (1) "How many blocks can you walk?" and (2) "How many flights of stairs can you climb?"[5] Poor exercise tolerance was defined by a patient's inability to walk four blocks or climb two flights of stairs without symptomatic limitation. Our results confirm that these questions are the most sensitive to identify patients who do not engage in moderate activity. However, current practice may differ from Reilly *et al.* in using different thresholds for a patient to be given a designation of an adequate functional capacity.[3,4,32] The modern approach to functional assessment is highly variable given the type of activities provided in the guidelines that are associated with more than 4 metabolic equivalents. Additionally, the guidelines do not specify a duration of time those activities need to be maintained for, further increasing uncertainty for clinicians performing perioperative risk assessment.[3]

We sought to clarify which, if any, physical activity question most effectively detects the ability to sustain a short duration of moderate-vigorous physical activity. Physical activity questions provide information about the patient's functional capacity and—more broadly—cardiorespiratory fitness. In response to such questions, patients may over- or underestimate their physical abilities when compared to objective measures for a variety of reasons, including poor recall, social desirability, and misinterpretation of the questions.[33] In the National Health and Nutrition Examination Survey database, although 51% of all adults self-reported adherence to national guidelines recommending at least 150 min of moderate-intensity physical activity per week, accelerometer-based measurements revealed that less than 5% were actually adherent.[14] In a large multicenter study by Wijeysundera *et al.,* subjective assessment of functional capacity did not predict cardiac complications in patients undergoing major noncardiac surgery; although subjective assessment was not standardized and conclusions about individual questions cannot be made.[16] In the same study, however, the Duke Activity Status Index did have moderate predictive ability to identify cardiac complications. The Duke Activity Status Index is a structured questionnaire that more formally estimates functional capacity and is also recommended by the guidelines.[3] Our results indicate that the answer to the question, "can you walk up 10 stairs?" has the best sensitivity with respect to an individual's ability to engage in moderate-vigorous physical activity. Further, when participants answered "no difficulty" to walking up 10 stairs and are able to walk two to three blocks, the posttest probability is almost 90% in their having engaged in a 2-min bout of moderate-vigorous physical activity.[29] However, our findings also suggest the overall predictive power of these questions to identify patients who do routinely perform short bouts of moderate-vigorous physical activity is poor.

Preoperative accelerometers or short exercise tests may provide a more objective assessment of functional capacity before noncardiac surgery. In our study, a 2-min bout of moderate-vigorous physical activity during the week of accelerometer wear time differentiated participants by overall activity level. However, it is unclear whether this difference is meaningful for preoperative risk stratification. In previous studies of preoperative accelerometer use, overall accelerometer activity was moderately correlated with cardiopulmonary exercise testing–derived peak oxygen consumption and ventilatory threshold.[34] In addition to accelerometers, short exercise tests may be utilized to identify patients at high risk of complications. Six-minute walk test distance is strongly correlated with the ventilatory threshold and distance can be used to risk stratify patients before major noncardiac surgery.[35,36] Additionally, Reddy *et al.* identified the time to complete an in-clinic stair climb test as the single strongest predictor of perioperative complications in patients undergoing major abdominal surgery, and this test outperformed the American College of Surgeons National Surgical Quality Program score (area under the curve, 0.81 *vs.* 0.62; $P < 0.0001$).[37] More objective measures of patient physical activity, either through patient worn accelerometers or brief exercise tests, may further improve perioperative risk stratification.

Limitations

Our study has several limitations. The National Health and Nutrition Examination Survey database is not a surgical database and may not generalize to surgical patients. Nonetheless, this cohort provides a nationally representative sample of physical activity and responses to physical function questions in participants who would be considered at increased risk of major adverse cardiac events. We did not relate the responses of physical activity questions directly to the results of formal cardiopulmonary exercise testing, the gold standard approach to functional capacity assessment. Rather, our primary outcome was the presence of a continuous 2-min bout of moderate-vigorous physical activity during the week of accelerometer wear time. Because the guidelines specifically emphasize the performance of activities associated with more than 4 metabolic equivalents, we believe our primary outcome is representative of this recommendation and a valid endpoint for analysis. Additionally, our choice of 2 min for the duration of continuous moderate-vigorous physical activity was not explicitly defined in the guidelines, as they do not specify any duration of activity associated with more than 4 metabolic equivalents of work.[3] Thus, we chose the shortest duration (2 min) that would approximate activities included in the practice guidelines (*e.g.,* walking two blocks at a rate of 4 mph). Our choice of accelerometer cut points may have been too conservative. However, we chose cut points that traditionally applied to the National Health and Nutrition Examination Survey dataset to classify moderate-vigorous physical activity. It is possible that this approach may have led to more participants classified as having a poor

functional capacity even though they may have met other criteria.[14,23] In our analysis, 27% of participants were classified as having an inadequate functional capacity, which is consistent with the distribution of previous studies looking at preoperative functional capacity assessment in this patient population.[6,16] Finally, not all of the participants included in the analysis completed all 7 days of the accelerometer protocol, which may introduce bias of being classified as having a poor functional capacity. The bias may have been further amplified by the use of our outcome variable as the presence or absence of a minimum threshold (2-min bout of moderate-vigorous physical activity); however, we did control for the number of weekend days and number of valid days as this can impact the overall results.[38]

Conclusions

In conclusion, we observed in a large nationally representative database that a standardized physical function question focused on walking up stairs was the most sensitive in identifying the presence of a 2-min bout of moderate-vigorous physical activity in participants with at least one Revised Cardiac Risk Index condition. Despite a high sensitivity, however, this single question remains insufficiently specific to identify patients with a poor functional capacity. Given the results of our study, future perioperative cardiovascular evaluation guidelines should consider recalibrating the method and role of subjective functional assessment for risk stratification. Accelerometers and brief exercise tests may improve preoperative risk stratification, but more research is needed to clarify their role.

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ANESTHESIOLOGY

Pharmacodynamic Interaction of Remifentanil and Dexmedetomidine on Depth of Sedation and Tolerance of Laryngoscopy

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ABSTRACT

Background: Dexmedetomidine is a sedative with modest analgesic efficacy, whereas remifentanil is an opioid analgesic with modest sedative potency. Synergy is often observed when sedative–hypnotics are combined with opioid analgesics in anesthetic practice. A three-phase crossover trial was conducted to study the pharmacodynamic interaction between remifentanil and dexmedetomidine.

Methods: After institutional review board approval, 30 age- and sex- stratified healthy volunteers were studied. The subjects received consecutive step-wise increasing target-controlled infusions of dexmedetomidine, remifentanil, and remifentanil with a fixed dexmedetomidine background concentration. Drug effects were measured using binary (yes or no) endpoints: no response to calling the subject by name, tolerance of shaking the patient while shouting the name ("shake and shout"), tolerance of deep trapezius squeeze, and tolerance of laryngoscopy. The drug effect was measured using the electroencephalogram-derived "Patient State Index." Pharmacokinetic–pharmacodynamic modeling related the administered dexmedetomidine and remifentanil concentration to these observed effects.

Results: The binary endpoints were correlated with dexmedetomidine concentrations, with increasing concentrations required for increasing stimulus intensity. Estimated model parameters for the dexmedetomidine EC50 were 2.1 [90% CI, 1.6 to 2.8], 9.2 [6.8 to 13], 24 [16 to 35], and 35 [23 to 56] ng/ml, respectively. Age was inversely correlated with dexmedetomidine EC50 for all four stimuli. Adding remifentanil did not increase the probability of tolerance of any of the stimuli. The cerebral drug effect as measured by the Patient State Index was best described by the Hierarchical interaction model with an estimated dexmedetomidine EC_{50} of 0.49 [0.20 to 0.99] ng/ml and remifentanil EC_{50} of 1.6 [0.87 to 2.7] ng/ml.

Conclusions: Low dexmedetomidine concentrations (EC$_{50}$ of 0.49 ng/ml) are required to induce sedation as measured by the Patient State Index. Sensitivity to dexmedetomidine increases with age. Despite falling asleep, the majority of subjects remained arousable by calling the subject's name, "shake and shout," or a trapezius squeeze, even when reaching supraclinical concentrations. Adding remifentanil does not alter the likelihood of response to graded stimuli.

(*ANESTHESIOLOGY* 2019; 131:1004–17)

EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Patients sedated with standard clinical doses of dexmedetomidine can be readily aroused
- Dexmedetomidine doses producing mild to deep sedation lack significant analgesic effect
- Remifentanil is an opioid analgesic with only modest sedative properties
- Addition of remifentanil to propofol sedation reduces the propofol concentration required to reach tolerance of shaking the patient while shouting their name and tolerance of laryngoscopy

What This Article Tells Us That Is New

- This three-phase crossover trial to study the pharmacodynamic interaction between remifentanil and dexmedetomidine in 30 age- and sex-stratified healthy volunteers found that, despite falling asleep, most subjects remained arousable by calling their name, shaking the subject while shouting their name, or a trapezius squeeze, even after reaching supraclinical concentrations
- Adding remifentanil to dexmedetomidine sedation did not affect the likelihood of response to graded stimuli
- Dexmedetomidine potency increased with increasing age

D EXMEDETOMIDINE is a sedative that acts through binding to the α2-adrenoceptor. Dexmedetomidine has the unusual property of providing significant seda-tion without cardiorespiratory compromise.[1] Additionally, patients sedated with dexmedetomidine can be readily aroused.[1] These features, combined with anxiolytic and amnestic effects, make dexmedetomidine useful in many procedures, such as procedural sedation, awake craniotomies, and postoperative and/or intensive care unit sedation. Side effects are mainly hemodynamic and include hypertension, hypotension, and bradycardia caused by vasoconstriction,

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sympatholysis, and baroreflex-mediated parasympathetic activation.[2,3]

In clinical anesthesia, hypnotics are frequently administered in combination with opioid analgesics. Combined drug effects can be synergistic, additive, or infraadditive.[4,5] The interaction is additive if the same drug effect is observed for a particular sum of the individual concentrations normalized to potency regardless of the ratio of the drugs to each other. Synergy is observed when the combination of two drugs, normalized to potency, produces a greater drug effect than an equivalent potency-normalized concentration of either drug alone. Rarely the combination of two drugs, normalized to potency, produces a lesser drug effect than an equivalent potency-normalized concentration of either drug alone. This is referred to as infraadditivity or antagonism. Quantifying drug interactions is important in the field of anesthesia and helps to develop better dosing regimens.

In previous interaction studies, dexmedetomidine has been shown to reduce requirements isoflurane,[6–8] sevoflurane,[9,10] propofol,[11–13] thiopental,[14–17] and fentanyl.[18] Studies investigating the sedative and analgesic properties of dexmedetomidine found that doses resulting in mild to deep sedation lack significant analgesic efficacy.[19,20] Therefore, to ensure patient comfort in painful procedures, dexmedetomidine is frequently combined with analgesic drugs. Remifentanil is an opioid analgesic with only modest sedative properties.[21] This trial was designed to study the pharmacodynamic interaction between dexmedetomidine and remifentanil and quantify the expected synergy to determine the combination of dexmedetomidine and remifentanil that (1) maintains an unarousable state of sedation and (2) allows subjects to tolerate noxious stimuli, including painful procedures, surgery, and laryngoscopy.

Materials and Methods

This investigator initiated trial was conducted at the Department of Anesthesiology at the University Medical Center Groningen, Groningen, The Netherlands, in accordance with the Declaration of Helsinki and in compliance with good clinical practice and the applicable regulatory requirements. Ethical approval was obtained from the independent medical ethics review committee (Medisch Ethische Toetsings Commissie) of the foundation Evaluation of Ethics in Biomedical Research (Stichting BEBO), Assen, The Netherlands. The study was registered in the ClinicalTrials.gov database (NCT03143972).

Patient Inclusion

After obtaining written informed consent and performing a standard health screening, 30 volunteers were included in this study. Subjects were stratified according to sex and age (18 to 34, 35 to 49, and 50 to 70 yr).

Exclusion criteria were a history of intolerance to dexmedetomidine or remifentanil, a body mass index greater than $30\,kg/m^2$ or less than $18\,kg/m^2$, pregnancy or currently breastfeeding, neurologic disorders, depression requiring treatment with anti-depressive drugs, psychosis, dementia, schizophrenia, alcohol or drug abuse, recent use of psychoactive medication, chronic use of more than $20\,g$ of alcohol daily, any significant cardiovascular disease or risk factor, bilateral nonpatent arteria ulnaris, or any other relevant medical condition.

Study Design

In this three-phase crossover study, each volunteer was scheduled for two study sessions, with a wash-out of at least 1 week between both days. During the first study session, volunteers received dexmedetomidine administered using target-controlled infusion with stepwise increasing effect site target concentrations of 1, 2, 3, 5, and 8 ng/ml dexmedetomidine. On their second study session, after an appropriate washout (more than 1 week), these volunteers received a stepwise increasing remifentanil infusion targeting effect site concentrations of 1, 2, 3, 5, and 7 ng/ml. After an interval for remifentanil washout, volunteers received dexmedetomidine *via* target-controlled infusion with an effect site target of 2 ng/ml. After an appropriate equilibration time, remifentanil was added with stepwise increasing effect site target concentrations of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 4.0 ng/ml, respectively. See also figure S1.1 in Supplemental Digital Content 1 (http://links.lww.com/ALN/C12) for a schematic overview of the infusion regimens. Throughout this work, these three study phases will be referred to as "dexmedetomidine phase," "remifentanil phase," and "interaction phase," respectively.

Study Procedures

Study participants were instructed not to use recreational drugs for 2 weeks before the study, not to smoke tobacco for 1 week, and not to consume alcohol for 2 days before each of their study days. All volunteers were instructed to fast from 6 h before the start of the planned study procedures. During the study a board-certified anesthesiologist and nurse anesthetist were responsible for the monitoring and safety of the volunteers, drug administration, and respiratory support. A complete anesthesia workstation was present in the room, as well as an anesthesia ventilator (specifications of indicated materials, devices, and software used in this study can be found in Supplemental Digital Content 2, http://links.lww.com/ALN/C13) enabling the anesthesia team to provide monitored anesthesia care, ventilatory support, and emergency care if needed. A research physician and research nurse were responsible for all other study procedures. Upon arrival, volunteers were connected to a vital sign monitor (Supplemental Digital Content 2, http://links.lww.com/ALN/C13). A 20-gauge IV cannula was placed for fluid administration, dexmedetomidine and remifentanil administration, and, if applicable, rescue

medication. A 20-gauge arterial cannula was placed under ultrasound guidance and local anesthesia for blood sampling and hemodynamic monitoring (Supplemental Digital Content 2, http://links.lww.com/ALN/C13). No premedication was administered. To monitor ventilation, including inspiratory oxygen fraction (Fio_2) and end-tidal carbon dioxide, volunteers breathed spontaneously through a tight-fitting face mask attached to a circular breathing system of an anesthesia ventilator (Supplemental Digital Content 2, http://links.lww.com/ALN/C13) with a Fio_2 set to 25%. If deemed necessary, the anesthesiologist supported respiration by verbal stimulation, chin lift or jaw trust, pressure supported spontaneous breathing, or positive pressure ventilation.

Measures of Drug Effect

The cerebral drug effect of dexmedetomidine was measured using a processed electroencephalographic measure, the Patient State Index (PSI-2) (Supplemental Digital Content 2, http://links.lww.com/ALN/C13). The PSI is an electroencephalogram–derived index to monitor the depth of anesthesia, with a PSI score of 100 representing the awake state and a PSI score of 0 denoting no detectable electrical brain activity.

Sedation was assessed using the Modified Observers Assessment of Alertness and Sedation (MOAA/S) score (table 1). MOAA/S assessments were performed by the attending anesthesiologist. The MOAA/S score was linked to our binary response/no response endpoints as follows:

1. No response to calling the subject by name was defined as a MOAA/S score of less than 3
2. Tolerance of shaking the patient while shouting the name ("shake and shout") was defined as a MOAA/S score less than 2
3. Tolerance of deep trapezius squeeze was defined as a MOAA/S score of 0
4. Tolerance of laryngoscopy was defined as a MOAA/S score of 0 and the ability to obtain a Cormack–Lehane score of 3 or less via direct laryngoscopy[22]

To standardize the pinch force used during the MOAA/S assessments of tolerance of trapezius squeeze to 20 lbs/inch,[2] the anesthesiologist trained himself or herself in squeezing force with a bedside pinch gauge (Supplemental Digital Content 2, http://links.lww.com/ALN/C13).

Volunteers were placed in supine position and were asked to stay in bed and not to engage in activities or spontaneous speech except for the required responses to MOAA/S assessments. Volunteers were not stimulated except for the MOAA/S assessments, and low ambient noise was ensured throughout the study period. Baseline measurements of vital parameters were taken for 5 min before drug infusion.

Drug Administration

Volunteers received dexmedetomidine via target-controlled infusion (Supplemental Digital Content 2, http://links.lww.com/ALN/C13). This target-controlled infusion was based

on the pharmacokinetic model developed by Hannivoort et al.[23] expanded with an equilibration rate constant (ke0) for the effect site of the MOAA/S estimated in the pharmacodynamic model by Colin et al.[24] To avoid hypertensive reactions, the infusion of dexmedetomidine was limited to 6 µg · kg^{-1} · h^{-1} for the first three infusion steps and was increased to 10 µg · kg^{-1} · h^{-1} for the two highest targets of 5 and 8 ng/ml. During the second study session, a computer-controlled infusion, based on the pharmacokinetic–pharmacodynamic model developed by Eleveld et al.,[25] was used to target remifentanil effect-site concentrations.

Before each increase in effect site target, MOAA/S was scored, a scheduled blood sample was taken for measurement of drug concentrations, and if the MOAA/S score was 0 or 1, tolerance of laryngoscopy was tested. The concentration targets and the measurements performed during a steady-state observation phase are shown schematically in Supplemental Digital Content 1, figures S1.1 and S1.2 (http://links.lww.com/ALN/C12).

Recovery Period

After reaching tolerance of laryngoscopy or after completion of all infusion steps, drug infusion was stopped, and the recovery period began. In addition, the infusion was stopped when one of the following stopping criteria was met and deemed clinically relevant by the attending anesthesiologist:

- 30% increase from baseline mean arterial blood pressure for more than 5 min
- 30% decrease from baseline mean arterial blood pressure for more than 5 min
- Heart rate below 40 beats/min for more than 5 min
- Changes in cardiac conduction or cardiac rhythm
- Any other safety reason as judged by attending anesthesiologist

If deemed necessary, a rescue dose of 0.5 mg of atropine was given, and the drug infusion was stopped. To maintain an acceptable blood pressure, volunteers were put in Trendelenburg position of approximately 15°. If this was not effective, a rescue dose of 5 mg of ephedrine was administered, and the drug infusion was stopped.

During the recovery period MOAA/S scores were assessed with a 2-min interval for the first 30 min and with a 10-min interval thereafter until volunteers reached two consecutive MOAA/S scores of 5 with a 10-min interval between measurements. Blood samples were collected at predefined time points (see Arterial Blood Sampling section below). If the volunteer met the discharge criteria of the hospital's postanesthesia care unit, he or she was discharged from the research unit after the last sample was taken.

Arterial Blood Sampling

Arterial blood samples were drawn at baseline and at pseudo-steady state before changing the target concentration. Once the infusion was stopped, dexmedetomidine samples were

drawn at 2, 5, 10, 20, 60, 120, 300, and 420 min in the first session. After the remifentanil-only session in the second study, blood samples were collected at 2, 5, 10, and 30 min after stopping the remifentanil infusion. After the dexmedetomidine and remifentanil interaction session in the second study, blood samples were collected at 2, 5, 10, 20, 30, 60, 120, and 300 min after stopping the infusions (at 60, 120, and 300 min, only dexmedetomidine assessment was performed).

Storage and Analysis of Blood Samples

Blood was collected in EDTA tubes (Supplemental Digital Content 2, http://links.lww.com/ALN/C13) and immediately stored on ice for a maximum of 60 min (dexmedetomidine) or 15 min (remifentanil). Afterwards, samples were centrifuged for 5 min at 1,754g at 4°C. Plasma was transferred into cryovials and stored at or below −20°C until analysis. For remifentanil, sample stability was improved by the addition of 1.5 µl of formic acid per milliliter of plasma before freezing.[26]

Dexmedetomidine and remifentanil concentrations were measured using ultra–high-performance liquid chromatography–mass spectrometry (Supplemental Digital Content 2, http://links.lww.com/ALN/C13).[23,26] The lower and upper limits of quantification were 0.05 and 20 ng/ml for both compounds with a coefficient of variation of less than 8% for dexmedetomidine and less than 9% for remifentanil (within the entire range of the quality control samples of 0.075 to 7.5 ng/ml). Samples that were thought to be above the upper limit of quantification were diluted with blank human plasma before the sample treatment.

Optimization of Trial Design

The design of the clinical trial was *a priori* optimized using optimal experimental design principles. As such, various trial designs and sample sizes were simulated and compared. The primary objective here was to find the minimal sufficient trial design that would allow us to estimate all parameters included in the hierarchical interaction model with sufficient precision. The hierarchical interaction model was used in trial design optimization, because this model structure was regarded most appropriate. This assumption is based on previous studies describing opioid–hypnotic interactions.[21,27] The hierarchical model implies that opioids on their own have no effect on tolerance of laryngoscopy but that opioids reduce the amount of hypnotic needed to reach tolerance of laryngoscopy.[21]

Simulations showed that using the currently proposed trial design, a trial population of 30 subjects undergoing two crossover study phases would suffice to meet the study objectives. The infusion scheme was based on an estimated EC50 of 4 ng/ml dexmedetomidine for tolerance of laryngoscopy as described by Kunisawa *et al.*[28] To account for potential differences between our study population and the population by Kunisawa *et al.*,[28] we chose to work with an adaptive trial design. Therefore, an interim analysis was planned after enrollment of the first five volunteers. The

adaptive design allowed us to change the drug infusion scheme to maximize the possibility of attaining informative drug concentrations and responses for pharmacokinetic–pharmacodynamic modeling based on the responses measured in these volunteers. See also Supplemental Digital Content 3 (http://links.lww.com/ALN/C14) for an extensive description of the adaptive trial design.

Data Handling

Measured arterial concentrations of samples drawn during pseudo–steady-state observation phases were used in pharmacodynamic modeling, because they are assumed to adequately reflect effect-site concentrations. Throughout this work these concentrations will be referred to as steady-state concentrations. MOAA/S scores and laryngoscopy observations during these steady-state phases were included in the final data set. MOAA/S scores were used to define the endpoints no response to name called, tolerance of shake and shout, and tolerance of trapezius squeeze. For PSI, the median value of a 60-s measurement interval before the start of the MOAA/S assessments was used in our analysis. This measurement period was chosen to circumvent the confounding effect of arousal caused by the MOAA/S assessments on the PSI value.

Pharmacodynamic Modeling Strategy

Nonlinear mixed effects modeling was used to study the relationship between measured steady-state concentrations and clinical endpoints no response to name called, tolerance of shake and shout, tolerance of trapezius squeeze, tolerance of laryngoscopy, and PSI (PSI-2).

Modeling of Binary Variables (No Response to Name Called, Tolerance of Shake and Shout, Tolerance of Trapezius Squeeze, and Tolerance of Laryngoscopy). For the binary dependent variables (no response to name called, tolerance of shake and shout, tolerance of trapezius squeeze, and tolerance of laryngoscopy) models were fitted to the data using the first-order estimation algorithm in NONMEM with the LIKELIHOOD option. The hierarchical interaction model, shown in equations 1 and 2, was selected as our base model.

$$U = \frac{C_D}{EC50_D} \cdot \left(1 + \left(\frac{C_R}{EC50_R}\right)^{\gamma_R}\right) \tag{1}$$

$$P_{\text{tolerance of a stimulus}} = \frac{U^{\gamma_D}}{1 + U^{\gamma_D}} \tag{2}$$

In this interaction model, U is the combined potency of the concentration (C) of both drugs (D for dexmedetomidine and R for Remifentanil) normalized over the concentration inducing half the maximal effect (EC50). With γ_R and γ_D representing the steepness of the curves, *P* is the probability of tolerating an applied stimulus.

First, for each stimulus (no response to name called, tolerance of shake and shout, tolerance of trapezius squeeze, and tolerance of laryngoscopy), a full structural model was constructed. For this, four parameters ($EC50_R$, $EC50_D$, γ_R, and γ_D) were estimated. Subsequently, we attempted to simplify this model. The full and reduced models were compared using the likelihood ratio test. Modifications to the structural and/or covariate model were accepted only if they resulted in a significant change in the objective function value. An increase in objective function value was judged statistically significant at the 5% level if exclusion of a parameter increased the objective function value by more than 3.84 points. Nonsignificant parameters were removed from the model one by one.

For the binary outcomes, our objective was to predict the population probability of no response to each stimulus. The emphasis is not on the concentration response relationship of an individual subject, and therefore interindividual variability was not included in the parameter estimation (*i.e.*, naive pooling approach). Once the final structural model was found, the influence of covariates was evaluated by inclusion of the covariates in the model on the $EC50_D$ parameter. Patient covariates considered for inclusion in the model were: age, height, weight, and sex.

Modeling of the Patient State Index. For the continuous dependent variable PSI, the first-order conditional estimation algorithm with interaction was used. Different previously published interaction models were fitted to the data. These models included: the hierarchical interaction model,[21] the Greco interaction model,[29,30] the sigmoid E_{max}, and the E_{max} model.[31] In contrast to the Greco model, the hierarchical interaction model assumes no effect of remifentanil in absence of dexmedetomidine. The sigmoid E_{max} and E_{max} model were fitted to test the hypothesis of no interaction effect, *i.e.*, these models assume that only dexmedetomidine exerts an effect on PSI.

These structural models were compared using the Akaike information criterion, the model with the lowest Akaike information criterion was chosen as our base model. Interindividual variability in the population was modeled using an exponential model. Additive, proportional, and combined residual error models were evaluated.

At each stage, the quality of the model was evaluated using change in objective function value, precision of parameter estimates, and shrinkage in empirical Bayes parameter estimates. Goodness–of–fit was graphically evaluated by graphs of the individual or population predicted *versus* observed responses and graphs of the conditionally weighted residuals *versus* individual predictions. As a safeguard against overparameterization, the condition number was calculated and compared across models.[32]

We first tested different structural models to account for the interaction between dexmedetomidine and remifentanil. Subsequently, covariates were tested by introducing them into the model. The covariates considered were age,

height, weight, and sex. We tested for significant covariates on the EC50 parameter(s) and the baseline PSI parameter. As a final check, log-likelihood profiling was performed using Pearl-speaks–NONMEM (Supplemental Digital Content 2, http://links.lww.com/ALN/C13).

Statistical Analysis

Estimated model parameters are documented as typical values with 90% confidence intervals. Model parameter estimation was done using NONMEM (Supplemental Digital Content 2, http://links.lww.com/ALN/C13), and confidence intervals were calculated based on 10,000 bootstrap samples for the models for no response to name called, tolerance of shake and shout, tolerance of trapezius squeeze, and tolerance of laryngoscopy. For the PSI model, confidence intervals were derived based on log-likelihood profiling using Pearl-speaks–NONMEM (Supplemental Digital Content 2, http://links.lww.com/ALN/C13). Clinical data are given as means and SD or as medians and ranges, where appropriate.

Results

We screened 48 healthy volunteers. Of these, we included 35 in the study. Five volunteers dropped out before the start of the second study session and were replaced. Two volunteers dropped out because of failure of arterial cannula placement at the start of the first study session, one because of failure of arterial cannula placement at the start of the second study session, one volunteer withdrew after the first study session, and one volunteer was too anxious during the first study session and was therefore taken out of the study. Our total of 30 volunteers completing both study sessions were stratified into three age categories (18 to 34, 35 to 49, and 50 to 70 yr) with five males and five females in each category. Volunteers ranged from 18 to 67 yrs of age, 49.2 to 98.3 kg, and 158 to 193 cm tall and had body mass indexes from 18.2 to 28.7 kg/m^2.

We collected 464 observations of MOAA/S, PSI, and concomitant plasma samples during steady-state observation phases for our analysis. This resulted in a median of 15.5 (range 6 to 20) MOAA/S scores per subject and a median of 2 laryngoscopy attempts (range 0 to 6) per subject. The raw data are shown in figure 1. In the dexmedetomidine phase, a total of 34 laryngoscopy attempts were performed in 22 subjects. Of those, tolerance of laryngoscopy was achieved in 13 subjects. In the interaction phase, a total of 43 laryngoscopy attempts were performed in 19 subjects. Of those, laryngoscopy was achieved in 15 subjects. The reasons for stopping the infusions are displayed in Supplemental Digital Content 1, figure S1.3 (http://links.lww.com/ALN/C12).

After the first five volunteers completed the study, an interim analysis was performed. In the dexmedetomidine phase, one of these five volunteers reached laryngoscopy at the 4 ng/ml target concentration, one reached laryngoscopy at 8 ng/ml, and three volunteers did not reach laryngoscopy.
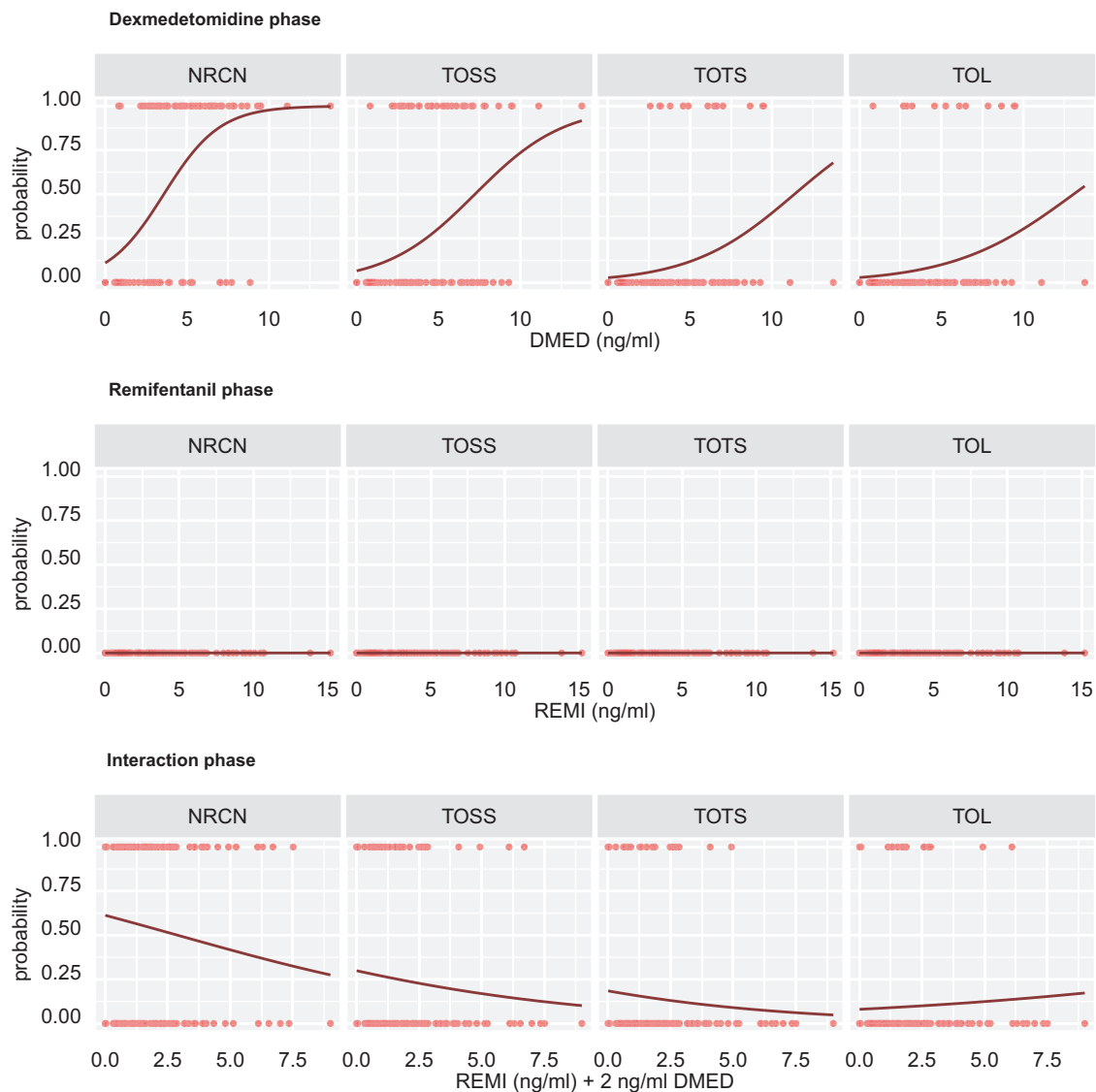
Fig. 1. All observations obtained during steady-state observation phases for no response to calling the subject by name (NRCN), tolerance of shake and shout (TOSS), tolerance of trapezius squeeze (TOTS), and tolerance of laryngoscopy (TOL), plotted *versus* measured dexmedetomidine (DMED) and remifentanil (REMI) concentrations. *Solid lines* denote a smoother obtained by fitting a generalized linear model through the data. For the interaction phase, remifentanil concentrations are plotted with 0 representing 0 ng/ml remifentanil but with a background concentration of 2 ng/ml dexmedetomidine being present.

According to the adaptive design flowchart (Supplemental Digital Content 3, figure S3.1 and tables S3.2, http://links. lww.com/ALN/C14), these results indicate a higher EC50 for tolerance to laryngoscopy than the 4 ng/ml estimated by Kunisawa *et al.*[28] and should have led to increasing the dexmedetomidine target concentrations. However, at the same time we observed long lasting hemodynamic side effects after stopping the dexmedetomidine infusion (hypotension and orthostatic hypotension). Therefore, for safety reasons, we decided not to increase target concentrations. Furthermore, because we noticed that our target-controlled

infusion model underpredicted dexmedetomidine plasma concentrations at the 8.0 ng/ml target concentration, it was decided to lower the highest target level in the dexmedetomidine phase from 8.0 to 6.0 ng/ml. Of the first five volunteers who completed the interaction phase, four volunteers reached laryngoscopy at 1, 1.5, 1.5, and 2 ng/ml remifentanil, respectively. The concentration range seemed appropriately chosen and was not adjusted (Supplemental Digital Content 3, figure S3.2 and tables S3.3 and S3.4, http://links.lww.com/ALN/C14). Throughout the rest of the study, the design was not adjusted.

Accuracy of the Target-controlled Infusion Models Used in the Study

The predictive performances of the target-controlled infusion models were assessed by comparing predictions against the measured concentrations and calculating performance criteria according to Varvel *et al.*[33] Median performance errors of 27 and 5.7% and median absolute performance errors of 34 and 20% were calculated for dexmedetomidine infusion in the dexmedetomidine phase and interaction phase, respectively. Because nonlinearity in the pharmacokinetics of dexmedetomidine at high concentration rates may be present, a subanalysis was performed on the samples in the clinically used concentration range up to 3 ng/ml. When plasma samples with dexmedetomidine target-controlled infusion targets above 3 ng/ml and samples collected during the recovery of these high infusion targets were excluded from the analysis, the median absolute performance errors were 22 and 24%, and the median performance errors were 3.4 and 16% for the dexmedetomidine and the interaction phase, respectively. For remifentanil infusion, median performance errors of 3.6 and 35% and median absolute performance errors of 32 and 45% were calculated for the remifentanil phase and interaction phase, respectively. *Post hoc*, we also compared the performance of the applied models for dexmedetomidine and remifentanil to other available models using the drug infusion profiles from our study (Supplemental Digital Content 1, table S1.1, http://links.lww.com/ALN/C12).

Adverse Events

During the dexmedetomidine phase, obstructive breathing and obstructive apnea occurred in five subjects, of whom four were managed with manual airway maneuvers and one with a nasopharyngeal airway. The dexmedetomidine infusion was stopped because of hypertension in one subject and bradycardia in four subjects (Supplemental Digital Content 1, figure S1.3, http://links.lww.com/ALN/C12). Hypotension was countered with the Trendelenburg position and IV fluids in four subjects. Three hypotensive subjects required ephedrine 5 mg, one of whom received two boluses of ephedrine 5 mg. Dexmedetomidine infusion was stopped in one subject because of hypotension. Most subjects experienced prolonged periods of (asymptomatic) hypotension during the recovery period. The volunteers were given intravenous fluids, drinks, and food, and mobilizing was done slowly and carefully. Eight subjects experienced symptomatic orthostatic hypotension when they started mobilizing; two subjects required atropine 0.5 mg because of vagal responses.

During the remifentanil phase, 25 subjects developed ventilatory insufficiency with bradypnea and/or apnea, resulting in desaturations. These were managed mostly by verbally stimulating subjects to keep breathing (11 subjects), pressure support (9 subjects), and an increased F_{IO_2} (15 subjects). One subject's ventilation was briefly assisted manually, and one subject received a nasopharyngeal airway.

Respiratory problems were a reason to stop the remifentanil infusion in six subjects. One subject developed ventilatory insufficiency with associated desaturation and developed severe opioid-induced muscle rigidity including thoracic rigidity, necessitating neuromuscular blockade, intubation, and sedation with propofol. All infusions were stopped, and she recovered uneventfully, but the interaction phase for this subject was cancelled. After the remifentanil infusion was stopped, 11 subjects developed nausea and received 4 mg ondansetron IV, and two subjects vomited.

During the interaction phase, obstructive breathing and apneas were recorded in 10 subjects, and ventilatory insufficiency with bradypnea and/or apnea was observed in two subjects. In this phase, drug infusions were stopped because of bradycardia in two subjects and because of hypotension in one subject. One subject received ephedrine 5 mg; the other nine cases of hypotension were managed with intravenous fluid and the Trendelenburg position. During the recovery period of the interaction phase, six subjects experienced symptomatic orthostatic hypotension, and two subjects experienced nausea.

Pharmacodynamic Models for No Response to Name Called, Tolerance of Shake and Shout, Tolerance of Trapezius Squeeze, and Tolerance of Laryngoscopy

First a complete hierarchical interaction model was fit to the data (equations 1 and 2), estimating four parameters per stimulus ($EC50_R$, $EC50_D$, γ_R, and γ_D). This resulted in an overparameterized model, with opioid γ and remifentanil EC50 parameters not simultaneously estimable. Therefore, the four opioid γ values were fixed to 1. The model was then further reduced by (1) estimating a single dexmedetomidine γ value (γ_D) across the different stimuli (where the change in objective function value was +1.74), (2) removal of the four interaction components from the model (where the change in objective function value was +3.85), and (3) fixing γ_D to 1 (where the change in objective function value was 0). The resulting structural model is shown in equation 3.

$$P_{\text{tolerance of a stimulus}} = \frac{\dfrac{C_D}{EC50_D}}{1 + \dfrac{C_D}{EC50_D}} \tag{3}$$

Figure 1 shows the proportion of volunteers tolerating a stimulus as a function of the measured remifentanil concentrations during sole administration of remifentanil and during the interaction phase of our study. In analogy to our model-based findings, the figure shows no obvious effects of remifentanil on the probability of tolerance of any of the stimuli. The decreasing probabilities of no response to name called, tolerance of shake and shout, and tolerance of trapezius squeeze in the interaction phase, likely reflect the long stimulus–free interval preceding baseline observations (remifentanil = 0), in which we waited an hour for the wash-out of remifentanil and consecutively the equilibration of dexmedetomidine to

2 ng/ml, whereas the remaining observations were collected every 12 min.

Introducing age as a covariate significantly reduced in the objective function value (where the change in objective function value was 83.6). With age, dexmedetomidine EC50 decreases, showing an increasing sensitivity for dexmedetomidine with increasing age (fig. 2). The final model is described by equations 3 and 4. Parameter estimates and 90% confidence intervals are shown in table 2.

$$EC50_D = EC50_{(Dtyp)} \cdot e^{(-0.0481 \cdot (AGE - 35))} \qquad (4)$$

Pharmacodynamic Model for PSI

PSI decreases with an increase in dexmedetomidine concentrations as shown in figure 3. For increasing remifentanil concentrations, the decrease in PSI is less pronounced. During the interaction phase, similar PSI values are seen across increasing remifentanil concentrations with a fixed dexmedetomidine background concentration of 2 ng/ml.

The Greco model had the lowest Akaike information criterion and was therefore initially retained for further model building. However, log-likelihood profiling consistently showed poor precision for the remifentanil EC_{50} and

γ parameters in this model. Therefore, we also considered other models as starting points for model building. Of all models tested, the hierarchical model with interindividual variability on the EC_{50} parameters of remifentanil and dexmedetomidine had the lowest objective function value and was therefore retained as our final model. Visual inspection of the *post hoc* estimates for dexmedetomidine EC_{50} and PSI baseline did not show significant correlations to any of the covariates. Addition of these covariates to the model did not result in an improved goodness of fit.

Our final pharmacodynamic model for PSI is shown in equations 5 and 6. Parameter estimates and associated confidence intervals are shown in table 2. The response surface is shown in figure 4 and log-likelihood profiles and goodness-of-fit plots are presented in Supplemental Digital Content 1, figures S1.6 and S1.7 (http://links.lww.com/ALN/C12).

$$PSI_{pred} = Base \cdot \left(1 - E_{max} \cdot \frac{U^{\gamma_D}}{1 + U^{\gamma_D}}\right) \qquad (5)$$

$$U = \frac{C_D}{EC_{50D}} \cdot \left(1 + \left(\frac{C_R}{EC_{50R}}\right)^{\gamma R}\right) \qquad (6)$$
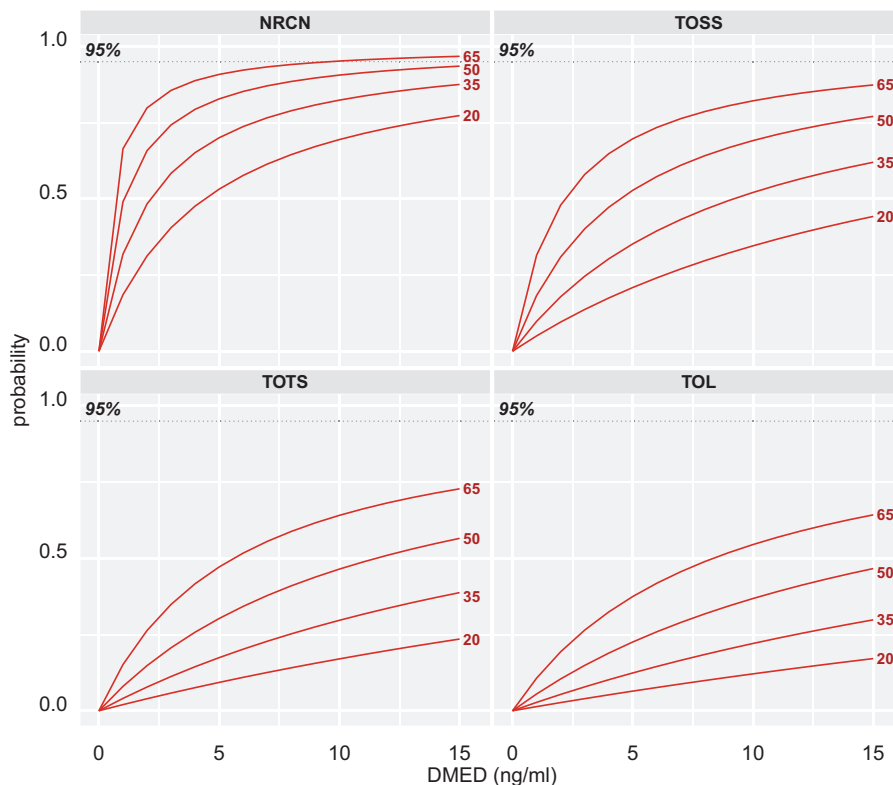
Fig. 2. Model predicted probabilities for no response to calling the subject by name (NRCN), tolerance of shake and shout (TOSS), tolerance of trapezius squeeze (TOTS), and tolerance of laryngoscopy (TOL) *versus* dexmedetomidine (DMED) concentrations. Probabilities are shown for 25-, 35-, 50-, and 65-yr-old subjects.

Table 1. Modified Observers' Assessment of Alertness and Sedation Score

Score	Response
5	Responds readily to name spoken in normal tone
4	Lethargic response to name spoken in normal tone
3	Responds only after name is called loudly and/or repeatedly
2	Responds only after mild prodding or shaking
1	Responds only after painful trapezius squeeze
0	No response after painful trapezius squeeze

For some volunteers in the remifentanil phase, the observed PSI values decreased below the PSI baseline predicted by the model. A graphical exploration of the data showed no clear relationship between PSI and measured remifentanil concentrations. We hypothesize that these measurements are due to remifentanil induced somnolence, a well known opioid side effect, causing the volunteers to fall asleep, thereby lowering the observed PSI values.

The Effect of Remifentanil on Arousability

For volunteers who tolerated laryngoscopy, the PSI values and MOAA/S scores recorded 5 min before and 2 to 3 min after laryngoscopy were compared. As shown in figure 5, PSI increased significantly after laryngoscopy in the dexmedetomidine phase, whereas a nonsignificant increase in PSI was seen in the interaction phase. In the dexmedetomidine phase, 7 of the 13 subjects who reached tolerance to laryngoscopy responded to name calling 2 to 3 min after the laryngoscopy. In the interaction phase, 4 of the 15 subjects who reached tolerance to laryngoscopy responded to name calling 2 to 3 min after the laryngoscopy attempt. Both the

PSI and MOAA/S scores suggest attenuation of the arousal brought on by laryngoscopy in the interaction phase.

Discussion

The aim of this study was to investigate the pharmacodynamic interaction of dexmedetomidine and remifentanil to guide dosing during anesthesia and sedation. In this study, the estimated EC_{50} values of dexmedetomidine were 2.1, 9.2, 24, and 35 ng/ml for no response to name called, tolerance of shake and shout, tolerance of trapezius squeeze, and tolerance of laryngoscopy, respectively. Surprisingly, we found no additional effect of remifentanil on the probability of no response to these stimuli. Age was inversely correlated with the dexmedetomidine EC_{50} for all four stimuli, suggesting that subjects become more sensitive to dexmedetomidine with increasing age. The depth of sedation as measured by PSI was described best by the hierarchical interaction model with an estimated dexmedetomidine EC_{50} of 0.49 ng/ml and remifentanil EC_{50} of 1.6 ng/ml. In contrast to the high dexmedetomidine EC_{50} concentrations for no response to name called, tolerance of shake and shout, tolerance of trapezius squeeze, and tolerance of laryngoscopy, relatively low plasma concentrations of dexmedetomidine are required to induce sedation/hypnosis as measured by PSI. Because the hierarchical model fitted the data best, this indicates that the effect of remifentanil administered without dexmedetomidine is negligible. The remifentanil EC_{50} of 1.6 ng/ml for PSI drug effect shows that there is an effect of clinical doses of remifentanil on PSI, despite remifentanil's lack of effect on the probability of no response to for no response to name called, tolerance of shake and shout, tolerance of trapezius squeeze, and tolerance of laryngoscopy.

In the European Medicines Agency–approved drug label, dexmedetomidine infusion rates of 0.7 to 1.4 μg ·

Table 2. Parameter Estimates of the Final Models

THETA	NRCN, TOSS, TOTS, and TOL	Estimate	90% CI Lower	90% CI Upper	PSI	Estimate	90% CI Lower	90% CI Upper	Shrinkage
	$EC50_{D typ}$								
	$EC50_{D\,NRCN}$	2.1	1.5	3.0	$EC50_D$	0.49	0.20	0.99	
	$EC50_{D\,TOSS}$	9.2	7.0	13	$EC50_R$	1.6	0.87	2.7	
	$EC50_{D\,TOTS}$	24	16	38	γ_D	1.0	0.76	1.4	
	$EC50_{D\,TOL\_}$	35	25	54	γ_R	2.3	1.5	3.5	
					Base PSI	82	79	84	
					E_{max}	0.75	0.73	0.77	
Covariate	Age	−0.048	−0.079	−0.028					
ETA					IIV_$EC50_D$	3.0	1.5	6.6	[10%]
					IIV_$EC50_R$	0.91	0.32	2.7	[32%]
RUV					Prop. error	0.041	0.036	0.048	[4%]

Base PSI, baseline PSI value; CI, confidence interval derived through bootstrap sampling (NRCN, TOSS, TOTS, and TOL model) and through log-likelihood profiling (PSI model); $EC50_{D/R}$, half-maximal effective concentration of dexmedetomidine/remifentanil; $EC50_{D typ}$, concentration dexmedetomidine at which the probability of a typical individual (35 yr old) tolerating a stimulus is 50%; ETA, interindividual variability on parameter estimates; IIV, interindividual variability; NRCN, no response to calling by name; Prop. error, proportional error; PSI, Patient State Index; RUV, residual unexplained variability; THETA, parameter estimates; TOL, tolerance of laryngoscopy; TOSS, tolerance of shake and shout; TOTS, tolerance of trapezius squeeze.
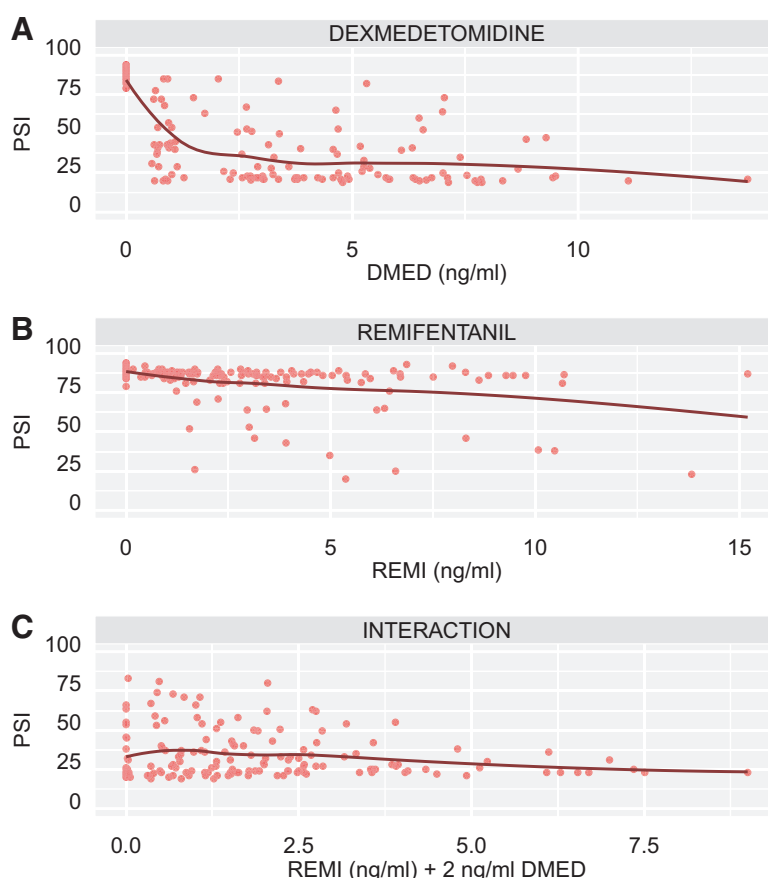
Fig. 3. All observations of the Patient State Index (PSI) obtained during steady-state observation phases plotted *versus* measured plasma concentrations of dexmedetomidine (DMED) and remifentanil (REMI). *Lines* are locally estimated scatterplot smoothers. Remifentanil concentrations are plotted in the interaction phases, with 0 representing a dexmedetomidine background concentration of 2 ng/ml with 0 ng/ml remifentanil.

kg^{-1} · h^{-1} are recommended, resulting in plasma concentrations of less than 2.5 ng/ml.[34] Within this range of dexmedetomidine concentrations, most subjects will appear asleep but remain arousable by name calling or a shake and shout stimulus (figs. 1 and 2). Of the estimated EC50 values for probability of tolerance of the applied stimuli, only the EC50 value (and not EC95) for no response to name called can be reached within clinical accepted concentrations. This is not surprising, because dexmedetomidine is known for its peculiarity that subjects, having fallen asleep, remain arousable within the clinical dose range. Previously it was stated that high concentrations of dexmedetomidine result in deep, unarousable sedation.[3,35] We were surprised that neither addition of remifentanil nor administration of supraclinical concentrations (up to 8 ng/ml) of dexmedetomidine could induce an unarousable state of sedation at a 95% probability of tolerance of laryngoscopy level, which is desirable for anesthetic induction. This study shows that although some volunteers reached an apparent unarousable

state (*i.e.*, tolerant of laryngoscopy), a significant proportion of volunteers remained arousable even with high concentrations of dexmedetomidine. Those who did appear unarousable and tolerated a laryngoscopy often also showed some delayed signs of arousal after all observations were done (fig. 5).

Although addition of remifentanil slightly increased the depth of sedation as measured by PSI, it did not increase the probability of no response to name called, tolerance of shake and shout, tolerance of trapezius squeeze, and tolerance of laryngoscopy. Considering the synergistic effect of remifentanil when added to propofol sedation, where remifentanil significantly reduces the concentration of propofol required to reach tolerance of shake and shout and laryngoscopy, our studied drug combination behaves in a very different way.[21] It seems contradictory that remifentanil, a potent analgesic, does not increase the probability of tolerance of trapezius squeeze and laryngoscopy. This can be explained by the fact that those painful stimuli were
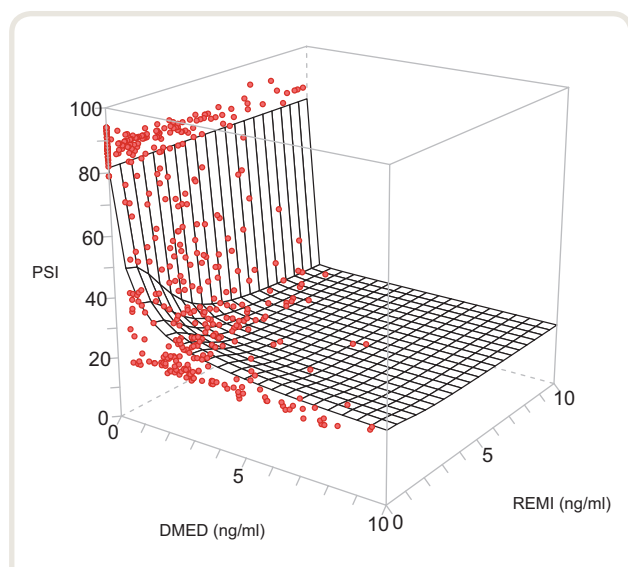
Fig. 4. Response surface for predicted Patient State Index (PSI) *versus* dexmedetomidine (DMED) and remifentanil (REMI) concentrations according to the final model. A window of 0 to 10 ng/ml is shown for both drugs to clarify the view. Observations are plotted as *red dots*.

only applied after no response to name called and shake and shout were tested as incorporated in the MOAA/S assessment. Because dexmedetomidine is known for inducing arousable sedation, subjects might already have been aroused to a certain degree by these preceding nonpainful stimuli before a painful stimulus was applied. Once a sufficient sedation level was reached for assessing tolerance of laryngoscopy, subjects who received remifentanil where

less aroused by this painful stimulus, compared with subjects who only received dexmedetomidine (fig. 5). Based on our data, it can be hypothesized that the coadministration of remifentanil during dexmedetomidine infusion, although not influencing the arousability, does decrease the intensity of a laryngoscopy. Earlier work by Kunisawa *et al.*[28] described a markedly lower dexmedetomidine EC50 for tolerance of "awake" laryngoscopy. Possibly subjects remained responsive but also became calm and cooperative. Therefore, although we found no interaction between remifentanil and dexmedetomidine on the pharmacodynamic endpoints tested in this trial, an interaction might be found when one looks at tolerance of awake laryngoscopy.

We found that dexmedetomidine potency increases with advancing age. Older volunteers tolerated noxious stimuli at lower concentrations of dexmedetomidine compared with younger volunteers. Within the clinically recommended dose range, concentrations up to 2.5 ng/ml can be achieved. At a concentration of 2.5 ng/ml, 83% of the 65-yr-old subjects were predicted to reach a sedative state in which they became unresponsive to calling their names. The probability of no response to calling their name for 20-yr-old volunteers at the same concentration is only 36%.

The main side effects observed during and after drug infusions were consistent with previously published adverse events for dexmedetomidine[34] and remifentanil.[36] Ventilatory adverse events (ventilatory depression, bradypnea, apnea) were mainly observed during the remifentanil step-up, whereas hemodynamic adverse events were mainly observed during the dexmedetomidine step-up. As previously shown by Colin *et al.*,[37] hypertension occurs at high dexmedetomidine plasma levels, whereas low plasma

Fig. 5. Patient State Index (PSI) values few minutes before and after subjects tolerated a laryngoscopy, in the dexmedetomidine phase (DMED) and the interaction phase (dexmedetomidine and remifentanil [DMED + REMI]).

concentrations of dexmedetomidine are associated with hypotension. The pharmacokinetic–pharmacodynamic model by Colin *et al.*[37] also shows that because of the slow onset of effects, the slow elimination of dexmedetomidine, and the low EC_{50} for the hypotensive effect, significant hypotension (and orthostatic hypotension) is expected during the recovery period.[37] Based on this, subjects were monitored at least 7 h after cessation of infusion in the dexmedetomidine phase and at least 5 h after the interaction phase. Symptomatic orthostatic hypotension was observed in the recovery period of 8 of 30 dexmedetomidine phases and 6 of 30 interaction phases. Despite our long recovery periods, 9 of the recovery periods needed to be extended because of persistent symptomatic orthostatic hypotension. In clinical practice, these long-lasting hemodynamic side effects are a major limitation for using dexmedetomidine in day care surgery.

We found acceptable performance of the Hannivoort model for dexmedetomidine and the Eleveld model for remifentanil compared with the other available models for these drugs. Median absolute performance errors between 20 and 30% and median performance errors below 20% are considered clinically acceptable and are in line with the performance of current pharmacokinetic–pharmacodynamic models used in target-controlled infusion pumps in anesthesia.[38] Absolute values of the performance criteria as published by Varvel *et al.*[33] have to be carefully interpreted in the context of this study, using supraclinical concentrations of dexmedetomidine and taking into account possible drug interactions. Using much lower concentrations of dexmedetomidine, Obara *et al.*[39] validated the Hannivoort model on their data and found also better performance of the Hannivoort model with a median absolute performance error of 18% and median performance error of 5.6%. When evaluating the performance of the Hannivoort model in the lower (clinical) concentration range, we concluded that no adjustments to the model need to be made for use in clinical practice as long as targets do not exceed 3 ng/ml. Whereas the Eleveld model performed well in the remifentanil phase, a remarkable increase in median performance error and median absolute performance error was seen in the interaction phase. An underlying pharmacokinetic interaction might be present, in which dexmedetomidine reduces remifentanil clearance. To avoid influence of these target-controlled infusion deviations, actual measured plasma concentrations obtained during apparent steady-state observation phases were used for modeling of pharmacodynamic outcomes. Data recorded during wash-out and recovery periods were not used in the modeling process, because during this phase there is no equilibrium between plasma and effect site concentrations.

From the PSI data (fig. 3), it seems that remifentanil alone has a slight effect on the PSI as well. It is not clear whether this reflects a real remifentanil effect or whether it reflects natural relaxation and sleepiness of the volunteers who were placed in supine position in a quiet area, with their eyes closed. The fact that the hierarchical model fitted the PSI data best suggests that the effect of remifentanil on the PSI is negligible. Because assessments of analgesic and sedative endpoints interfere with each other, this study design focused mainly on sedative endpoints. Regarding analgesia, the degree of interaction between remifentanil and dexmedetomidine remains unclear.

In conclusion, low plasma concentrations of dexmedetomidine are required to induce a gradually increasing sedative effect as is measured by PSI (dexmedetomidine EC_{50} = 0.49 ng/ml). However, although they become sedated and appear to be asleep, the majority of subjects remain arousable when stimulated by calling their names, shaking the patient while shouting their names, trapezius squeeze, and laryngoscopy, even when reaching supraclinical dexmedetomidine concentrations. Sensitivity to dexmedetomidine increases with age. Adding remifentanil, although it might reduce the intensity of a painful stimulus, did not alter the arousability of subjects to clinical stimuli. Therefore, although the combination dexmedetomidine and remifentanil might be useful in "conscious sedation" procedures, dexmedetomidine alone cannot be considered suitable to completely replace an intraoperative hypnotic. To ensure deep unarousable sedation as needed for most anesthetic inductions, different (or additional) hypnotics will be required.

Research Support

Competing Interests

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ANESTHESIOLOGY

Acoustic Shadowing Facilitates Ultrasound-guided Radial Artery Cannulation in Young Children

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Anesthesiology 2019; 131:1018–24

ABSTRACT

Background: Arterial cannulation in young children can be challenging. Ultrasound guidance using focused acoustic shadowing may be suitable for guiding radial artery puncture in young children. The present research tested the hypothesis that ultrasound guidance using focused acoustic shadowing helps increase the success rate of radial artery cannulation in this population.

Methods: In a double-blinded, parallel-group trial, 79 young children undergoing surgery under general anesthesia were randomly assigned to two groups (1:1 ratio): the traditional ultrasound group and the novel ultrasound group. Young children in the traditional group underwent conventional ultrasound-guided radial artery puncture, whereas those in the novel ultrasound group underwent radial artery puncture guided by acoustic shadowing ultrasound with double developing lines. All radial artery punctures were performed using the short-axis out-of-plane approach. The primary endpoint was the success rate of cannulation at the first attempt. The secondary endpoints included cannulation failure rate, ultrasound location time, and puncture time.

Results: The success rate of cannulation at the first attempt in the novel ultrasound group (35 of 39 [90%]) was significantly higher than that in the traditional ultrasound group (24 of 40 [60%]; difference: 30% [95% CI, 12 to 48%], $P = 0.002$). None of the patients in the ultrasound with acoustic shadowing group experienced failure of radial artery puncture and cannulation. The ultrasound location time and puncture time in the ultrasound acoustic shadowing group were significantly lower than that in the traditional ultrasound group (location time: median [interquartile range]: 6 [5, 8] *vs.* 18 [15, 21] s; puncture time: 24 [15, 41] *vs.* 40 [23, 56] s).

Conclusions: Acoustic shadowing *via* the use of double developing lines significantly improved the success rate of radial artery puncture in young children, compared with that achieved with the use of traditional ultrasound guidance.

(*Anesthesiology* 2019; 131:1018–24)

R adial artery cannulation is commonly performed in the operating room, intensive care unit, and emergency room for invasive arterial pressure monitoring and arterial blood gas analysis to guide the treatment of shock and electrolyte disturbance.[1] Radial artery cannulation is particularly challenging in young children owing to the small caliber of the artery. Multiple attempts attributable to failure of cannulation may lead to an arterial hematoma.[2] Radial artery cannulation is traditionally performed using anatomic landmarks and palpation of the radial pulse. The successful use of this technique requires experience, especially in young children. Some studies have shown that ultrasound-guided cannulation is more successful than the palpation technique.[3,4] In a randomized trial, critically ill children who required radial artery puncture for invasive monitoring were randomly divided into an ultrasound-guided group and a palpation technique group. The success rate of radial artery cannulation at the first attempt in the ultrasound-guided group was significantly greater than that in the palpation technique group.[4] However, the ultrasound-guided radial artery cannulation was not faster than the traditional palpation technique.[4] Although the success rate of puncture has improved with the ultrasound-guided technique, the success rate is largely dependent on the ultrasound operator's experience and skills. This is largely attributable to the two-dimensional nature of ultrasound imaging. The operator requires good hand–eye coordination, technical skills, and some experience to overcome this shortcoming of ultrasound, which limits the advantages of

EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Arterial cannulation in infants and young children is particularly challenging
- Ultrasound guidance facilitates arterial cannulation, but the success rate is still highly dependent on operator experience and skills

What This Article Tells Us That Is New

- This prospective, randomized trial in young children shows that a modified ultrasound-guided approach, using focused acoustic shadowing, results in a higher success rate and shorter cannulation time of the radial artery when compared with traditional ultrasound guidance

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ultrasound-guided vascular puncture, especially for operators with insufficient experience.[5–7] We adopted a new technique involving the use of double developing lines on the ultrasound probe to ameliorate this shortcoming of ultrasound. We hypothesized that ultrasound-guided vascular puncture with double developing lines could help increase the success rate of radial artery puncture in young children. The primary endpoint was the success rate of cannulation in the first attempt. The secondary endpoints were ultrasound localization time and puncture time.

Materials and Methods

This study was approved by the institutional review board of the Beijing You'An Hospital, Capital Medical University, Beijing, China. Written informed consent was obtained from the guardians of all participants before their enrollment. This trial was registered in the Chinese Clinical Trial Register (ChiCTR–INR–17011421; principal investigator: Zhefeng Quan; date of registration: May 17, 2017; http://www.chictr.org.cn/). This manuscript adheres to the reporting requirements specified in the Consolidated Standards of Reporting Trials guidelines.

Sample Size Calculation

The sample size of this study was based on the preliminary experiment, in which the success rate of the first puncture in the group with double developing lines was 85%, whereas the success rate in the control group was 55%. The test level α was taken as 0.05, Z0.05/2 = 1.96. The power level, 1 − β, was taken as 0.8. Therefore, a sample size of 34 was required in each group. Taking into account the rate of withdrawal (~15%), the final sample size for each group was identified as 40, necessitating a total study population of 80 patients.

Radial Artery Puncture

Young children undergoing elective surgical procedures that required continuous arterial pressure monitoring between May 12, 2017 and January 16, 2019 at the Beijing You'an Hospital and Beijing Friendship Hospital of Capital Medical University were eligible for inclusion. The inclusion criteria were: age range, 4 to 24 months; weight range, 4 to 10 kg; and American Society of Anesthesiologists grades I to IV. The exclusion criteria were: negative Allen's test; abnormal ulnar artery; infection near the radial artery puncture site; congenital heart disease; hemorrhagic or cardiogenic shock; patients who had undergone arterial puncture within the 1-month period immediately preceding the commencement of the trial. The young children who were enrolled were randomly assigned to the traditional ultrasound or ultrasound with double developing lines group (hereafter referred to as the novel ultrasound group) by sealed envelope (1:1 ratio) randomization assignment; envelopes prepared by statisticians were opened by the research assistant at the time of enrollment. All patients were enrolled

by the researchers, and data were recorded by residents who were blinded to the group allocation.

In the operating room, electrocardiogram, noninvasive arterial blood pressure, and peripheral oxygen saturation were monitored with IntelliVue MP70 (Philips, The Netherlands). After induction of general anesthesia, the left hand was chosen for radial artery puncture. All punctures were performed by attendants who had performed more than 50 ultrasound-guided punctures. Data were recorded by residents who were blinded to the group allocation.

The forearm was raised by 3 cm and the wrist was extended over a roll, with the hand positioned in dorsiflexion. Aseptic preparation of the skin around the site of insertion (2 cm proximal to the wrist joint on the palmar side of the forearm) was undertaken with povidone iodine. The ultrasonic probe with disposable sterile covers was connected to an ultrasound system (MINDRAY Medical International Company, China). The probe with a frequency of 11 MHz and a depth of 1 cm was adjusted to optimize the image. All radial artery punctures were performed using the short-axis out-of-plane procedure. Young children in the traditional group underwent conventional ultrasound-guided radial artery puncture, whereas radial artery puncture in the novel group was guided by ultrasound with double developing lines.

The metal-containing strand taken from x-ray–detectable surgical gauze was bound parallelly (interval: 2 mm) to the ultrasound probe and positioned perpendicular to the long axis of the probe. An ultrasonic couplant and sterile 3M membrane were applied over the ultrasound probe to fix the double developing lines (fig. 1). The probe was adjusted such that the radial artery was positioned between these shadows on the ultrasound image. These two lines produced two low-density shadows in the ultrasound image. Subsequently, the needle was inserted at an angle of 30° to the skin, between the two lines on the probe, and along the edge of the probe. The needle tip, which appeared as a white spot on the ultrasound screen, was directed toward the arterial lumen while remaining between the two small acoustic shadows on the screen (fig. 2) Once the needle had entered the radial artery, the angle of the needle was reduced from 30° to 15° and the needle was slowly pushed forward for 1 to 2 mm. The dynamic positions of the double developing lines, the needle tip, and the radial artery on the ultrasound screen during puncture are shown in figure 3. After retracting the needle a few millimeters into the cannula, the latter was fully introduced into the radial artery. Then the cannula was fixed and the blood pressure was monitored with a pressure transducer.

Observation Indices

The patient's general condition was recorded along with the measurement of the depth between the skin and the radial artery and the internal diameter of the radial artery. The primary endpoint was the success rate of cannulation at first attempt. The secondary endpoints were ultrasound localization
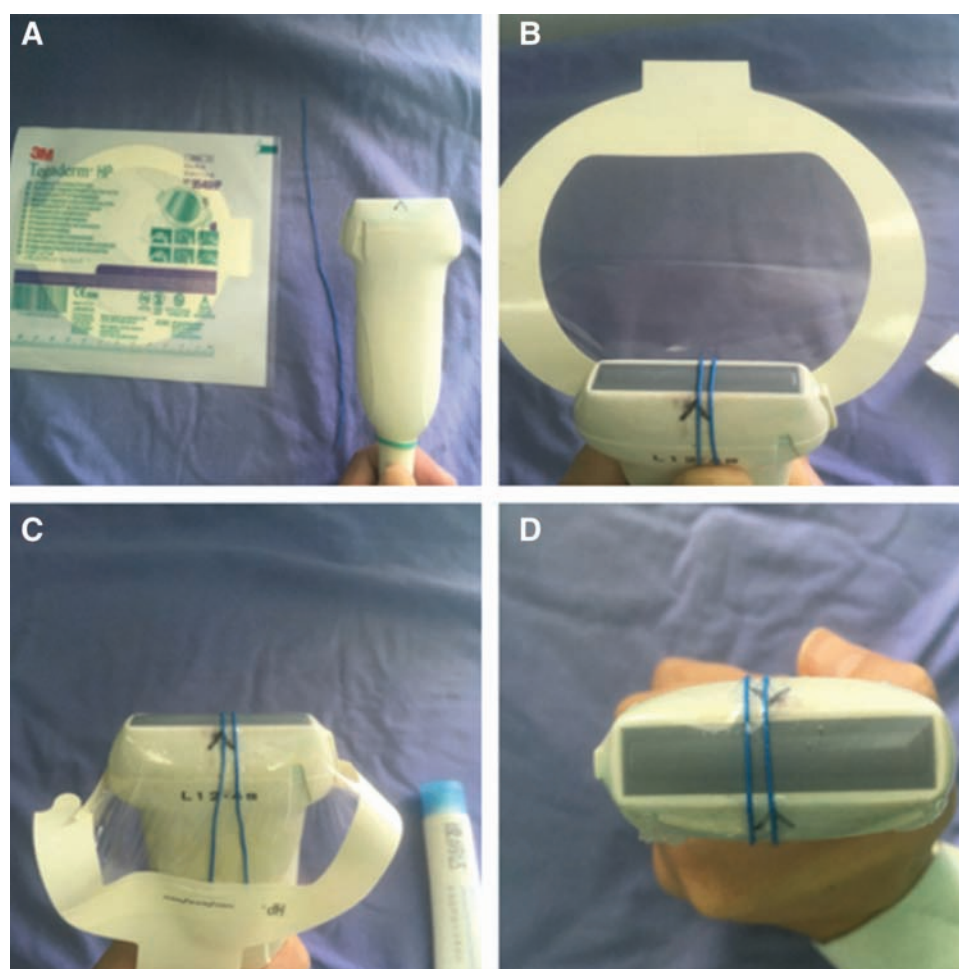
Fig. 1. The double developing lines on the ultrasound probe. Double parallel developing lines were bound to the midpoint of the ultrasound probe perpendicular to its long axis. (*A*) The ultrasound probe, the developing line, and the sterile 3M membrane. (*B*) The developing line was bound as double parallel lines on the midpoint of the ultrasound probe perpendicular to its long axis. The ultrasonic couplant was added on the ultrasound probe. (*C*) Sterile 3M membrane was used to cover the ultrasound probe. (*D*) The front view of double developing lines on the ultrasound probe.

time and puncture time. Successful puncture was considered to be a smooth insertion of the cannula into the radial artery. Failure of cannulation of the radial artery after three or more attempts was defined as puncture failure. Ultrasound localization time was defined as the time from the placement of the ultrasound probe over the skin to the penetration of the puncture needle into the skin. Puncture time was defined as the time that elapsed between the penetration of the puncture needle into the skin to the time of its penetration into the radial artery. The incidence of vascular complications, including bleeding and hematoma formation, was recorded.

Data Analysis

Statistical analysis was undertaken using Minitab 16.0 (Minitab Inc., USA). Data pertaining to normally distributed variables are presented as mean $\pm$ SD. Nonnormally distributed variables are presented as the median with the interquartile range. The Anderson–Darling test was used to test normality of data. The t test (two-sample, independent, unpaired) or Mann–Whitney U test was used to assess between-group differences. Data on the successful puncture rate and the incidence of adverse reactions were analyzed using Fisher exact test. Differences in incidence and the associated 95% CI were calculated. Two-tailed tests were used; P values less than 0.05 were considered indicative of statistically significant difference.

Results

Eighty young children were randomly divided into two groups. However, one patient dropped out because of cancellation of the surgery. The remaining 79 young children completed the study. There were no missing data. There

Fig. 2. The positions of ultrasound probe with double developing lines and needle. (*A*) Positions of the ultrasound probe with double developing lines and the needle. (*B*) Ultrasonic image of the radial artery (*red arrow*) and the low-density shadow of the double developing lines (*yellow arrow*).

(interquartile range: 15, 21) in the traditional ultrasound group ($P < 0.001$). The median puncture time was 24 (interquartile range: 15, 41) s in the novel ultrasound group and 40 (interquartile range: 23, 56) s in the traditional ultrasound group ($P = 0.012$; table 2). Moreover, 12 of 40 (30%) young children in the traditional group and 4 of 39 (10%) young children in the novel group experienced bleeding at the puncture point (difference: 20% [95% CI, 2.65 to 36.84%], $P = 0.029$). There was no significant between-group difference with regard to the incidence of hematoma (7 of 40 [18%] *vs.* 3 of 39 [8%], $P = 0.190$; table 3).

Discussion

In the novel ultrasound group, the success rate of radial artery puncture at first attempt as well as the location time and puncture time were approximately 30% higher and 13 and 16 s shorter, respectively, than in the traditional ultrasound group. This may be related to the fast positioning and accurate guidance provided by ultrasound with double developing lines.

The procedure of radial artery puncture can be divided into three steps.[8] The first step involves localization of the puncture point, whereas the second step involves the puncture of the artery. The final step involves the insertion of the cannula into the radial artery. The double developing lines technique helps locate the projection point of the midpoint of the radial artery on the skin surface to enable quick and accurate determination of the puncture point. During the puncture, the direction of the needle is maintained at the same level as that of the developing lines of the ultrasound probe, which helps guide the puncture. This novel ultrasound technique isolates the contact between the ultrasound probe and the skin through the double developing lines on the ultrasound probe, thereby displaying a vertical low-density shadow in the ultrasound image. Thus, the needle can be positioned accurately prior to skin penetration, which indirectly compensates for the shortcomings of the method guided by two-dimensional ultrasound.

Nakayama *et al.* found that the success rate of ultrasound-guided radial artery puncture is highest when the depth of the radial artery is 2 to 4 mm.[9] The proximity to the skin does not improve the success rate of ultrasound-guided puncture. This finding is related to the shortcomings of two-dimensional ultrasound because the puncture needle can only be discovered by the two-dimensional ultrasound probe when it reaches a certain depth.[9]

The dynamic needle-tip positioning technique has been shown to significantly improve the clinically relevant aspects of radial artery catheterization.[10,11] However, this technique relies on the experience and skill of the ultrasound operator.

In this study, the radial artery was placed between the two low-density shadows of double developing lines by adjusting the ultrasound probe. Then the puncture needle was placed at the intersection of the double developing lines and the skin, which is the projection point of the radial

were 40 patients in the traditional ultrasound group and 39 patients in the novel ultrasound group. The baseline characteristics of patients are shown in table 1. There were no significant between-group differences with regard to the depth of the radial artery from the skin (2 ± 0 *vs.* 2 ± 0 mm, $P = 0.732$) or the inner diameter of the radial artery (2 ± 0 *vs.* 2 ± 0 mm, $P = 0.947$; table 1). In the novel ultrasound group, the success rate of cannulation at the first attempt (35 of 39 [90%]) was significantly greater than that in the traditional ultrasound group (24 of 40 [60%]; difference: 30% [95% CI, 12% to 48%], $P = 0.002$). Four patients experienced failure of cannula insertion in the traditional ultrasound group, as against none in the novel ultrasound group. The median ultrasonic location time in the novel ultrasound group was 6 s (interquartile range: 5, 8), compared with 18 s

Fig. 3. The process of radial artery puncture guided by acoustic shadowing ultrasound with double developing lines. The developing lines are made of metal-containing strands taken from x-ray–detectable surgical gauze. The *silver line* indicates the needle tip. The *black circle* indicates the radial artery. (*A*) The needle tip is about to pierce the skin; (*B*) the needle tip has pierced the skin but has not reached the vessel; (*C*) the needle tip pierces deeper; (*D*) the needle tip is in the vessel.

Table 1. Baseline Characteristics of the Study Subjects

Parameter	Traditional Ultrasound Group (n = 40)	Novel Ultrasound Group (n = 39)	P Value
Age, months	9 ± 3	10 ± 2	0.876
Weight, kg	7 ± 1	7 ± 1	0.285
Height, cm	67 ± 4	66 ± 5	0.209
ASA Grade II, n	3 (7%)	2 (5%)	0.872
ASA Grade III, n	25 (63%)	27 (69%)	
ASA Grade IV, n	12 (30%)	10 (26%)	
Male, n	17 (43%)	18 (46%)	0.744
Mean arterial pressure, mmHg	55 ± 6	53 ± 5	0.109
Depth from skin to artery, mm	2 ± 0	2 ± 0	0.732
Inner diameter of artery, mm	2 ± 0	2 ± 0	0.947

Data presented as mean ± SD or as frequency and percentage (%). ASA, American Society of Anesthesiologists.

Table 2. Comparison of Success Rate of Cannula Insertion at First Attempt, Failure Rate, Location Time, and Puncture Time between the Two Groups

Parameter	Traditional Ultrasound Group (n = 40)	Novel Ultrasound Group (n = 39)	P Value	Proportion Difference (95% CI)
Success rate of cannula insertion at first attempt	24 (60%)	35 (90%)	0.002	−0.297 (−0.477 to −0.118)
Failure rate of cannula insertion	4 (10%)	0	0.359	0.1 (0.007 to 0.193)
Ultrasound location time, s	18 (15, 21)	6 (5, 8)	< 0.001	
Puncture time, s	40 (23, 56)	24 (15, 41)	0.012	

Data presented as number and percentage (%) or median (interquartile range).

Table 3. Vascular Complications during Radial Artery Puncture

Parameter	Traditional Ultrasound Group (n = 40)	Novel Ultrasound Group (n = 39)	P Value	Proportion Difference (95% CI)
Bleeding, n (%)	12 (30%)	4 (10.3%)	0.029	0.197 (0.027 to 0.368)
Hematoma, n (%)	7 (17.5%)	3 (7.7 %)	0.190	0.098 (−0.046 to 0.243)

artery on the surface. This technique greatly shortens the ultrasonic location time. After the localization of the puncture point, when the needle stays at the same level as the double developing lines, it is easy to obtain the image of the vertical direction of the needle, which helps shorten the puncture time. Another advantage of this method is that the operator can focus on the two low–density shadows instead of staring at the full screen of the ultrasound monitor. When the needle stays at the same level as that of the double developing lines, the needle is between the two low-density shadows. An experienced operator can visualize the needle tip indenting the anterior wall of the radial artery within this two–line shadow. For inexperienced operators or those who are unable to visualize the position of the needle tip, the insertion of the needle into the artery can be ascertained by observing blood return in the needle hub.

In this study, the incidence of bleeding in the novel ultrasound group was lower than that in the traditional ultrasound group, which is likely attributable to the higher initial success rate of the puncture in the former group. There were no significant between-group differences with regard to the incidence of other adverse reactions.

A limitation of this study was that it was not a double-blind trial. During the puncture, the operator needs to simultaneously pay attention to the ultrasound image to ensure the needle stays at the same level as the double developing lines. Additionally, only skilled attendants who have carried out more than 50 ultrasound-guided punctures participated in this study. Therefore, we cannot determine how much the level of expertise influenced the success rate. Given the special anatomic characteristics of young children, it may not be appropriate to generalize these data to other populations, which is another potential limitation of this study.

Conclusions

The double developing lines technique not only helps shorten the ultrasound location and puncture time, but also improves the success rate of radial artery puncture at the first attempt in young children, and thereby facilitates radial artery cannulation in young children.

Research Support

Competing Interests

The authors declare no competing interests.

Reproducible Science

Full protocol available at: 15811190059@126.com. Raw data available at: 15811190059@126.com.

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ANESTHESIOLOGY

A Population-based Comparative Effectiveness Study of Peripheral Nerve Blocks for Hip Fracture Surgery

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Hip fractures are the most frequent indication for emergency surgery in older adults.[1] More than 300,000 hip fracture surgeries are performed annually in the United States,[2] more than 65,000 in the United Kingdom,[3] and more than 20,000 in Canada.[4] Individuals requiring hip fracture surgery are typically older and are often medically complex.[5,6] Mortality and adverse events are common after hip fracture surgery; one in five individuals experience a complication and 1-yr mortality rates exceed 25%.[7–9] Given the rapid aging of the population,[10,11] hip fractures will continue to be a significant contributor to adverse individual and population-level health outcomes. While many studies and reviews have evaluated the role of neuraxial *versus* general anesthesia in hip fracture care,[12–14] further anesthetic strategies to improve outcomes and decrease healthcare resource use after hip fracture surgery are urgently needed.

The advanced age and high baseline illness severity typical of hip fracture surgery patients may leave them vulnerable to adverse effects of systemic opioid analgesics, which are used to treat fracture- and surgery-related pain.[15,16] However, poorly treated acute pain is also associated with negative clinical outcomes.[16,17] Therefore, alternate analgesic strategies, such as peripheral nerve blocks, could help to improve postoperative clinical outcomes by decreasing requirements for systemic opioids and reducing

ABSTRACT

Background: Adverse outcomes and resource use rates are high after hip fracture surgery. Peripheral nerve blocks could improve outcomes through enhanced analgesia and decreased opioid related adverse events. We hypothesized that these benefits would translate into decreased resource use (length of stay [primary outcome] and costs), and better clinical outcomes (pneumonia and mortality).

Methods: The authors conducted a retrospective cohort study of hip fracture surgery patients in Ontario, Canada (2011 to 2015) using linked health administrative data. Multilevel regression, instrumental variable, and propensity scores were used to determine the association of nerve blocks with resource use and outcomes.

Results: The authors identified 65,271 hip fracture surgery patients; 10,030 (15.4%) received a block. With a block, the median hospital stay was 7 (interquartile range, 4 to 13) days *versus* 8 (interquartile range, 5 to 14) days without. Following adjustment, nerve blocks were associated with a 0.6-day decrease in length of stay (95% CI, 0.5 to 0.8). This small difference was consistent with instrumental variable (1.1 days; 95% CI, 0.9 to 1.2) and propensity score (0.2 days; 95% CI, 0.2 to 0.3) analyses. Costs were lower with a nerve block (adjusted difference, −$1,421; 95% CI, −$1,579 to −$1,289 [Canadian dollars]), but no difference in mortality (adjusted odds ratio, 0.99; 95% CI, 0.89 to 1.11) or pneumonia (adjusted odds ratio, 1.01; 95% CI, 0.88 to 1.16) was observed.

Conclusions: Receipt of nerve blocks for hip fracture surgery is associated with decreased length of stay and health system costs, although small effect sizes may not reflect clinical significance for length of stay.

(*Anesthesiology* 2019; 131:1025–35)

EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Many observational analyses and ongoing randomized trials have evaluated the potential value of neuraxial *versus* general anesthesia for hip fracture surgery
- The association between peripheral nerve blocks and outcomes after hip fracture surgery is less well studied

What This Article Tells Us That Is New

- Among elderly patients undergoing emergency hip fracture surgery in Ontario, Canada, peripheral nerve blocks may be associated with slightly decreased postoperative lengths of stay and health system costs
- The use of peripheral nerve blocks was not associated with a difference in postoperative pneumonia rates

opioid-related adverse effects. A recent Cochrane review that included 31 trials and 1,760 participants[18] found high-quality

evidence that supports the efficacy of peripheral nerve blocks in improving acute pain scores; moderate-quality evidence supports the role of peripheral nerve blocks in decreasing pneumonia and time to first mobilization. Inadequate data were available to estimate the impact of peripheral nerve blocks on other outcomes. Evidence supporting the generalizability of these findings, as well as the population-level impact of peripheral nerve blocks on clinical outcomes or resource use after hip fracture surgery are lacking.

We hypothesized that older hip fracture surgery patients who receive a peripheral nerve block would be less likely to experience opioid-related adverse events, however, in-hospital use of these drugs and related adverse events are not routinely coded in population-level data. Therefore, we further hypothesized that decreasing opioid-related adverse events should translate into decreased resource use, such as shorter length of stay (a priority outcome for older people[19]) and lower healthcare costs (a secondary outcome). We also hypothesized that the odds of clinical outcomes validly available in population-level data, such as all-cause mortality and pneumonia, might also be lower when peripheral nerve blocks were used.

Materials and Methods

Design and Setting

Following protocol registration (osf.io/ts658/), we conducted a population-based cohort study in Canada's most populous province (Ontario; more than 13 million inhabitants), which provides universal physician and hospital health insurance coverage. Healthcare data in Ontario are collected using standardized methods and are stored at ICES.[20] For the current study, data were linked deterministically using encrypted, patient-specific identifiers across the following databases: the Discharge Abstract Database (acute care hospitalization details including diagnoses, procedures and length of stay); the Ontario Health Insurance Plan (physician service claims); the National Ambulatory Care Reporting System (all emergency and outpatient care); the Continuing Care Reporting System (long-term and respite care); the Ontario Drug Benefits Database (prescription drug claims for residents greater than or equal to 65 yr); and the Registered Persons Database, which captures all death dates for residents of Ontario. Because data used for this study were routinely collected and deidentified, it was legally exempt from research ethics review.

Cohort

We identified all Ontario residents age 66 yr or older on the day of their hip fracture surgery using Canadian Classification of Interventions codes for hip fracture surgery (diagnostic code S72 for hip fracture plus an emergency hospital admission that included procedural codes 1VA53, 1VA74, 1VC74, or 1SQ53).[21] Reabstraction studies demonstrate high levels of agreement when identifying hip fracture surgery patients (κ = 0.95; positive predictive value,

0.95 [95% CI, 0.94 to 0.97]).[22] This was a patient-level analytic data set and included surgeries occurring from January 1, 2011 to December 31, 2015. Use of this start date placed all data after an update to peripheral nerve block physician billing codes that occurred in 2008. Data from 2008 to 2009 were excluded to allow normalization of billing code use, while instrumental variable analysis required a 1-yr lookback, making 2011 the earliest available start date. The end date reflects the most recent availability of complete data.

Exposure

We identified peripheral nerve blocks 1 day before, on the day of, or 1 day after, surgery using Ontario Health Insurance Plan physician billing codes (G260: major plexus block [could include 3-in-1 block, lumbar plexus, or sacral plexus block]; G060: major peripheral nerve block [could include fascia iliaca or femoral nerve block]; G061: minor peripheral nerve block [could include block of terminal branches, such as lateral femoral cutaneous]; G279: percutaneous nerve block catheter) that have been validated against a clinical reference to demonstrate their accuracy (positive likelihood ratio, 16.83; negative likelihood ratio, 0.03; sensitivity, 97%; specificity, 94%) for correctly identifying the true presence (or absence) of a peripheral nerve block.[23] Peripheral nerve blocks were coded as provided or not provided based on the presence (or absence) of a billing code within one day of surgery.

Outcomes

Our primary outcome was postoperative length of stay, measured from the Discharge Abstract Database as the number of days from surgery to hospital discharge.[22] Our secondary resource use outcome was 30-day health system costs (calculated from the day of surgery to 30 days after surgery from the perspective of the healthcare system). We used patient-level validated costing algorithms,[24,25] standardized to 2016 Canadian dollars. This approach includes all direct costs (those where costs are directly and specifically available and attributable to the patient such as physician service claims, diagnostic and laboratory testing, pharmaceuticals, equipment or medical devices, home care) and indirect costs (*i.e.,* health system utilization of inpatient and outpatient hospital care, emergency care, inpatient rehabilitation, complex continuing care, and long-term care, calculated by accounting for an individual's resource intensity weight, case-mix group and duration of care) to the health system. This approach includes the cost of surgery but lacks the granularity to specifically account for materials such as regional anesthesia supplies (the anesthesiologist's fee for placing the peripheral nerve block is included). Patient costs incurred outside of those covered by the health system are not accounted for. Clinical outcomes were in-hospital pneumonia, identified using validated *International Classification of Diseases, Tenth Revision*

codes J10 through J18, flagged as arising during hospitalization from the index the Discharge Abstract Database,[26] and 30-day all-cause mortality (from the Discharge Abstract Database and Registered Persons Database). *Post hoc* we identified discharge disposition from the Discharge Abstract Database, which was categorized as institutional (nursing home, continuing care facility, rehabilitation) *versus* home (back to prefracture place of residence).

Covariates

We measured baseline covariates that we postulated—based on clinical and epidemiologic knowledge—could influence receipt of a peripheral nerve block, as well as outcome risk. Demographics were identified from the Discharge Abstract Database and from the Canadian Census. Standard methods were used to identify Elixhauser comorbidities using *International Classification of Diseases, Tenth Revision* codes from the Discharge Abstract Database in in the 3 yr preceding surgery.[27] Preoperative residence in a long-term care facility was identified from the Continuing Care Reporting System. We calculated each individuals' hospital-patient one-year mortality risk score.[28] Prescription drugs in the 6 months before surgery were identified (opioids, anticoagulants, antiplatelet agents, antipsychotics, benzodiazepines, and dementia medications). The Johns Hopkins Adjusted Clinical Groups system was used to identify healthcare resource utilization bands and frailty-defining diagnoses.[29] The specific surgical procedure, and a unique identifier for each hospital, was recorded from the Discharge Abstract Database. We identified the year of surgery as a potential confounder as, in 2013, evidence-based provincial recommendations[30] for hip fracture care were published that recommended nerve block use. We also identified whether each patient was admitted to a trauma service (as opposed to an orthopedic surgery admission) by identifying the specialty of the physician service listed as most responsible for the admission in the Discharge Abstract Database.

Sample Size

This was a population-based study; therefore, all eligible members of the Ontario population were included. All tests of significance were two-tailed with alpha = 0.05.

Missing Data

No exposure, outcome, or covariate data was missing.

Statistical Analysis

All analyses were performed using SAS 9.4 (SAS Institute, USA). Baseline covariate data was compared between those with and without a peripheral nerve block using absolute standardized differences; values greater than 0.10 were considered to represent substantial imbalance.[31]

Unadjusted outcome rates were compared between exposure levels. Because our length of stay and cost distributions were skewed, but our regression approach is based on transformed mean values, we reported summary statistics using both means and SD and medians and interquartile ranges. Our primary adjusted analysis used multilevel, multivariable, generalized linear regression with a log link and γ response distribution (which is recommended for surgical data to account for the skewed length of stay distribution).[32] Cost data were analyzed in the same manner as length of stay.[33] When exponentiated, the β coefficient from a log-γ model can be interpreted as the ratio of means. A generalized linear model with a logit link and binomial distribution (*i.e.,* logistic regression) was used for mortality and pneumonia. Differences in continuous outcomes (length of stay, costs) on the absolute difference scale were calculated using predicted outcome estimates from the adjusted model, generated across 1,000 bootstrap samples (created using 1:1 resampling with replacement).[34] The median, 97.5th and 2.5th percentiles of the bootstrap distribution were used to define the effect size and 95% CI.

All adjusted regression models used generalized estimating equation methods to account for clustering of patients in hospitals, and included the exposure of interest (peripheral nerve block), age (restricted cubic spline with five knots), biologic sex (binary), neighborhood income quintile (five-level categorical variable), rurality (binary), procedure (categorical), hospital-patient one-year mortality risk score (continuous linear), each Elixhauser comorbidity (binary), each specified drug class (binary), year of surgery (categorical), resource utilization band (categorical), frailty (binary), and preoperative long-term care residence (binary).

Prespecified Sensitivity Analyses

Two prespecified additional approaches were used to analyze the association of receipt of peripheral nerve block with length of stay. Because even with a robust set of measured confounders, unmeasured differences between patients who did or did not receive a peripheral nerve block could still exist and influence outcome, our first approach was to account for the likely presence of unmeasured confounding variables using an instrumental variable analysis (Supplemental Digital Content 1, http://links.lww.com/ALN/C47). First, we performed a Durbin–Wu–Hausman test of endogeneity to evaluate whether unmeasured confounding may be present.[35–37] Next, we performed the instrumental variable analysis, which leverages a source of natural variation (sometimes called quasi-randomization) to help block the influence of unmeasured variables, while adjusting for measured confounders. A common type of instrumental variable is called the hospital preference instrumental variable, which has been used in previous studies in perioperative medicine.[38] This approach assumes that the local practice pattern (in our case the proportion of hip fracture patients at each participant's hospital who received a peripheral nerve block

in the year before each participant's surgery) influences receipt of the intervention (*i.e.,* the peripheral nerve block), without otherwise influencing the outcome. This approach is built on three key assumptions: (1) patients who happen to receive care at a hospital that provides a high proportion of peripheral nerve blocks to its hip fracture patients is more likely to receive a peripheral nerve block than someone who presents to a low peripheral nerve block–use hospital, regardless of their personal characteristics; (2) going to a high peripheral nerve block use hospital does not influence outcome, except through receipt of a peripheral nerve block; and (3) going to a high peripheral nerve block use hospital is not influenced by a patient's unmeasured characteristics. The first two assumptions are verifiable by looking at the strength of correlation between the instrumental variable and receipt of a peripheral nerve block (which should be strong) and with outcome (which should be negligible). The last assumption is not directly verifiable, although determining that measured patient characteristics between high and low peripheral nerve block–use hospitals are similar supports the validity of the assumption, as does content knowledge, like the fact that a patient requiring emergency surgery is unlikely to choose their hospital based on regional anesthesia practice.[39] The second additional approach was a propensity score matched analysis (Supplemental Digital Content 2, http://links.lww.com/ALN/C47).

Subgroup Analyses

We tested for the presence of effect modification for length of stay by adding the following prespecified multiplicative interaction terms to the primary adjusted regression model: (1) peripheral nerve block × chronic obstructive pulmonary disease (as regional anesthesia techniques may be more effective in people with respiratory disease[40]); (2) peripheral nerve block × sex (as sex and gender based analyses are recommend in health services research[41]); (3) peripheral nerve block × dementia (as opioids are associated with delirium in older hospitalized patients[42]); (4) peripheral nerve block × frailty (as individuals with frailty are vulnerable to adverse health outcomes[43]); (5) peripheral nerve block × preoperative opioid prescription (as peripheral nerve blocks may be particularly beneficial in individuals with preexisting chronic pain[44]). *Post hoc* we tested a peripheral nerve block × trauma admission interaction term. Where an interaction term had a *P* value less than 0.05, we calculated the effect estimate at each level of the effect-modifying variable.

Post Hoc Sensitivity Analyses

Following completion of our primary analyses, we performed several *post hoc* analyses to test assumptions in our primary analysis. First, because death in-hospital could decrease length of stay in a biased manner, we repeated the primary analysis limited to individuals discharged alive from hospital. Next, because neuraxial (*vs.* general) anesthesia is

a regional anesthesia technique associated with decreased length of stay but is a covariate that could precede receipt of a peripheral nerve block (therefore acting as a confounder), we repeated the primary analysis with the addition of a reviewer-recommended categorical variable representing receipt of a neuraxial anesthetic (spinal or epidural), general anesthetic, or combined general and neuraxial anesthesia. Peer reviewers also recommended that we perform a fixed effects regression analysis with a hospital identifier entered as a categorical variable (as opposed to accounting for each hospital using a clustered, generalized estimating equation approach as we did in our primary analysis; Supplemental Digital Content 3, http://links.lww.com/ALN/C47).

Results

We identified 65,271 hip fracture surgery patients; 10,030 received a peripheral nerve block (15.4%). Of people with a peripheral nerve block, 62.2% were billed as a plexus block and 37.8% as a major nerve block, and no one had a minor block billed as the sole peripheral nerve block; only 0.6% of patients had a continuous catheter inserted. Standardized differences for all measured covariates suggested non-substantial differences between exposure groups except for a larger proportion peripheral nerve block receivers being from a nonrural residence and having surgery in 2015 (table 1). *Post hoc* we identified that 9,054 (13.9%) patients were admitted to a trauma service.

Peripheral Nerve Blocks and Postoperative Length of Stay

The mean and median postoperative length of stay for people who received a peripheral nerve block were 11.5 ± 17.7 and 7 (interquartile range, 4 to 13) days; for people without a peripheral nerve block the mean and median length of stay were 12.5 ± 19.0 and 8 (interquartile range, 5 to 14) days. Before covariate adjustment, receipt of a peripheral nerve block was associated with a decrease in length of stay (1-day decrease; ratio of means, 0.92; 95% CI, 0.90 to 0.94). After multilevel multivariable adjustment, receipt of a peripheral nerve block remained associated with decreased length of stay (0.6-day decrease; 95% CI, 0.5 to 0.8; ratio of means, 0.96; 95% CI, 0.93 to 0.99). The fully-adjusted regression model is presented in table 2.

The proportion of hip fracture patients who received a peripheral nerve block at each participant's hospital in the year before the index surgery met the two verifiable assumptions required for a valid instrumental variable: (1) it was strongly associated with receipt of a peripheral nerve block (*F*-statistic, 45.5); and (2) it was uncorrelated with length of stay (correlation coefficient, −0.02). Measured covariate data for patients at high *versus* low (cut off greater than 8% *vs.* less than or equal to 8%) peripheral nerve block–use hospitals is provided in the Supplemental Digital Content (http://links.lww.com/ALN/C47), showing that

Table 1. Baseline Characteristics of Study Population by Peripheral Nerve Block Status

	Peripheral Nerve Block (n = 10,030)	No Peripheral Nerve Block (n = 55,241)	ASD*
Demographics			
Age at surgery (yr; mean ± SD)	79 ± 13	78 ± 14	0.07
Female	7,015 (69.9)	37,581 (68.0)	0.04
Income quintile			
1 (lowest)	2,178 (21.7)	12,167 (22.0)	0.01
2	2,216 (22.1)	11,069 (20.0)	0.05
3	1,985 (19.8)	10,912 (19.8)	0.00
4	1,913 (19.1)	10,633 (19.3)	0.01
5 (highest)	1,738 (17.3)	10,460 (18.9)	0.04
Rural	1,019 (10.2)	7,826 (14.2)	0.12
Year of surgery			
2011	1,640 (16.4)	10,587 (19.2)	0.07
2012	1,839 (18.3)	11,205 (20.0)	0.04
2013	2,070 (20.6)	11,571 (21.0)	0.01
2014	1,949 (19.5)	11,365 (20.6)	0.03
2015	2,532 (25.3)	10,693 (19.4)	0.14
Comorbidities			
Alcohol abuse	283 (2.8)	2,031 (3.7)	0.05
Atrial arrhythmia	835 (8.3)	4,783 (8.7)	0.01
Blood loss anemia	1,673 (16.7)	10,241 (18.5)	0.05
Cardiac valve disease	321 (3.2)	1,829 (3.3)	0.01
Coagulopathy	223 (2.2)	1,492 (2.7)	0.03
Chronic obstructive pulmonary disease	1,104 (11.0)	6,574 (11.9)	0.03
Cerebrovascular disease	458 (4.6)	2,726 (4.9)	0.01
Disease of pulmonary circulation	216 (2.2)	1,387 (2.5)	0.02
Dementia	1,780 (17.8)	9,944 (18.0)	0.01
Depression	460 (4.6)	2,729 (4.9)	0.01
Deficiency anemia	56 (0.6)	352 (0.6)	0.00
Diabetes mellitus without complications	1,377 (13.7)	7,505 (13.6)	0.00
Diabetes mellitus with complications	1,450 (14.5)	7,668 (13.9)	0.02
Dialysis	166 (1.7)	803 (1.5)	0.02
Drug abuse	68 (0.7)	477 (0.9)	0.02
Heart failure	2,142 (21.4)	11,601 (21.0)	0.01
Hemiplegia	72 (0.7)	479 (0.9)	0.02
Hypertension without complications	4,002 (39.9)	23,202 (42.0)	0.04
Hypertension with complications	95 (1.0)	493 (0.9)	0.01
Liver disease	125 (1.3)	815 (1.3)	0.00
Malignancy	630 (6.3)	3,687 (6.7)	0.02
Metastases	180 (1.8)	1,127 (2.0)	0.00
Obesity	128 (1.3)	886 (1.6)	0.03
Peptic ulcer disease	139 (1.4)	852 (1.4)	0.00
Peripheral vascular disease	231 (2.3)	1,328 (2.4)	0.01
Psychoses	91 (0.9)	560 (1.0)	0.01
Renal disease	408 (4.1)	2,259 (4.1)	0.00
Rheumatic disease	96 (1.0)	701 (1.3)	0.03
Venous thromboembolism	67 (0.7)	436 (0.8)	0.01
Weight loss	338 (3.4)	1,857 (3.4)	0.00
Frail	6,050 (60.3)	33,621 (60.9)	0.01
Hospital One-year Mortality Risk score (mean ± SD)	37 ± 6	37 ± 7	0.01
Medications			
Anticoagulant	1,240 (12.4)	7,258 (13.1)	0.02
Antiplatelet agent	797 (8.0)	4,074 (7.4)	0.02
Antipsychotic	1,165 (11.6)	5,891 (10.7)	0.03
Benzodiazepine	1,771 (17.7)	9,328 (16.9)	0.02
Opioid	2,228 (22.2)	12,233 (22.1)	0.00
Dementia medication	755 (7.5)	4,228 (7.8)	0.01
Healthcare resource use			
Long-term care facility	1,571 (15.7)	8,383 (15.2)	0.01
Resource utilization band			
2 (lowest)	205 (2.0)	1,220 (2.2)	0.01
3	1,370 (13.7)	7,587 (13.7)	0.00
4	2,465 (24.6)	13,097 (23.7)	0.02
5 (highest)	5,990 (59.7)	33,337 (60.4)	0.01
Procedure			
Implantation of internal device, pelvis	16 (0.2)	147 (0.3)	0.02
Implantation of internal device, hip joint	3,449 (34.4)	20,914 (37.9)	0.07
Fixation, hip joint	2,233 (22.3)	10,403 (18.8)	0.09
Fixation, femur	4,332 (43.2)	23,777 (43.0)	0.00

All column values indicate n (%) unless otherwise indicated.

*Values greater than 0.10 indicate a substantial difference.

ASD, absolute standardized difference.

Table 2. Adjusted Regression Model for Length of Stay

	RoM	95% CI
Covariate		
Peripheral nerve block *vs.* none	0.96	0.93–0.99
Year of surgery		
2015	Ref	1.00–1.00
2014	1.08	1.04–1.12
2013	1.10	1.06–1.15
2012	1.16	1.11–1.21
2011	1.20	1.13–1.27
Age linear segment	0.99	0.98–0.99
RCS segment 1	1.02	1.01–1.02
RCS segment 2	0.90	0.84–0.96
RCS segment 3	1.31	1.05–1.63
Rural (*vs.* not rural)	1.25	1.17–1.33
Neighborhood income quintile		
1 (lowest)	1.04	1.01–1.08
2	1.03	1.00–1.06
3	1.03	0.99–1.07
4	1.02	0.99–1.05
5 (highest)	Ref	1.00–1.00
Comorbidities		
Alcohol abuse (*vs.* not/none)	0.99	0.93–1.05
Atrial arrhythmia (*vs.* not/none)	0.91	0.87–0.95
Blood loss anemia (*vs.* not/none)	1.17	1.14–1.20
Cardiac valve disease (*vs.* not/none)	0.97	0.92–1.02
Cerebrovascular disease (*vs.* not/none)	0.90	0.85–0.95
Chronic obstructive pulmonary disease (*vs.* not/none)	0.96	0.93–0.99
Coagulopathy (*vs.* not/none)	1.05	0.99–1.11
Deficiency anemia (*vs.* not/none)	1.04	0.92–1.18
Dementia (*vs.* not/none)	1.14	1.09–1.18
Depression (*vs.* not/none)	1.19	1.12–1.27
Diabetes mellitus without complications (*vs.* not/none)	0.95	0.91–0.98
Diabetes mellitus with complications (*vs.* not/none)	1.03	1.00–1.07
Dialysis (*vs.* not/none)	1.18	1.06–1.33
Disease of pulmonary circulation (*vs.* not/none)	1.16	1.09–1.23
Drug abuse (*vs.* not/none)	0.97	0.84–1.11
Frail (*vs.* not/none)	1.57	1.50–1.65
Heart failure (*vs.* not/none)	1.07	1.04–1.11
Hemiplegia (*vs.* not/none)	1.09	0.98–1.22
Hypertension without complications (*vs.* not/none)	0.95	0.93–0.98
Hypertension with complications (*vs.* not/none)	0.92	0.83–1.03
Liver disease (*vs.* not/none)	0.90	0.83–0.98
Malignancy (*vs.* not/none)	0.87	0.83–0.91
Metastases (*vs.* not/none)	0.94	0.88–1.01
Obesity (*vs.* not/none)	1.22	1.14–1.30
Peptic ulcer disease (*vs.* not/none)	0.98	0.91–1.06
Peripheral vascular disease (*vs.* not/none)	0.94	0.88–1.01
Psychoses (*vs.* not/none)	1.40	1.19–1.64
Renal disease (*vs.* not/none)	0.88	0.83–0.94
Rheumatic disease (*vs.* not/none)	0.89	0.81–0.98
Venous thromboembolism (*vs.* not/none)	1.03	0.91–1.16
Weight loss (*vs.* not/none)	1.16	1.09–1.23
Hospital One-year Mortality Risk score (per 1-unit increase)	1.03	1.02–1.03
Healthcare resource use		
Long-term care before admission	0.63	0.61–0.66
Resource utilization band		
2 (lowest)	0.72	0.66–0.79
3	0.80	0.76–0.85
4	0.85	0.83–0.87
5 (highest)	Ref	1.00–1.00
Medications		
Anticoagulant (*vs.* not/none)	1.03	1.00–1.06
Antiplatelet agent (*vs.* not/none)	0.95	0.92–0.98
Antipsychotic (*vs.* not/none)	1.01	0.97–1.06
Benzodiazepine (*vs.* not/none)	0.97	0.94–1.00
Opioid (*vs.* not/none)	0.95	0.93–0.97
Dementia medication (*vs.* not/none)	0.92	0.87–0.98
Procedure		
Implantation of internal device, hip joint	1.08	0.92–1.27
Fixation, hip joint	0.96	0.93–0.98
Fixation, femur	0.95	0.92–0.97
Implantation of internal device, pelvis	Ref	1.00–1.00

RCS, restricted cubic spline; Ref, reference; RoM, ratio of means.

only having surgery in 2015 was substantively different between groups (more common in high peripheral nerve block–use hospitals). The Durbin–Wu–Hausman test was significant ($P < 0.0001$), supporting the role of an instrumental variable analysis. Following two-stage residual inclusion analysis, receipt of a peripheral nerve block was associated with a 1.05-day decrease in length of stay (95% CI, 0.87 to 1.19; ratio of means, 0.77; 95% CI, 0.73 to 0.82).

We successfully matched 8,261 (82.4%) peripheral nerve block patients to a similar patient without a peripheral nerve block. Following matching, receipt of a peripheral nerve block was associated with a 0.2-day (95% CI, 0.2 to 0.3) decrease in length of stay.

Effect Modifiers

Significant effect modification was identified between receipt of a peripheral nerve block and frailty ($P = 0.041$) and age ($P < 0.0001$). The association of peripheral nerve blocks with length of stay was greater in people without frailty (ratio of means, 0.93; 95% CI, 0.89 to 0.97) than in people with frailty (ratio of means, 0.97; 95% CI, 0.94 to 1.01). The association of peripheral nerve block with length of stay was stronger at younger ages (fig. 1). No significant effect modification was found between receipt of a peripheral nerve block and chronic obstructive pulmonary disease, sex, history of dementia, or trauma admission.

Peripheral Nerve Blocks and Secondary Outcomes

In the 30 days after surgery, peripheral nerve blocks were associated with a decrease in health system costs before and

Fig. 1. Effect modification by age on the association of peripheral nerve blocks with length of stay. This figure demonstrates the effect size (*red line*) and 95% CI (*shaded area*) for receipt of a nerve block with length of stay at each age between 66–100 yr. The *solid black line* at 1.0 represents the null value for the association; values where the *line* and *shaded area* are below the *black line* represent a significant association. These values were calculated using the primary adjusted multilevel regression model for length of stay.

after covariate adjustment (greater than $1,400; table 3). There was no unadjusted or adjusted difference in the odds of mortality or pneumonia between people with or without a peripheral nerve block (table 3).

Post Hoc Sensitivity Analyses

In the analysis limited to people who were discharged alive from hospital, the adjusted ratio of means for length of stay was 0.95 (95% CI, 0.92 to 0.98). In the analysis that included anesthesia type as a categorical variable, the adjusted ratio of means from the regression model was 0.96 (95% CI, 0.93 to 0.99); when this categorical anesthesia type was used in the IV analysis, the peripheral nerve block–attributable decrease in length of stay was estimated to be 1.0 day (95% CI, 0.9 to 1.2). When the hospital identifier was entered into our primary adjusted regression model as a categorical fixed effect, the ratio of means was 0.97 (95% CI, 0.95 to 0.99). There was no significant adjusted difference in the odds of institutional discharge between people with and without a peripheral nerve block (odds ratio, 0.97; 95% CI, 0.89 to 1.09).

Discussion

In this population-based cohort of older people having emergency hip fracture surgery, the receipt of a peripheral nerve block was associated with a small decrease in length of postoperative hospital stay (that was statistically significant, but of questionable clinical significance) and decreased health system costs (approximately 5% lower). These findings were consistent across a variety of analytic approaches, including instrumental variable and propensity score analyses and are consistent with the positive effects of peripheral nerve blocks previously described in a systematic review of small randomized trials.[18] Given the increasing number of older people in Western populations, these findings are promising, however, the effect sizes varied notably (largest upper confidence limit, 1.2 days; smallest lower confidence limit, 0.2 days). This suggests that future multicenter randomized trials are likely required to provide more definitive causal data and should include patient-centered outcomes.

Hip fractures are the most common surgical indication for hospitalization in older people, and although rates of hip fracture are decreasing, the aging of the population means that hip fractures will continue to be a major driver of emergency healthcare utilization by older adults.[1,2,45] Additionally, the comorbidity burden of older people who experience a hip fracture is increasing,[2] leaving many hip fracture patients vulnerable to the adverse effects of systemic analgesic therapies.[15,16] A recent Cochrane review found that peripheral nerve blocks may offer advantages in hip fracture patients, with high quality evidence supporting a clinically relevant decrease in pain on movement immediately after block placement (−3.4 on a 10-point scale), and moderate evidence of decreased time to first mobilization

Table 3. Unadjusted and Adjusted Secondary Outcomes

Outcomes	Peripheral Nerve Block (n = 16,162)	No Peripheral Nerve Block (n = 6,499)	Crude Effect Estimate† (95% CI)	Adjusted Effect Estimate*† (95% CI)
30-day health system costs, mean ± SD	$20,158 ± $21,746	$22,221 ± $30,186	0.91 (0.89–0.92)	0.95 (0.92–0.98)
30-day mortality, n (%)	685 (6.8)	3,683 (6.7)	0.94 (0.89–1.00)	0.99 (0.89–1.11)
In-hospital pneumonia, n (%)	245 (2.4)	1,492 (2.7)	0.90 (0.79–1.04)	1.01 (0.88–1.16)

*All adjusted analyses included: age, neighborhood income quintile, rurality, procedure, Hospital One-Year Mortality score, each Elixhauser comorbidity, each specified drug class, year of surgery, resource utilization band, frailty, and long-term care residence;
†Effect estimate for costs is a ratio or means, for mortality and pneumonia an odds ratio.

(11 h shorter), reduced rates of pneumonia (relative risk, 0.41), and lower analgesic costs.[18] However, despite the large number of hip fracture surgeries that occur each year, findings from the Guay *et al.*'s Cochrane review were limited; most were small and single center and few reported key outcomes. For example, only three studies (total n = 131) evaluated pneumonia, and only two studies (total n = 155) evaluated time to mobilization.

Findings from our study build on Guay *et al.*'s Cochrane review, as the beneficial patient-level impacts of peripheral nerve blocks identified in randomized trials appear to translate into beneficial health system outcomes at a population level, including more than 7,000 potential hospital days saved per year if applied across 13,000 surgeries annually. Causally, improved pain control and earlier ambulation could translate into earlier discharge readiness and decreased healthcare resource use. The potential causality (*i.e.,* internal validity) of our findings are further supported by the minimal differences in patient characteristics between individuals who received a peripheral nerve block and those who did not, as well as by the consistency of the directional effect across three different analytic approaches, including an instrumental variable analysis (which may address issues of unmeasured confounding more effectively than traditional methods in observational research[46,47]). The fact that effect sizes differed between each approach likely reflects the slightly different question addressed by each analysis.[34,48,49] Our primary approach (*i.e.,* regression analysis) provides an estimate of average treatment effect; in other words, what would happen if the entire population received a peripheral nerve block compared to no one receiving a nerve block. In contrast, the instrumental variable analysis provides a local average treatment effect estimate (*i.e.,* what is the treatment effect in people who were eligible and willing to have or not have a peripheral nerve block). In this case, this local average treatment effect cannot be extrapolated to the types of patients who would never receive a peripheral nerve block (such as someone with an absolute contraindication or totally unwilling to have a peripheral nerve block), or someone who would almost always receive a peripheral nerve block. However,

in the setting of a peripheral nerve block for hip fracture surgery the local average treatment effect may be practice- and policy-relevant as very few absolute contraindications to peripheral nerve blocks exist, and few patients would always be expected to receive a peripheral nerve block. Finally, the propensity score matched analysis provides an estimate of the average effect of treatment in the treated, which reflects the impact of a peripheral nerve block in the subset of the population who actually received a block. The positive impact of peripheral nerve blocks was also reflected in an estimated reduction in health system costs of greater than $1,400 CAD per patient. Considering that more than 13,000 hip fractures per year are treated surgically on Ontario, these savings could translate into more than $18 million dollars in yearly health system savings.

The inconsistency between our findings related to pneumonia (no decrease in the odds of pneumonia after receipt of a peripheral nerve block) and systematic review findings (decreased pneumonia risk with a peripheral nerve block) must also be considered and could reflect numerous issues. First, across the three trials included in the Cochrane review, only 25 pneumonias were identified.[50–52] A single study drove results with a control group respiratory infection rate of 43%.[50] Therefore, these findings may be fragile.[53] Furthermore, these three trials were conducted between 1980 and 2003, and may not reflect contemporary practice. Finally, although the pneumonia definition used has been validated,[26] misclassification bias is always a risk in observational research. Whether the causal pathway involves other postulated benefits of peripheral nerve blocks, such as early mobilization or reduced incidence of delirium, these outcomes cannot be accurately captured in administrative data. Specifically, functional data are not routinely captured and diagnostic codes for delirium suffer from substantial misclassification bias.[54,55] Therefore, prospective multicenter trials are needed to address patient-centered outcomes and to elucidate specific causal pathways.

Strengths and Limitations

Our findings should be considered in the context of the study's strengths and limitations. First, as an observational

study, we could only estimate an association (as opposed to causation) between peripheral nerve blocks and outcomes. We also used health administrative data that were not initially collected for research purposes and are therefore at risk of misclassification bias. However, our exposure and outcome variables have been validated and demonstrate a high level of accuracy relative to clinical data. We were not able to capture outcomes considered as typical opioid-related adverse events (*e.g.,* nausea, pruritis, respiratory depression), therefore our findings provide no insights into how peripheral nerve blocks impact these relatively common clinical outcomes. We did not capture postdischarge opioid use either. Our adjusted analyses included a large set of measurable postulated confounding variables, accounted for clustering at the hospital level, and had consistent results across analytic approaches. Furthermore, while observational research is at risk of unmeasured confounding, our instrumental variable analysis (which can help to account for unmeasured confounders) estimated a larger treatment effect than our regression-based analysis. We were unable to account for exactly what type of peripheral nerve block was placed (*e.g.,* fascia iliac *vs.* femoral), how successful each peripheral nerve block placement was in establishing effective analgesia, or whether adjuncts (such as local anesthesia additives or additional analgesics like intrathecal opioids) were provided; therefore, our effect estimates reflect a pragmatic (as opposed to explanatory) research question.[56] We also limited our analyses to perioperative blocks (within 1 day of surgery); therefore, the role of blocks placed on arrival in the emergency department were not captured. We must also acknowledge that small differences in length of stay may not be as relevant at the individual patient level as at the health system level. Finally, the external validity of these results beyond Ontario will require confirmation in future research.

Conclusions

In a population-based cohort study of older people having emergency hip fracture surgery, receipt of a peripheral nerve block was consistently associated with reduced postoperative length of stay and reduced health system costs. These findings suggest that peripheral nerve blocks could contribute to improved population-level health system outcomes for hip fracture surgery patients. An appropriately powered multicenter trial will be required to estimate a causal relationship with patient-centered outcomes.

Research Support

Competing Interests

The authors declare no competing interests.

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ANESTHESIOLOGY

Effect of a Cognitive Aid on Reducing Sugammadex Use and Associated Costs

A Time Series Analysis

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Sugammadex provides rapid and effective reversal of neuromuscular blocks but is expensive

What This Article Tells Us That Is New

- The investigators tested the hypothesis that a cognitive aid to guide selective use of sugammadex reduced use
- They conducted a segmented regression (interrupted time series) retrospective analysis before and after implementing the cognitive aid and informational meetings for their department
- Sugammadex use and associated costs, which were increasing, decreased substantially after introduction of the cognitive aid

ABSTRACT

Background: The authors observed increased pharmaceutical costs after the introduction of sugammadex in our institution. After a request to decrease sugammadex use, the authors implemented a cognitive aid to help choose between reversal agents. The purpose of this study was to determine if sugammadex use changed after cognitive aid implementation. The authors' hypothesis was that sugammadex use and associated costs would decrease.

Methods: A cognitive aid suggesting reversal agent doses based on train-of-four count was developed. It was included with each dispensed reversal agent set and in medication dispensing cabinet bins containing reversal agents. An interrupted time series analysis was performed using pharmaceutical invoices and anesthesia records. The primary outcome was the number of sugammadex administrations. Secondary outcomes included total pharmaceutical acquisition costs of neuromuscular blocking drugs and reversal agents, adverse respiratory events, emergence duration, and number of neuromuscular blocking drug administrations.

Results: Before cognitive aid implementation, the number of sugammadex administrations was increasing at a monthly rate of 20 per 1,000 general anesthetics ($P < 0.001$). Afterward, the monthly rate was 4 per 1,000 general anesthetics ($P = 0.361$). One month after cognitive aid implementation, the number of sugammadex administrations decreased by 281 per 1,000 general anesthetics (95% CI, 228 to 333, $P < 0.001$). In the final study month, there were 509 fewer sugammadex administrations than predicted per 1,000 general anesthetics (95% CI, 366 to 653; $P < 0.0001$), and total pharmaceutical acquisition costs per 1,000 general anesthetics were $11,947 less than predicted (95% CI, $4,043 to $19,851; $P = 0.003$). There was no significant change in adverse respiratory events, emergence duration, or administrations of rocuronium, vecuronium, or atracurium. In the final month, there were 75 more suxamethonium administrations than predicted per 1,000 general anesthetics (95% CI, 32 to 119; $P = 0.0008$).

Conclusions: Cognitive aid implementation to choose between reversal agents was associated with a decrease in sugammadex use and acquisition costs.

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Cognitive aids implemented in the perioperative environment are associated with an improvement in quality and patient safety.[1–3] The most widely used cognitive aid is the surgical safety checklist, which has been shown to decrease morbidity and mortality associated with surgery in multiple settings globally.[4] Another cognitive aid that has been instrumental in improving patient safety is the emergency manual, which has been shown to improve implementation of best known practices during critical events in various healthcare settings.[2,5–7] While the mechanism behind the success of cognitive aids is not entirely understood, a recent study[8] describes factors that are associated with the successful implementation of cognitive aids. Some of the factors include completing more implementation steps, leadership support, having an implementation champion, and institution-specific customization of the cognitive aid. Ultimately, one of the most important features of a cognitive aid is that it can result in improved practice processes.[1,4]

In April 2016, sugammadex was introduced in our institution. As its use and subsequent pharmacy acquisition costs increased, our department was asked to review

neuromuscular blockade reversal practices with the intent to implement a practice change to decrease the use of sugammadex and associated costs. A review of a random sample of 87 anesthesia records demonstrated that 71% of patients received sugammadex more than 1 h after the administration of rocuronium (see Supplemental Digital Content 1, fig. S1, showing the sugammadex usage in August 2017; http://links.lww.com/ALN/C44). While duration from last dose of rocuronium does not determine the need for reversal of neuromuscular blockade, based on a previous study[9] we estimated that more than half of the patients would likely have had sufficient recovery from neuromuscular blockade to allow for the administration of neostigmine with glycopyrrolate.

Given the previous use of checklists and emergency manuals to guide clinician behavior, and the request to implement a practice change to decrease the use of sugammadex, we sought to implement a cognitive aid to guide management of neuromuscular blockade reversal agents in the operating room setting. A previous study[10] used a cognitive aid, "Neostigmine Dosing Guide," to help change neuromuscular blockade reversal agent administration practices in an institution where only neostigmine was available at the time. We adapted the "Neostigmine Dosing Guide" cognitive aid to take into account sugammadex and the use of only peripheral nerve stimulators in our institution. The goal of our cognitive aid was to guide management of neuromuscular blockade reversal agents in an evidence-based, safe, and cost-effective manner.

The purpose of this study was to determine if implementation of the cognitive aid resulted in a change in sugammadex use in the operating room by performing an interrupted time series analysis. Our hypothesis was that the cognitive aid would decrease sugammadex use in the operating room, which would be associated with decreased total acquisition costs of neuromuscular blocking drugs and reversal agents while maintaining safe and timely patient care.

Materials and Methods

Setting

A waiver of informed consent for medical record review was obtained from the institutional review board. This study was conducted at an urban tertiary care, academic medical center with 415 inpatient beds. The institution performs more than 22,000 anesthetic cases annually, of which approximately 26% are general anesthetics utilizing neuromuscular blockade. Multiple clinical information systems are used throughout the institution, with Anesthesia Touch (Plexus Technology Group, LLC, USA) being the primary system used for anesthesia record documentation, and Pharmacy Clinical Workstation (Cerner Corporation, USA) being the primary clinical and drug distribution pharmacy platform that is integrated with point-of-care and financial systems.

Two systems are in place for dispensing medications in the operating room: password-protected medication dispensing cabinets (Omnicell, USA), and manually retrieved standardized sets of medications directly from a pharmacist. For each dispensing system, both neuromuscular blockade reversal agents are simultaneously available for use by the anesthesia clinicians. This manuscript adheres to the guidelines of the Standard for Quality Improvement Reporting Excellence.

Study Intervention

A cognitive aid similar to a previously described model[10] was developed to guide management of neuromuscular blockade reversal agents (fig. 1). Dosing strategies for sugammadex and neostigmine were made based on a review article[11] that outlines available evidence on the topic of neuromuscular blockade and reversal. A "notes" section was included on the lower portion of the cognitive aid to remind clinicians of subtle details and certain exceptions. The cognitive aid was presented at a departmental conference on November 16, 2017, during which attending physicians, resident physicians, and nurse anesthetists offered feedback and suggestions for enhancements. The following day, a departmental email was sent to solicit additional feedback from clinicians who may have missed the conference. After making changes suggested by our clinicians, the cognitive aid was formally adopted by our department on November 27, 2017. In operating rooms with a medication dispensing cabinet, the cognitive aid was placed in the same bin as sugammadex and neostigmine, with the goal of prompting the clinician to use the cognitive aid when reaching for these drugs. In operating rooms with standard medication sets manually dispensed by the pharmacist, the cognitive aid was included with each medication set containing sugammadex and neostigmine.

Peripheral Nerve Stimulators

Before October 26, 2017, three different peripheral nerve stimulators were available: MiniStim★ MS-IVB (Halyard Health, United Kingdom), EZstim II (Life-Tech, Inc., USA), and DigiStim 2 Plus (Neuro Technology, Inc., USA). There were several units of each device type, and there was no particular pattern in which they were distributed. Our perioperative administration purchased the EZstim★ III (Halyard Health) peripheral nerve stimulators and placed them in all anesthetizing locations on October 26, 2017. The previously used peripheral nerve stimulators were then placed into storage to be used only in the event that the EZstim★ III should be unavailable.

Data Sources

Data on the number of administrations of drugs and acquisition costs were collected from accounting data in the Pharmacy Clinical Workstation system. For every month, each vial or prefilled syringe that was removed and not

Neuromuscular Blockade Reversal Cognitive Aid

Documented Twitches	Dosing
0-1 twitches	4 mg/kg sugammadex
2 twitches	2 mg/kg sugammadex
3 twitches	50 mcg/kg neostigmine
4 twitches with fade	40 mcg/kg neostigmine
4 twitches without fade	20 mcg/kg neostigmine

Notes
Dose based on *ideal body weight*
Dose can be *approximated* (e.g. if calculate 220mg sugammadex, 200mg is ok)
The maximum dose of *neostigmine* is 5 mg
Remember to dose neostigmine with an *anticholinergic* (e.g. glycopyrrolate)
Administer neostigmine *as early as possible*, but when surgically appropriate
Patients at high risk for postoperative *respiratory complications* (e.g. severe COPD) may receive sugammadex instead of neostigmine regardless of twitches
Sugammadex should only be used to reverse *rocuronium* and/or *vecuronium*
Atracurium should always be reversed with *neostigmine*

Fig. 1. The cognitive aid implemented in our institution. COPD, chronic obstructive pulmonary disease.

returned to the pharmacy was counted as an administration, such that all drugs were counted regardless of dose used or wasted. The acquisition cost of each vial or prefilled syringe was obtained from the monthly pharmacy invoices. Total acquisition cost of neuromuscular blocking drugs and reversal agents was calculated by summing the individual cost of each drug counted every month, such that it reflects the total pharmacy invoice and takes into account different prices between outpatient and inpatient anesthetics. Costs differed between outpatient and inpatient anesthetics because the 340B Drug Pricing Program of the U.S. Department of Health and Human Services (Washington, DC) allows our institution to obtain discounted prices for outpatient drugs. The median per vial or prefilled syringe costs were as follows: sugammadex 200 mg vial, $89; neostigmine 3 mg syringe, $31; glycopyrrolate 1 mg vial, $12; rocuronium 100 mg vial, $4; vecuronium 10 mg vial, $5; atracurium 100 mg vial, $15; and suxamethonium 200 mg syringe, $35.

Anesthesia record data were documented in the electronic system Anesthesia Touch. Using the Practice Management Intelligence Reporting System (Plexus Technology Group, LLC, USA), data were extracted for all anesthetics documented during the study period. Specific data points extracted included names of adverse events, surgery end and emergence times (to calculate emergence duration), type of surgery, American Society of Anesthesiologists (ASA) physical status, surgery start and end times (to calculate surgery duration), patient age, airway management technique, and type of anesthesia. All cases documented as ASA physical status VI or VIE, cases that were cancelled on the day of surgery before induction, and those documented as only labor epidural analgesia were removed. Adverse events were filtered to only include adverse respiratory events (reintubation, respiratory insufficiency, difficult airway, bronchospasm, and laryngospasm), which are documented when they occur in the operating room or the postanesthesia care unit.

Primary and Secondary Outcomes

The primary outcome was the number of sugammadex administrations per 1,000 general anesthetics per month as assessed by interrupted time series analysis.

Secondary outcomes included the following:

1. Total acquisition costs of neuromuscular blocking drugs and reversal agents (rocuronium, vecuronium, atracurium, suxamethonium, sugammadex, neostigmine, and glycopyrrolate) per 1,000 general anesthetics per month
2. The number of adverse respiratory events per 1,000 general anesthetics per month
3. The emergence duration (median minutes from surgery end to emergence) in patients undergoing general anesthesia

4. The number of administrations of neuromuscular blocking drugs, including rocuronium, vecuronium, atracurium, and suxamethonium, per 1,000 general anesthetics per month

Sensitivity Analyses

We performed several sensitivity analyses to verify the robustness of our statistical models. We assessed three drugs not associated with neuromuscular blockade that should not have been affected by the cognitive aid but would be subject to the same threats to internal validity as the primary and secondary outcomes. We ran models adjusted for several risk factors for perioperative respiratory complications[12–14] to evaluate if time-varying confounding affected the number of adverse respiratory events. We also assessed the number of supraglottic airway techniques and general anesthetics to evaluate for possible time-varying confounding on sugammadex use.

Statistical Analysis

We performed an interrupted time series analysis to investigate the effects of our cognitive aid on the number of drug administrations, total pharmaceutical acquisition costs, adverse respiratory events, and emergence duration. A formal statistical power analysis was not performed. Rather, we used previous research[15] that suggested that data from at least 8 months before and after our intervention would be sufficient to estimate the level and slope parameters.

For outcomes that were monthly counts (*e.g.*, number of drug administrations, number of adverse respiratory events, *etc.*), data were analyzed using segmented Poisson and Negative Binomial models. We fit, for each outcome, a previous specified model of $\hat{Y}_t = \beta_0 + \beta_1 \cdot t_s + \beta_2 \cdot X_t + \beta_3 \cdot t_p$, where $\hat{Y}_t$ is the predicted value at time t, β_0 is the intercept, β_1 is the baseline trend, β_2 is the level change, and β_3 is the trend change after the cognitive aid was implemented. The times t_s and t_p are the time in months (defined as 30-day periods, except for the first and last months, which were 15-day periods) from the beginning of the study and the time after cognitive aid implementation; X_t is a scalar (0 for precognitive aid, and 1 for postcognitive aid) as described in a previous study.[16]

Data for a 1-month period (November 16, 2017, to December 15, 2017) surrounding the initial implementation of the cognitive aid were excluded from the fitting of models. The count models included an offset term of the natural logarithm of the number of general anesthetics per month. Estimates of the parameters of interest ($\beta_0, \beta_1, \beta_2, \beta_3$) were presented from the model with the best fit based on the lowest corrected Akaike Information Criteria.

For emergence duration, which was not a count that was dependent on the number of anesthetics per month, we used a linear regression approach allowing autoregressive terms to enter the model if they improved model performance,

which would account for the possible autocorrelation of the time series nature of these data. These linear regression time series selection models were also run on the monthly count data standardized by the number of general anesthetics per month as sensitivity analyses. These models were estimated using the SAS/ETS (version 14.1; SAS Institute Inc., USA) procedure "Autoreg" with maximum likelihood estimation. To account for autocorrelation, we used a stepwise selection to allow up to six lagged dependent variables to enter the model and calculated generalized Durbin–Watson statistics for the final model for each outcome.

Estimates of the postcognitive aid trend ($\beta_1 + \beta_3$) were also calculated by reparameterizing the above model. Resulting models were used to estimate mean predicted values and 95% CIs for each time point given two scenarios: first, assuming the cognitive aid was implemented, and second, under the counterfactual assumption that the cognitive aid had not been implemented, by setting $X_t = 0$ for the prediction. Estimates of the variances for the pre- and postpredictions were calculated from the span of the 95% CIs, and then used to estimate the variance of the difference, which was used to estimate the 95% CI for the estimated mean difference (precognitive aid minus postcognitive aid) at each time point. SAS version 9.04.01M3 was used with SAS Enterprise Guide (version 7.15HF3; SAS Institute Inc., USA) for the time series analysis.

The number of drug administrations and general anesthetics was summarized for the entire cohort for pre- and postcognitive aid periods. Standardized differences between pre- and postcognitive aid characteristics and corresponding 95% CIs were calculated using Hedge's g for interval data and Cliff's delta for ordinal and dichotomous data with the effsize function (effsize_07.1) running on R software (R version 3.5.3 [2019-03-11] through R Studio (R Studio Version 1.1.463; RStudio, Inc., USA).

Results

Between December 1, 2016, and July 31, 2018, there were a total of 12 time periods before and 8 time periods after the intervention that were included in the analysis. Table 1 reports the number of monthly general anesthetics and drug administrations.

Figure 2 presents the time series for sugammadex, total acquisition cost of neuromuscular blocking drugs and reversal agents, adverse respiratory events, and emergence duration, while figure 3 presents the times series for the neuromuscular blocking drugs, with the fitted pre- and postcognitive aid trends, as well as the extrapolated precognitive aid trends that were used to calculate the counterfactual differences.

The number of sugammadex administrations in the first month of the study period was 238 per 1,000 general anesthetics. Before cognitive aid implementation, the number of sugammadex administrations was increasing at a monthly rate of 20 per 1,000 general anesthetics ($P < 0.001$). After

Table 1. Number of Monthly General Anesthetics and Drug Administrations

Month	General Anesthetics (n)	Sugammadex (n)	Rocuronium (n)	Vecuronium (n)	Atracurium (n)	Suxamethonium (n)
1*	609	145	426	95	21	149
2	869	244	640	108	14	247
3	1,082	363	826	136	23	249
4	1,061	403	733	147	41	227
5	959	396	770	121	26	267
6	1,142	451	777	131	20	303
7	1,059	401	700	129	18	223
8	939	416	732	124	33	182
9	1,110	521	815	141	22	205
10	1,048	480	765	138	17	204
11	1,071	509	805	148	16	215
12	1,079	512	832	142	26	193
13†	1,149	293	811	162	25	218
14	952	242	671	138	20	171
15	1,164	275	844	163	47	234
16	1,102	285	784	202	46	263
17	1,049	280	745	142	31	252
18	1,083	294	741	160	32	213
19	1,166	321	807	188	33	247
20	1,068	258	693	164	26	215
21*	598	172	424	79	16	117

*Half-month period (15 days). †Intervention month.

cognitive aid implementation, the monthly rate decreased by 16 sugammadex administrations per 1,000 general anesthetics ($P = 0.032$), with a postintervention monthly rate of four sugammadex administrations per 1,000 general anesthetics ($P = 0.361$). Implementation of the cognitive aid was associated with an immediate decrease of 281 sugammadex administrations per 1,000 general anesthetics in the postintervention month (95% CI, 228 to 333; $P < 0.001$). In the final study month, there were 509 fewer sugammadex administrations than predicted per 1,000 general anesthetics (95% CI, 366 to 653; $P < 0.0001$), and total pharmaceutical acquisition costs per 1,000 general anesthetics were $11,947 less than predicted (95% CI, $4,043 to $19,851; $P = 0.003$).

Implementation of our cognitive aid did not significantly affect the number of adverse respiratory events, emergence duration, or number of administrations of rocuronium, vecuronium, or atracurium. In the final month, there were 75 more suxamethonium administrations than predicted per 1,000 general anesthetics (95% CI, 32 to 119; $P = 0.0008$).

Sensitivity Analyses

Results of the sensitivity analyses are presented in Supplemental Digital Content 1, figure S2 (http://links. lww.com/ALN/C44) and Supplemental Digital Content 2, tables S1, S2, and S3 (http://links.lww.com/ALN/C45). Covariate adjustment did not improve model fit for any of the study variables. The estimated coefficients from the segmented regression analysis and the combined intercept and slope changes associated with the cognitive aid, expressed as counterfactual differences at 8 months postimplementation,

are also found in Supplemental Digital Content 2, tables S1, S2, and S3 (http://links.lww.com/ALN/C45).

Discussion

In this interrupted time series analysis, we found that implementation of an evidence-based cognitive aid with criteria for selecting sugammadex *versus* neostigmine and providing dosing recommendations based on train-of-four count was associated with a decrease in the number of sugammadex administrations and total pharmaceutical acquisition cost of neuromuscular blocking drugs and reversal agents. We did not observe a clinically meaningful change in the number of adverse respiratory events or the emergence duration. We found a small, clinically insignificant increase in the number of administrations of suxamethonium, but not of rocuronium, vecuronium, or atracurium.

The means by which our cognitive aid exerted its effect to decrease the number of sugammadex administrations may be related to a variety of factors. First, the cognitive aid was immediately available to providers at the point of care. As part of the implementation process, our pharmacists placed the cognitive aid in the same bin as sugammadex and neostigmine in medication dispensing cabinets and dispensed the cognitive aid in conjunction with each medication set containing sugammadex and neostigmine. While this approach facilitated easy access to the cognitive aids, this strategy alone would likely have been insufficient.

Several studies have demonstrated that implementation of cognitive aids is a complex process, which results in varying levels of success depending on how the implementation process occurs.[8,17,18] An implementation champion has been
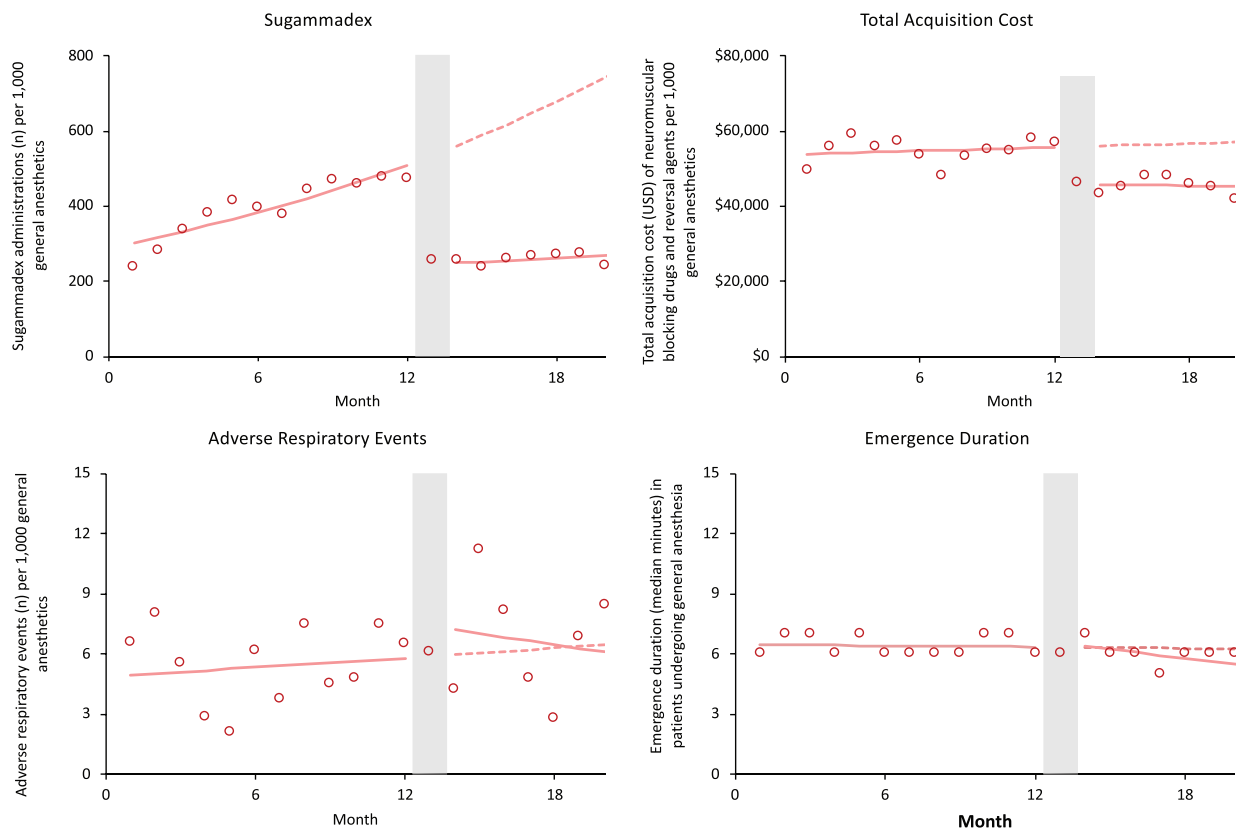
Fig. 2. Interrupted time series analysis for primary and secondary outcomes. Interrupted time series analysis for sugammadex, total acquisition cost of neuromuscular blocking drugs and reversal agents, adverse respiratory events, and emergence duration. For each plot, the *vertical gray area* is a 1-month period surrounding implementation of the cognitive aid, the *red circles* are the raw data, the *solid line* is the fitted regression line (Poisson for sugammadex and adverse respiratory events; Negative Binomial for total acquisition cost; Linear for emergence duration), and the *dashed line* is the projected trend assuming there was no cognitive aid (counterfactual). USD, U.S. dollars.

identified[17] as an important component of successful implementation of cognitive aids. In our department, the role of the implementation champion was to develop the cognitive aid, solicit feedback from members of the department, and oversee the printing and appropriate distribution of the cognitive aid. In addition, the role included reminding clinicians about the cognitive aid at departmental meetings, during which the rationale behind implementation of the cognitive aid was explained and demonstrations of how to use the cognitive aid were performed, both of which function to build understanding and buy-in from department members.[17] Furthermore, there was leadership support from the department chair, which primarily took the form of encouraging the implementation champion and providing dedicated time to train staff in the use of the cognitive aid, both of which further facilitate use of the cognitive aid.[8,18] Importantly, while the implementation champion and the department chair promoted adoption of the cognitive aid, there was no financial incentive or other pressure (*e.g.*, evaluation of individual use as a clinical performance indicator)[10] to influence use of the cognitive aid.

Second, we designed and customized the cognitive aid to be user-friendly within our institutional context: Neuromuscular blockade reversal agent choice and dosing recommendations were based on qualitative train-of-four count and fade. While a more comprehensive assessment of neuromuscular blockade would include assessment of a train-of-four ratio, we intentionally did not include these criteria because our institution does not have a quantitative train-of-four monitor. Furthermore, we hoped that the cognitive aid would add value to the anesthesia clinician in the management of neuromuscular blockade reversal. At the time sugammadex was initially added to the hospital formulary, sugammadex was a relatively new drug unfamiliar to our clinicians with some uncertainty regarding its clinical use. Several studies have emphasized that simplicity of design encourages and promotes use,[19,20] and that customization makes cognitive aids more relevant to the local institution.[3]

Finally, we used a multidisciplinary and collaborative approach to create the cognitive aid: A departmental meeting was held in which the idea for the cognitive aid was introduced, an email soliciting comments was sent to all department
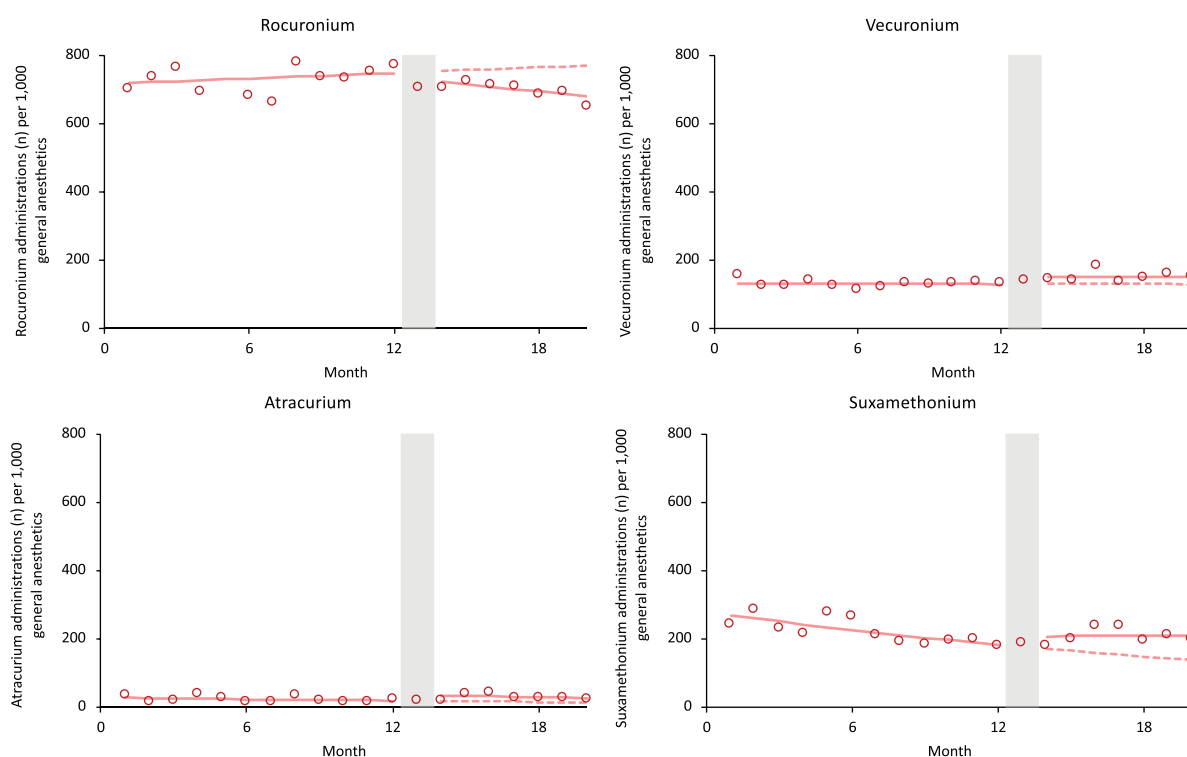
Fig. 3. Interrupted time series analysis for neuromuscular blocking drugs. Interrupted time series analysis for rocuronium, vecuronium, atracurium, and suxamethonium. For each plot, the *vertical gray area* is a 1-month period surrounding implementation of the cognitive aid, the *red circles* are the raw data, the *solid line* is the fitted regression line (Poisson for rocuronium and vecuronium; Negative Binomial for atracurium and suxamethonium), and the *dashed line* is the projected trend assuming there was no cognitive aid (counterfactual).

members, and feedback was integrated before implementation of the final version. In addition, we invited one of the pharmacists to give feedback on the cognitive aid and discuss strategies for implementation. Several studies have identified that such an approach improves cognitive aid implementation because it gives clinicians an opportunity to be engaged and become part of the adoption and implementation process.[1,8]

While a common quality improvement approach to achieve drug prescribing practice changes among clinicians is the audit-feedback cycle, we did not choose this method because it is time- and resource-intensive, its effects are generally small, and changes are often not sustained.[21] For example, Todd et al.[22] found that audit-feedback cycles combined with the provision of peripheral nerve stimulators in all operating rooms increased the rate of appropriate use of neuromuscular blockade reversal agents and decreased the rate of residual neuromuscular blockade and reintubations. However, after cessation of audit-feedback cycles, the authors found an increase in inappropriate use of neuromuscular blockade reversal agents.[23] Similarly, Colombet et al.[24] found that while audit-feedback cycles decreased the rate of intravenous proton pump inhibitors, usage patterns eventually returned to baseline when audit-feedback cycles were no longer in place.

Concurrent with decreased sugammadex use, we found a decrease in the total acquisition costs of neuromuscular blocking drugs and reversal agents. To determine if the changes in neuromuscular blockade reversal agent usage patterns resulted in any negative impact, we studied the number of adverse respiratory events and the emergence duration, as an increase in these outcomes would likely negate any cost savings associated with decreased sugammadex use. Significant changes for adverse respiratory events were not observed in any of the parameters of the segmented regression analysis or the counterfactual differences at 8 months postimplementation. Similarly, we did not observe an increase in the emergence duration. While the overall number of adverse respiratory events was small and additional factors could be considered, implementation of the cognitive aid did not appear to have a readily observable negative effect on the number of adverse respiratory events or emergence duration.

While our cognitive aid contains some basic information about reversal of neuromuscular blockade to guide clinicians, there are several elements that could be added to enhance the cognitive aid depending on institutional practices and resources. A note indicating that the ideal location for measuring the train-of-four count is the adductor

pollicis[25,26] could be added in the "notes" section. Another useful reminder may be to indicate that greater doses of sugammadex are required for reversal of vecuronium-induced compared to rocuronium-induced neuromuscular blockade given the greater neuromuscular blocking potency of vecuronium and lesser affinity of sugammadex for vecuronium.[27] Finally, while we did not include train-of-four ratio monitoring in our cognitive aid because we do not have quantitative monitoring available in our institution, the addition of this information should be considered if quantitative monitoring is available, because even protocolized administration of neostigmine does not guarantee complete reversal of neuromuscular blockade.[28,29] For an example of such a cognitive aid, see Supplemental Digital Content 3 (http://links.lww.com/ALN/C46), a modified version of the Neuromuscular Blockade Reversal Cognitive Aid.

Our study has several important limitations. First, the interrupted time series statistical design does not allow for conclusions of causality, and it is unclear if confounding factors could have influenced the outcomes. For example, knowing that the leadership is observing the pharmaceutical usage patterns (the Hawthorne effect[30]) could have itself contributed to a change in practice patterns. Second, each operating room was supplied with a single type of qualitative peripheral nerve stimulator approximately 1 month before the cognitive aid was implemented. While the increased availability of a single type of peripheral nerve stimulator could have affected the usage patterns of neuromuscular blockade reversal agents, previous studies[22,31] have found that availability of peripheral nerve stimulators alone is not sufficient to affect practice changes. In addition, given the retrospective nature of our data, it was not possible to accurately determine how often the peripheral nerve stimulators were used. Finally, while our cognitive aid cards could have sustainable effects, over time they could get damaged or lost. Long-term effects of the cognitive aid are unclear. An electronic-based cognitive aid, such as the electronic preanesthetic induction patient safety checklist used by Wetmore et al.,[19] might be more sustainable as it would not be lost or destroyed over time unless intentional changes in software are made.

In summary, through this interrupted time series analysis, we observed that the implementation of an evidence-based cognitive aid was associated with a decrease in the number of sugammadex administrations and total acquisition costs of neuromuscular blocking drugs and reversal agents, without an increase in adverse respiratory events or emergence duration. These findings could be useful to institutions that are considering adding sugammadex to their formulary and for those that already have sugammadex. Some institutions may choose not to introduce sugammadex because of previous studies showing that its adoption results in tripling of pharmaceutical acquisition costs associated with neuromuscular blockade.[32] However, the adoption and implementation of a cognitive aid could help clinicians using an evidence-based approach determine when to use sugammadex *versus* neostigmine in a cost-effective manner. If a cognitive aid is to be used at institutions with quantitative monitoring devices available, the modified version should be considered because quantitative train-of-ratio monitoring would offer a more thorough protocol for reversal of neuromuscular blockade. Nevertheless, it is important to emphasize that the implementation process of the cognitive aid is critical to its successful use.

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ANESTHESIOLOGY REFLECTIONS FROM THE WOOD LIBRARY-MUSEUM

From Jerking Teeth to Clerking Harvard: Dr. Hiram Whitney and His Patients' Ethereal Requests
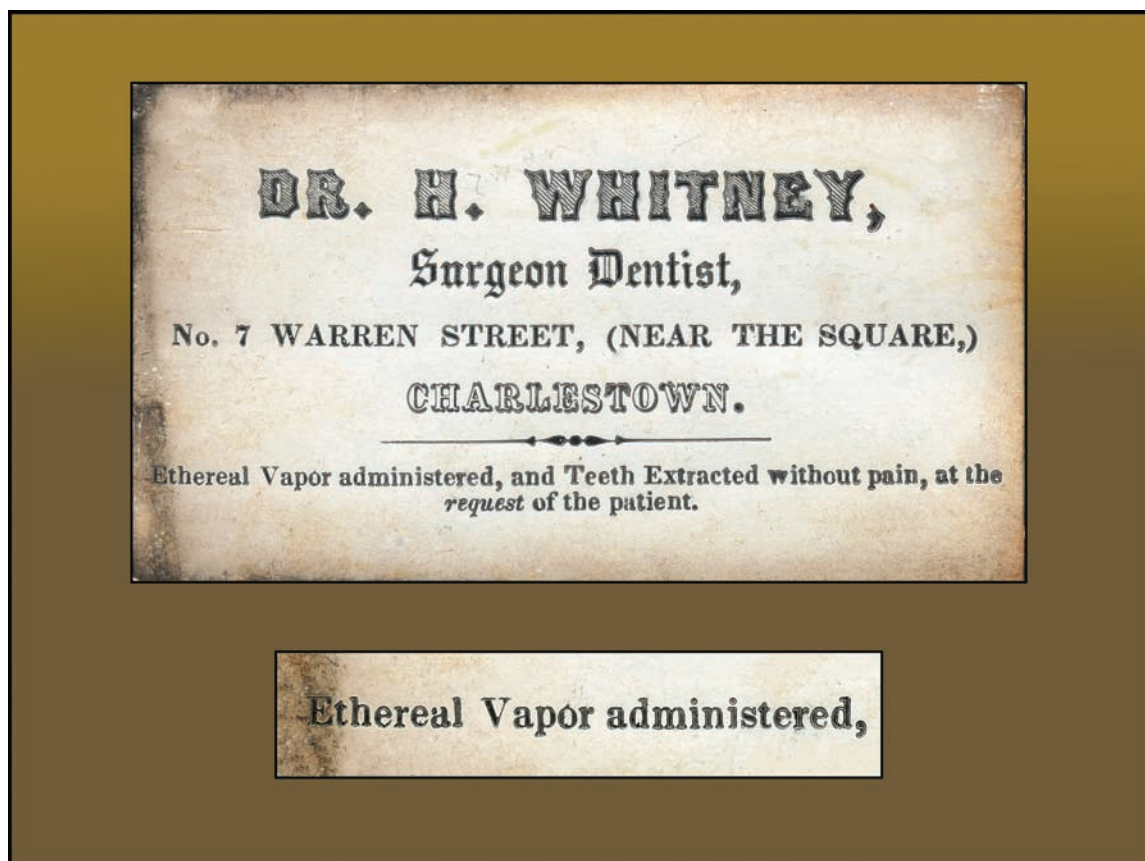
A lifelong native of Harvard, Massachusetts, Dr. Hiram Whitney (1815 to 1879) served as both a popular dentist and, for his final 13 yr, as the town clerk. Just 1 yr after Morton's public demonstration of surgical etherization, the 1848 "Environs of Boston" directory listed Dr. Whitney at the Warren Street address printed on this business card (*above*) from the Wood Library-Museum's Ben Z. Swanson Collection. In those early years of etherizing patients, many dentists were discouraged by the ethereal legacy of day-long accumulation of drowsy, nauseated patients in dental offices. Perhaps that is why Dr. Whitney would only administer ether "at the *request* of the patient." (Copyright © the American Society of Anesthesiologists' Wood Library-Museum of Anesthesiology.)

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ANESTHESIOLOGY

Intraoperative Mechanical Ventilation and Postoperative Pulmonary Complications after Cardiac Surgery

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Modern ventilation approaches use a bundle of lower tidal volumes, lower driving pressures, and positive end-expiratory pressure
- The contributions of each component to reducing postoperative pulmonary complications in an adult cardiac surgical population is not known

What This Article Tells Us That Is New

- In this retrospective analysis, the intraoperative ventilation bundle was associated with a lower rate of postoperative pulmonary complications
- Lower modified driving pressure was independently associated with fewer pulmonary complications

ABSTRACT

Background: Compared with historic ventilation strategies, modern lung-protective ventilation includes lower tidal volumes (V_T), lower driving pressures, and application of positive end-expiratory pressure (PEEP). The contributions of each component to an overall intraoperative protective ventilation strategy aimed at reducing postoperative pulmonary complications have neither been adequately resolved, nor comprehensively evaluated within an adult cardiac surgical population. The authors hypothesized that a bundled intraoperative protective ventilation strategy was independently associated with decreased odds of pulmonary complications after cardiac surgery.

Methods: In this observational cohort study, the authors reviewed nonemergent cardiac surgical procedures using cardiopulmonary bypass at a tertiary care academic medical center from 2006 to 2017. The authors tested associations between bundled or component intraoperative protective ventilation strategies (V_T below 8 ml/kg ideal body weight, modified driving pressure [peak inspiratory pressure − PEEP] below 16 cm H_2O, and PEEP greater than or equal to 5 cm H_2O) and postoperative outcomes, adjusting for previously identified risk factors. The primary outcome was a composite pulmonary complication; secondary outcomes included individual pulmonary complications, postoperative mortality, as well as durations of mechanical ventilation, intensive care unit stay, and hospital stay.

Results: Among 4,694 cases reviewed, 513 (10.9%) experienced pulmonary complications. After adjustment, an intraoperative lung-protective ventilation bundle was associated with decreased pulmonary complications (adjusted odds ratio, 0.56; 95% CI, 0.42–0.75). *Via* a sensitivity analysis, modified driving pressure below 16 cm H_2O was independently associated with decreased pulmonary complications (adjusted odds ratio, 0.51; 95% CI, 0.39–0.66), but V_T below 8 ml/kg and PEEP greater than or equal to 5 cm H_2O were not.

Conclusions: The authors identified an intraoperative lung-protective ventilation bundle as independently associated with pulmonary complications after cardiac surgery. The findings offer insight into components of protective ventilation associated with adverse outcomes and may serve as targets for future prospective interventional studies investigating the impact of specific protective ventilation strategies on postoperative outcomes after cardiac surgery.

(*ANESTHESIOLOGY 2019; 131:1046–62*)

Postoperative pulmonary complications, a well–documented group of complications after cardiac surgery, are associated with a fourfold increase in mortality,[1,2] extended intensive care unit (ICU) and hospital lengths of stay,[2,3] and more than $20,000 in institutional expenses per event.[3–5] In the cardiac surgery population, measurable derangements in pulmonary function occur in nearly all patients,[6,7] and approximately 10% to 25% develop postoperative pulmonary complications requiring substantial healthcare resource utilization.[1,6]

Cardiopulmonary bypass (CPB), mechanical ventilation, and surgical manipulation of the thoracic cavity each

play major roles in the evolution of pulmonary injury.[1] Preoperative, intraoperative, and postoperative factors impact a patient's ability to cope with these insults.[7,8] Several externally validated risk scores incorporating these factors have been developed to improve risk stratification for postoperative pulmonary complications after cardiac surgery.[9,10] Despite rigorous model development, shortcomings of postoperative pulmonary complication prediction models remain evident. One recent multicenter study demonstrated that a large proportion of variation in pneumonia rates remains unexplained by prediction models focused on surgical technique and underlying patient risk, suggesting that other unmeasured practices may account for the differences observed.[11] One such process of care associated with postoperative pulmonary complications, yet not accounted for in current prediction models, is the practice of intraoperative lung-protective ventilation. Compared with historic intraoperative ventilation techniques, modern lung-protective ventilation strategies use lower tidal volumes (V_T),[1,4,5,12–15] lower driving pressures,[16–18] and positive end-expiratory pressure (PEEP).[13,15,19] These techniques have already gained acceptance in ICUs after large studies have demonstrated reduced morbidity and mortality.[18,20] However, the contributions of each component to an overall intraoperative lung-protective ventilation strategy aimed at reducing postoperative pulmonary complications (postoperative pulmonary complications) have not been comprehensively studied in an adult cardiac surgical population.

Although ICU ventilation after cardiac surgery has been assessed,[21,22] scarce data currently exist evaluating the relationship between intraoperative ventilator management during cardiac surgery, postoperative pulmonary complications, and mortality. Because the post–CPB intraoperative period represents a unique transition from often nonventilated to ventilated lungs, optimizing respiratory mechanics to reduce lung injury is of critical concern. To better characterize this currently understudied relationship, we performed an observational cohort study using the Society of Thoracic Surgeons and Multicenter Perioperative Outcomes Group databases at our institution. We hypothesized that a bundled intraoperative lung-protective ventilation strategy (*i.e.*, lower V_T, driving pressure, and application of PEEP) was independently associated with decreased odds of postoperative pulmonary complications after cardiac surgery, when adjusted within a novel, robust multivariable model leveraging data uniquely available from each database. We additionally hypothesized that when studied as separate exposures, components of the intraoperative bundled lung-protective ventilation strategy had differential associations with postoperative pulmonary complications.

Materials and Methods

We obtained Institutional Review Board approval (HUM00132314) for this observational cohort study performed at our academic quaternary care center; the requirement for informed patient consent was waived. We adhered to the Strengthening the Reporting of Observational Studies in Epidemiology checklist for reporting observational studies. Study methods including data collection, outcomes, and statistical analysis were established prospectively and presented at an institutional peer–review committee on January 20, 2016; a revised finalized proposal was registered before accessing study data.[23]

Patient Population

Inclusion criteria for the study were adult (at least 18 yr old) patients who underwent elective or urgent cardiac surgical procedures with full CPB, limited to coronary artery bypass grafting, valve, and aortic procedures, performed in isolation or in combination. We reviewed patients over a continuous 11-yr study period from January 1, 2006 to June 1, 2017. Exclusion criteria were preoperative mechanical ventilation within 60 days of surgery, use of a double-lumen endotracheal tube or one-lung ventilation, American Society of Anesthesiologists class V or VI physical status, preoperative extracorporeal membrane oxygenation support, ventricular assist device implantation procedures (planned and unplanned), reoperative cardiac surgical procedures, transcatheter procedures, or procedures using partial- or left-heart bypass. At our institution, surgical techniques for the study cohort commonly included direct aortic cannulation *via* full sternotomy, and rarely, axillary or femoral cannulation or direct cannulation *via* mini-sternotomy. No robotic procedures or minimally invasive direct coronary artery bypass procedures were performed.

Data Collection

We collected study data from three sources: the Multicenter Perioperative Outcomes Group electronic anesthesia database, the Society of Thoracic Surgeons Adult Cardiac Surgery Database, and our hospital enterprise electronic health record. Within the Multicenter Perioperative Outcomes Group database, physiologic monitors including vital signs and ventilator settings and measurements are collected in automated fashion every 60 s and stored in an electronic intraoperative anesthesia record for all cases. Templated intraoperative script elements—including case times, medications and fluids administered, and anesthetic interventions such as airway management techniques—are additionally routinely recorded within the anesthesia record for all cases. Within the Society of Thoracic Surgeons database, patient history, surgical procedure, and outcome data are similarly stored as discrete concepts for all adult cardiac surgical procedures performed within our institution. To maintain high rates of interobserver agreement across cases, data are standardized using detailed prespecified definitions, and are collected (Society of Thoracic Surgeons database)[24] or validated (Multicenter Perioperative Outcomes Group database) by nurses with completed training in data definitions used. Detailed methods for data entry, validation,

and quality assurance are described elsewhere,[25–27] and have been used for multiple published studies.[28–31] Within the Multicenter Perioperative Outcomes Group and Society of Thoracic Surgeons databases, local datasets were linked *via* unique codified surgical case and patient identifiers; data extraction and analysis were performed on a secure server. Finally, local electronic health record data (Epic Systems Corporation, USA) were used to determine postoperative arterial blood gas values and ICU ventilator data, as necessary for components of outcome variables described below; these data were similarly linked to the final analytic dataset. The quality of local electronic health record data used for this study was verified *via* manual review by an anesthesiologist investigator (M.R.M.) of all cases experiencing the primary outcome, all cases with outlier data, and 10% of cases not experiencing the primary outcome.

Clinical Processes of Care

Perioperative anesthetic management for all cases was at the discretion of the attending cardiac anesthesiologist, who directs an anesthesia care team of anesthesiology fellows and residents. Routinely, anesthetic agents included induction with midazolam, propofol, or etomidate; analgesia with fentanyl or morphine; neuromuscular blockade with rocuronium, vecuronium, or cisatracurium; and maintenance with isoflurane, transitioned to a propofol or dexmedetomidine infusion before transport to ICU. In addition to standard monitoring, intraoperative hemodynamic management was routinely guided by invasive arterial line, central venous pressure, and pulmonary artery catheter monitors, as well as transesophageal echocardiography and arterial/mixed venous blood gas measurements. Fluids, blood products, vasoactive infusions, and inotropic infusions were managed at the discretion of the attending anesthesiologist in communication with the cardiac surgeon, with typical hemodynamic targets including a mean arterial pressure greater than 65 mmHg, cardiac index greater than 2.2 l/min/m², mixed venous oxygen saturation greater than 65%, hematocrit greater than 21%, and echocardiographic assessment of post-CPB ventricular systolic function unchanged to improved compared with pre-CPB function.

Ventilator settings in the operating room were managed by the attending anesthesiologist. Intubation was performed with a 7.5- or 8.0-mm–internal-diameter endotracheal tube. Mechanical ventilation was performed using Aisys CS2 anesthesia workstations (General Electric Healthcare, USA). Providers typically employed a pressure-controlled volume-guaranteed ventilation mode (default setting) throughout the entire study period, targeting normocapnia or mild hypocapnia, and avoiding hypoxemia. Of note, default settings on ventilators used included $V_T = 500$ ml and PEEP = 0 cm H_2O; the default PEEP setting was subsequently changed to PEEP = 5 cm H_2O in March 2007. Ventilation was paused during CPB; the ventilator circuit remained connected to the patient, but with no application

of PEEP. Before discontinuation of CPB, it was resumed after providing recruitment maneuvers. After transport to ICU, a structured handoff detailing intraoperative management, including final ventilator settings and plan for extubation, was communicated to an ICU team of intensivists, nurses, and respiratory therapists. Ventilator weaning, extubation, and management of complications were made at the discretion of the ICU team, as based on local protocols and targeting goals discussed during postoperative handoff.

Outcomes

The primary outcome was occurrence of a postoperative pulmonary complication, predefined as a composite of pulmonary complications recorded in the Society of Thoracic Surgeons database and adjudicated by nurses trained in outcome definitions, or recorded in our enterprise electronic health record and adjudicated by an anesthesiologist (M.R.M.). These included any one of the following: prolonged initial postoperative ventilator duration longer than 24 h (Society of Thoracic Surgeons database), pneumonia (Society of Thoracic Surgeons database), reintubation (Society of Thoracic Surgeons database), or postoperative partial pressure of oxygen to fractional inspired oxygen (PaO_2/FIO_2) below 100 mmHg within 48 h postoperatively while intubated (local electronic health record, Appendix 1).

We selected a threshold of PaO_2/FIO_2 below 100 mmHg as a postoperative pulmonary complication component based on previously validated assessments of pulmonary dysfunction associated with mortality after cardiac surgery.[32–34] Given varied mechanisms of pulmonary injury, and the distinction between pneumonia *versus* other pulmonary complications as described in recent consensus guidelines,[35,36] each component of the postoperative pulmonary complication composite outcome was also separately analyzed as a secondary outcome. Additional predefined secondary outcomes included 30-day postoperative mortality, initial postoperative mechanical ventilation duration, minimum PaO_2/FIO_2 within 48 h postoperatively while intubated (as a continuous variable), length of ICU stay, and length of hospital stay. All secondary outcomes were similarly adjudicated by trained Society of Thoracic Surgeons nurse reviewers with the exception of minimum PaO_2/FIO_2 which was adjudicated by an anesthesiologist (M.R.M.).

Exposure Variables – Lung-protective Ventilation

The primary exposure variable studied was a bundled intraoperative lung-protective ventilation strategy, comprising median V_T below 8 ml/kg predicted body weight and median driving pressure below 16 cm H_2O and median PEEP at or above 5 cm H_2O. Varying lung-protective cutoffs for each ventilator component are currently described in the literature, ranging from V_T 6 to 10 ml/kg predicted body weight,[1,13,15] driving pressure 8 to 19 cm H_2O,[16,18,37] and PEEP 3 to 12 cm H_2O.[13,15] Given these ranges, our

cutoffs were selected by inspection of previously collected ventilation practice institutional data, targeting upper quartiles (approximately 75% compliance for each component) to ensure class balance between cases with lung-protective ventilation *versus* non-lung-protective ventilation and to improve multivariable model discrimination.[5,13,28,38–40]

Predicted body weight (in kg) was calculated as: $50 + 2.3$ • (height [in] − 60) for men; $45 + 2.3$ • (height [in] − 60) for women.[41] Modified airway driving pressure was calculated as (peak inspiratory pressure − PEEP). As performed in previous studies,[42] we used modified driving pressure for all cases, given the lack of ventilator plateau pressure data available within our electronic medical record necessary for a true driving pressure calculation. To adjust for decisions to maintain normoxia rather than a lung-protective ventilation strategy (otherwise favoring lower F_{IO_2} and moderate PEEP), intraoperative oxygen saturation measured by pulse oximetry and F_{IO_2} were included as covariates. To summarize each ventilator variable on a per-case basis, median values while mechanically ventilated were calculated. Ventilator parameters while on CPB, during which ventilators were routinely paused, were excluded from the median value calculation. For descriptive purposes, ventilator parameters were additionally subdivided into median value pairs, separated into the pre-CPB and post-CPB periods. In cases with multiple instances of CPB, post-CPB ventilator parameters were analyzed after the final CPB instance.

Covariate Data

For descriptive purposes and to adjust for confounding variables potentially associated with the exposure variables or study outcomes, a range of perioperative characteristics were included as covariates within our study. Patient anthropometric, medical history, anesthetic, surgical, and laboratory testing/study variables were selected as available within the Multicenter Perioperative Outcomes Group and Society of Thoracic Surgeons databases. All variables used in several existing scores for calculating risk of complications including postoperative pulmonary complications after cardiac surgery were included (*e.g.*, cardiac surgery type, bypass times, comorbidities, *etc.*), in addition to other relevant descriptive covariates (table 1).[9,10,43] To evaluate for changes in practice and Society of Thoracic Surgeons database reporting over the study time period, the Society of Thoracic Surgeons Adult Cardiac Surgery Database version was included as a covariate; this resulted in four time periods for adjustment (1/1/2006–12/31/2007; 1/1/2008–6/30/2011; 7/1/2011–6/30/2014; 7/1/2014–5/31/2017) To account for variation in unmeasured intraoperative practices attributable to the attending anesthesiologist and potentially associated with postoperative pulmonary complications, we characterized attending anesthesiologists by tertiles of low/medium/high frequency of bundled intraoperative lung-protective ventilation use.

Statistical Analysis

All statistical analyses were performed using SAS version 9.3 (SAS Institute, USA). Normality of continuous variables was graphically assessed using histograms and Q-Q plots. Continuous data were presented as mean ± SD or median and interquartile range; binary data were summarized via frequency and percentage. Comparisons of continuous data were made using a two-tailed independent *t* test or a Mann–Whitney *U* test, and categorical data were compared by a Pearson chi-square or Fisher's exact test, as appropriate. Trend analyses of the components of the lung-protective ventilation bundle were completed using the Cochran–Armitage test. A *P* value less than 0.05 denoted statistical significance.

Before any multivariable analysis, collinearity among covariates was assessed using the variance inflation factor; variables with a variance inflation factor greater than 10 were excluded. To target development of a clinically usable reduced-fit postoperative pulmonary complication multivariable model avoiding overfitting, covariates meaningfully describing the study population but not used in existing cardiac surgery risk score models were additionally excluded from multivariable analysis. Missing data were handled via a complete case analysis. To further aid in covariate selection, we used the least absolute shrinkage and selection operators technique and restricted covariates to the number of outcomes divided by 10, while also accounting for the lung-protective ventilation bundle as well as lung-protective ventilation bundle components (V_T, driving pressure, and PEEP). We chose this variable selection technique, given its ability to perform regularization and variable selection to improve model accuracy and interpretability, particularly among analyses with a relatively large number of covariates and modest number of outcomes.[44] Using a multivariable logistic regression model, we characterized the risk-adjusted association between the primary exposure of intraoperative lung-protective ventilation bundle and the primary outcome of postoperative pulmonary complication. Additionally, we repeated our multivariable analysis to assess independent associations between each lung-protective ventilation bundle component and postoperative pulmonary complications. Overall model discrimination of logistic regression models was assessed using the c statistic. Secondary outcomes were assessed using multivariable linear regression models. Goodness-of-fit for linear regression models was summarized via R-squared; such models were evaluated using varied distributional assumptions (*i.e.*, linear *versus* logarithmic transformations) for continuous secondary outcomes. Multilevel modeling clustering at the provider level was not possible because of limited sample size per provider; instead, the previously mentioned fixed covariate of anesthesiology attending lung-protective ventilation frequency tertile was used.

In addition to analyzing independent associations between an overall lung-protective ventilation strategy and

Table 1. Perioperative Patient Characteristics and Univariate/Bivariate Associations with Postoperative Pulmonary Complications

Characteristic	Entire Cohort N = 4694 n (%) or Mean ± SD/Median [Interquartile Range]	Postoperative Pulmonary Complication		P Value	% Cases with Complete Data
		No, N = 4181 (89.1%) n (%) or Mean ± SD/Median [Interquartile Range]	Yes, N = 513 (10.9%) n (%) or Mean ± SD/Median [Interquartile Range]		
Preoperative characteristics					
Age	62 ± 14	62 ± 14	64 ± 14	< .0001	100
Sex, male	3,024 ± 64.4	2,730 ± 65.3	294 ± 57.3	0.0004	100
Race, non-white	515 ± 11.0	442 ± 10.6	73 ± 14.2	0.0127	99.9
Height, cm	172 ± 11	172 ± 10	170 ± 11	< .0001	100
Actual body weight, kg	86.9 ± 21.0	87.0 ± 20.8	85.8 ± 22.8	0.2624	100
Predicted body weight, kg	65.8 ± 11.1	66.1 ± 11.0	63.6 ± 11.6	< .0001	100
Body mass index, kg/m^2				0.1389	100
Underweight (< 18.5)	52 ± 1.1	45 ± 1.1	7 ± 1.4		
Normal weight (18.5–24.9)	1,090 ± 23.2	971 ± 23.2	119 ± 23.2		
Overweight (25–29.9)	1,724 ± 36.7	1,551 ± 37.1	173 ± 33.7		
Class I obesity (30–34.9)	1,065 ± 22.7	954 ± 22.8	111 ± 21.6		
Class II obesity (35–39.9)	448 ± 9.5	392 ± 9.4	56 ± 10.9		
Class III obesity (≥ 40)	315 ± 6.7	268 ± 6.4	47 ± 9.2		
Current smoker	630 ± 13.4	551 ± 13.2	79 ± 15.4	0.1637	100
Chronic lung disease*	543 ± 11.6	444 ± 10.6	99 ± 19.3	< .0001	100
Recent pneumonia within one month	55 ± 1.2	45 ± 1.1	10 ± 2.0	0.0829	100
Sleep apnea	490 ± 10.4	442 ± 10.6	48 ± 9.4	0.3957	100
Pulmonary hypertension	1,447 ± 30.8	1,296 ± 31.0	151 ± 29.4	< .0001	99.5
Moderate (PA systolic pressure 31–55 mmHg)	1,185 ± 25.2	1,088 ± 26.1	97 ± 19.1		
Severe (PA systolic pressure > 55 mmHg)	262 ± 5.6	208 ± 5.0	54 ± 10.7		
New York Heart Association Class				< .0001	99.0
I	3,807 ± 81.9	3,448 ± 83.3	359 ± 70.8		
II	305 ± 6.6	272 ± 6.6	33 ± 6.5		
III	409 ± 8.8	326 ± 7.9	83 ± 16.4		
IV	125 ± 2.7	93 ± 2.3	32 ± 6.3		
Recent myocardial infarction < 21 days	329 ± 7.0	275 ± 6.6	54 ± 10.5	0.0009	100
Preoperative left ventricular ejection fraction, %	60 [55, 65]	60 [55, 65]	60 [50, 65]	0.0003	100
Poor mobility†	2,353 ± 50.1	2,030 ± 48.6	323 ± 63.0	< .0001	100
Extracardiac arteriopathy	931 ± 19.8	798 ± 19.1	133 ± 25.9	0.0002	100
Peripheral arterial disease	333 ± 7.1	278 ± 6.7	55 ± 10.7	0.0007	
Carotid disease	602 ± 12.8	519 ± 12.4	83 ± 16.2	0.0161	
Amputation for arterial disease	69 ± 1.5	57 ± 1.4	12 ± 2.3	0.0830	
Previous major vascular surgical intervention	224 ± 4.8	188 ± 4.5	36 ± 7.0	0.0115	
Dyslipidemia	2,703 ± 57.6	2,419 ± 57.9	284 (55.4)	0.2803	100
Arrhythmia‡	735 ± 15.7	626 ± 15.0	109 ± 21.3	0.0002	100
Renal Impairment					
Creatinine clearance, ml · min^{-1} · 1.73 m^{-2}§	76.5 ± 24.5	77.9 ± 23.8	65.2 ± 27.4	< .0001	99.8
Dialysis requirement	104 ± 2.2	72 ± 1.7	32 ± 6.2	< .0001	100
Diabetes treated with Insulin	374 ± 8.0	312 ± 7.5	62 ± 12.1	0.0003	100
Liver disease	77 ± 1.6	69 ± 1.7	8 ± 1.6	0.8785	100
Cancer	225 ± 4.8	204 ± 4.9	21 ± 4.1	0.4318	100
Active endocarditis	238 ± 5.1	197 ± 4.7	41 ± 8.0	0.0014	100
Critical preoperative state	410 ± 8.7	300 ± 7.2	110 ± 21.4	< .0001	100
Preoperative ventilation	(exclusion)	(exclusion)	(exclusion)	(exclusion)	(exclusion)
Preoperative inotropic support	366 ± 7.8	266 ± 6.4	100 ± 19.5	< .0001	100
Cardiogenic shock	24 ± 0.5	18 ± 0.4	6 ± 1.2	0.0268	100
Intra-aortic balloon pump	58 ± 1.2	34 ± 0.8	24	< .0001	100
Hemoglobin, g/dl	13.5 ± 1.9	13.6 ± 1.9	12.6 ± 2.2	< .0001	99.7
Platelet count, K/ul	225 ± 69	225 ± 68	223 ± 77	0.6178	99.7
White blood cell count, K/ul	6.8 [5.7, 8.3]	6.8 [5.7, 8.2]	7.4 [6.0, 9.1]	< .0001	99.7
International normalized ratio	1.0 [1.0, 1.1]	1.0 [1.0, 1.0]	1.0 [1.0, 1.1]	< .0001	99.6
Preoperative SpO$_2$, %	97 [96, 98]	97 [96, 98]	97 [95, 98]	< .0001	100
Preoperative respiratory rate	16 [16, 18]	16 [16, 18]	16 [16, 18]	0.0015	97.0
Acuity				< .0001	100
Elective	3,740 ± 79.7	3,409 ± 81.5	331 ± 64.5		
Urgent	954 ± 20.3	772 ± 18.5	182 ± 35.5		
Surgical procedure type				< .0001	100
Aortic	100 ± 2.1	84 ± 2.0	16 ± 3.1		
Valve + Aortic	927 ± 19.8	807 ± 19.3	120 ± 23.4		
Valve + Aortic + CABG	84 ± 1.8	63 ± 1.5	21 ± 4.1		

(Continued)

Table 1. (Continued)

Characteristic	Entire Cohort N = 4694 n (%) or Mean ± SD/Median [Interquartile Range]	Postoperative Pulmonary Complication		P Value	% Cases with Complete Data
		No, N = 4181 (89.1%) n (%) or Mean ± SD/Median [Interquartile Range]	Yes, N = 513 (10.9%) n (%) or Mean ± SD/Median [Interquartile Range]		
Isolated CABG	969 ± 20.6	892 ± 21.3	77 ± 15.0		
Isolated Valve	2,078 ± 44.3	1,902 ± 45.5	176 ± 34.3		
Valve + CABG	536 ± 11.4	433 ± 10.4	103 ± 20.1		
Admission type				< .0001	100
Admit	3,488 ± 74.3	3,197 ± 76.5	291 ± 56.7		
Inpatient	1,206 ± 25.7	984 ± 23.5	222 ± 43.3		
Date of surgery by STS version				< .0001	100
2.52 (Jan 2006 through Dec 2007)	349 ± 7.4	312 ± 7.5	37 ± 7.2		
2.61 (Jan 2008 through June 2011)	1,286 ± 27.4	1,106 ± 26.5	180 ± 35.1		
2.73 (July 2011 through June 2014)	1,679 ± 35.8	1,482 ± 35.5	197 ± 38.4		
2.81 (July 2014 through May 2017)	1,380 ± 29.4	1,281 ± 30.6	99 ± 19.3		
ASA physical status				< .0001	100
III	1,476 ± 31.4	1,366 ± 32.7	110 ± 21.4		
IV	3,218 ± 68.6	2,815 ± 67.3	403 ± 78.6		
Intraoperative characteristics					
Perfusion time, h	2.2 ± 1.0	2.1 ± 1.0	2.9 ± 1.3	< .0001	100
Aortic crossclamp time, h	1.7 ± 0.8	1.7 ± 0.8	2.2 ± 1.1	< .0001	99.7
Anesthesia duration, h	6.5 [5.4, 7.9]	6.4 [5.3, 7.7]	7.7 [6.4, 9.4]	< .0001	100
Anesthesia provider¶				0.9443	100
Low LPV user	2,489 ± 53.0	2,214 ± 53.0	275 ± 53.6		
Medium LPV user	1,844 ± 39.3	1,646 ± 39.4	198 ± 38.6		
High LPV user	361 ± 7.7	321 ± 7.7	40 ± 7.8		
Intraoperative albuterol	57 ± 1.2	46 ± 1.1	11 ± 2.1	0.0416	100
Intraoperative diuretic‖	2,898 ± 61.7	2,588 ± 61.9	310 ± 60.4	0.5179	100
Intraoperative vasopressor infusion (phenylephrine, norepinephrine, vasopressin)	4,294 ± 91.5	3,815 ± 91.3	479 ± 93.4	0.1036	100
Intraoperative inotrope infusion (epinephrine, dobutamine, milrinone, isoproterenol, dopamine)	1,723 ± 36.7	1,397 ± 33.4	326 ± 63.6	< .0001	100
Total intraoperative opioid, oral morphine equivalents	300 [270, 360]	300 [270, 360]	300 [240, 375]	0.0177	99.9
Total intraoperative crystalloid, liter	3.0 [2.0, 4.3]	3.0 [2.0, 4.1]	3.4 [2.3, 5.3]	< .0001	98.8
Total intraoperative colloid, liter	0 [0, 0.5]	0 [0, 0.5]	0 [0, 0.5]	0.3498	100
Intraoperative packed red blood cells, units	0 [0,2]	0 [0, 2]	2 [0, 4]	< .0001	100
Intraoperative red blood cell salvage, liter	0 [0,0]	0 [0, 0]	0 [0, 0]	0.9154	100
Intraoperative fresh frozen plasma, units	0 [0,0]	0 [0, 0]	0 [0, 2]	< .0001	100
Intraoperative platelets, units	0 [0, 0]	0 [0, 0]	0 [0, 2]	< .0001	100
Intraoperative cryoprecipitate, units	0 [0, 0]	0 [0, 0]	0 [0, 2]	< .0001	100
Total urine output, liter	1.9 ± 1.2	1.9 ± 1.1	1.9 ± 1.4	0.4361	99.1
Pre-CPB ventilation/respiratory parameters					
Tidal volume, ml/kg predicted body weight	7.8 ± 1.5	7.8 ± 1.5	8.1 ± 1.7	0.0001	100
Peak inspiratory pressure, cm H_2O	17 [15, 20]	17 [15, 20]	19 [16, 22]	< .0001	100
Positive end-expiratory pressure, cm H_2O	5 [4, 5]	5 [4, 5]	5 [2, 5]	0.0359	100
Driving pressure, cm H_2O	13 [11, 16]	13 [11, 16]	15 [12, 18]	< .0001	100
SpO_2, %	99 [98, 100]	99 [98, 100]	99 [99, 100]	< .0001	100
Inspired FIO_2, %	97 [96, 98]	97 [95, 98]	97 [96, 98]	< .0001	100
Post-CPB ventilation/respiratory parameters					
Tidal volume, ml/kg predicted body weight	7.8 ± 1.5	7.7 ± 1.4	8.1 ± 1.6	< .0001	100
Peak inspiratory pressure, cm H_2O	18 [15, 20]	17 [15, 20]	20 [17, 23]	< .0001	100
Positive end-expiratory pressure, cm H_2O	5 [4, 5]	5 [4, 5]	5 [4, 5]	0.7216	100
Driving pressure, cm H_2O	13 [11, 16]	13 [11, 16]	16 [13, 19]	< .0001	100
SpO_2, %	100 [99, 100]	100 [99, 100]	100 [98, 100]	0.4913	100
Inspired FIO_2, %	97 [96, 98]	97.0 [96, 98]	97 [96, 98]	0.0033	100
Overall ventilation					
Bundled LPV strategy**	1,913 ± 40.8	1,787 ± 42.7	126 ± 24.6	< .0001	100

P value from independent t test, Mann–Whitney U test, or chi-square test, as appropriate.

*Defined by chronic lung disease at or above moderate or bronchodilator therapy within STS; or COPD at or above moderate on preoperative anesthesia history and physical. †Defined by functional capacity – Low (at or below four metabolic equivalents of task) on preoperative anesthesia history and physical. ‡Defined via STS as a history of any of the following: atrial fibrillation, atrial flutter, third degree heart block, ventricular fibrillation, or ventricular tachycardia. §Calculated using the Chronic Kidney Disease – Epidemiology Collaboration equation. ¶Defined as the frequency of primary anesthesiology attending using a bundled LPV strategy, as a proportion of all cardiac cases performed by the anesthesiology attending among the study population, transformed into tertiles. ‖Defined as intraoperative administration of furosemide, bumetanide, or mannitol. **Defined as intraoperative median values of tidal volume less than 8 ml/kg predicted body weight, positive end-expiratory pressure at or above 5 cm H_2O, and driving pressure less than 16 cm H_2O.

ASA, American Society of Anesthesiologists; CABG, coronary artery bypass graft; CPB, cardiopulmonary bypass; FIO_2, fraction of inspired oxygen; LPV, lung-protective ventilation; PA, pulmonary artery; SpO_2, oxygen saturation measured by pulse oximetry; STS, Society of Thoracic Surgeons.

the postoperative pulmonary complication primary outcome, we performed several sensitivity analyses, including an analysis of lung-protective ventilation separated into component parts: V_T below 8 ml/kg predicted body weight, driving pressure below 16 cm H_2O, or PEEP at or above 5 cm H_2O, and analysis of lung-protective ventilation strategies separately examined before and after CPB.

We also performed a sensitivity analysis, using a model that further restricted the number of covariates to the number of outcomes divided by 20.[45] Additionally, we compared our multivariable postoperative pulmonary complication model developed using least absolute shrinkage and selection operator for covariate selection with a multivariable postoperative pulmonary complication model including all noncollinear covariates with P less than 0.10. Finally, we performed subgroup analyses stratified by salient clinical characteristics.

Results

Of the 5,365 cardiac surgical cases reviewed, 4,694 met study inclusion criteria (fig. 1). Among these cases, 513 (10.9%) experienced a postoperative pulmonary complication. Individual nonmutually exclusive components of postoperative pulmonary complications included pneumonia (121 cases, 23.6% of postoperative pulmonary complications), prolonged ventilation longer than 24 h, (302, 58.9% of postoperative pulmonary complications), reintubation (115, 22.4% of postoperative pulmonary complications), and PaO_2/F_{IO_2} below 100 mmHg (164, 32.0% of postoperative pulmonary complications).

Patient Population – Baseline Characteristics and Univariate Analyses

As described in table 1, our study population had a median age of 62 yr, and 64% were men. Cardiac surgeries performed included coronary artery bypass grafting (20.6%), valve (44.3%), aorta (2.1%), and combination (33.0%). Cases were primarily elective (79.7%); remaining cases were urgent (20.3%). Our study population included cases across four time partitions by Society of Thoracic Surgeons Adult Cardiac Surgery Database version, including 349 (7.4%) from 1/1/2006 to 12/31/2007; 1,286 (27.4%) 1/1/2008 to 6/30/2011; 1,679 (35.8%) 7/1/2011 to 6/30/2014; 1,380 (29.4%) 7/1/2014 to 5/31/2017. An overall lung-protective ventilation strategy was used in 1,913 cases (40.8%); among components of a lung-protective ventilation strategy, a V_T below 8 ml/kg predicted body weight was achieved in 64% of cases, modified driving pressure below 16 cm H_2O in 71% of cases, and PEEP at or above 5 cm H_2O in 63% of cases. Adherence to varying thresholds and independent associations with postoperative pulmonary complications are provided in Supplemental Digital Content 1A through 1C (http://links.lww.com/ALN/C26). Crude incidence of postoperative pulmonary complications among cases using an overall lung-protective ventilation strategy was 6.6%,

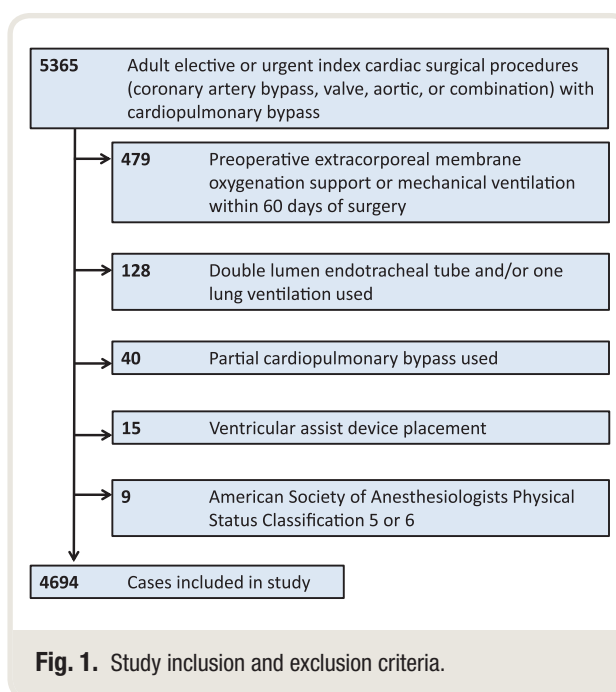| 5365	Adult elective or urgent index cardiac surgical procedures (coronary artery bypass, valve, aortic, or combination) with cardiopulmonary bypass
479	Preoperative extracorporeal membrane oxygenation support or mechanical ventilation within 60 days of surgery
128	Double lumen endotracheal tube and/or one lung ventilation used
40	Partial cardiopulmonary bypass used
15	Ventricular assist device placement
9	American Society of Anesthesiologists Physical Status Classification 5 or 6
4694	Cases included in study

Fig. 1. Study inclusion and exclusion criteria.

compared with 13.9% among cases without an overall lung-protective ventilation strategy (table 2). Postoperative pulmonary complications were associated with increased postoperative mortality as well as longer postoperative mechanical ventilation, ICU stay, and hospital stay (table 3). Patients receiving a lung-protective ventilation strategy were more commonly tall, nonobese, male, and nonsmokers (Supplemental Digital Content 2, http://links.lww.com/ALN/C27).

Intraoperative Ventilator Management

Patients were ventilated with a cohort mean $\pm$ SD V_T of 7.8 $\pm$ 1.5 ml/kg predicted body weight, median (interquartile range) driving pressure of 13 (11 to 16) cm H_2O, and PEEP of 5 (4 to 5) cm H_2O. Compared with pre-CPB ventilator parameters, we observed no significant differences in post-CPB parameters (table 1). We observed distributions of overall per-case median ventilator parameters to be unimodal and rightward-skewed for V_T and driving pressure, *versus* a bimodal distribution (0 cm H_2O and 5 cm H_2O) for PEEP (fig. 2). Over the study period, we observed significant linear trends in ventilation practices: providers used decreasing V_T and driving pressure, and increasingly used PEEP ($P < 0.001$ for all trends; fig. 3).

Impact of Ventilator Parameters–Multivariable Analyses

Of the 4,694 cases studied, we observed data completeness rates greater than 99% for all but two risk adjustment variables, preoperative respiratory rate (97.0%) and total intraoperative crystalloid (98.8%). Peak inspiratory pressure and weight were removed from the model due to

Table 2. Summary of Primary Study Outcomes, Primary Outcome Components, and Bundled Lung-protective Ventilation Strategy

| | Entire Cohort N = 4694 | Bundled LPV Strategy* | | |
		No, 59.3% (n = 2781)	Yes, 40.7% (n = 1913)	P Value
Postoperative pulmonary complication	513 (10.9)	387 (13.9)	126 (6.6)	< .0001
Pneumonia	121 (2.6)	99 (3.6)	22 (1.2)	< .0001
Prolonged postoperative ventilation†	302 (6.4)	226 (8.1)	76 (4.0)	< .0001
Reintubation	115 (2.6)	85 (3.5)	30 (1.6)	< .0001
$PaO_2/FIO_2 < 100$ mmHg‡	164 (3.8)	131 (5.2)	33 (1.9)	< .0001

P value from independent t test or chi-square test, as appropriate.
*Defined as intraoperative median values of below 8 ml/kg predicted body weight, positive end-expiratory pressure at or above 5 cm H_2O, and driving pressure below 16 cm H_2O.
†Defined as initial postoperative mechanical ventilation more than 24 h. ‡Within 48 h postoperatively while intubated.
FIO_2, fraction of inspired oxygen; LPV, lung-protective ventilation; PaO_2, arterial partial pressure of oxygen.

Table 3. Summary of Primary Study Outcomes, Secondary Study Outcomes, and Bundled Lung-protective Ventilation Strategy

| | Entire Cohort N = 4,694 | Postoperative Pulmonary Complication | | | Bundled LPV Strategy* | | |
		No, 89.1% (n = 4,181)	Yes, 10.9% (n = 513)	P Value	No, 59.3% (n = 2781)	Yes, 40.7% (n = 1913)	P Value
30-day postoperative mortality	49 (1.0)	19 (0.5)	30 (5.9)	< .0001	35 (1.3)	14 (0.7)	0.0810
Postoperative durations							
Total postoperative ventilator, h	17.2 (77.3)	7.1 (5.2)	99.4 (216.5)	< .0001	21.5 (96.1)	10.9 (34.3)	< .0001
Total ICU, h	73.9 (115.0)	57.6 (51.6)	207.1 (282.1)	< .0001	79.7 (137.6)	65.4 (69.3)	< .0001
Total hospital length of stay, days	7.5 (6.7)	6.5 (3.9)	15.7 (14.4)	< .0001	8.1 (7.8)	6.7 (4.4)	< .0001

P value from independent t test or chi-square test, as appropriate.
*Defined as intraoperative median values of below 8 ml/kg predicted body weight, positive end-expiratory pressure at or above 5 cm H_2O, and driving pressure below 16 cm H_2O.
ICU, intensive care unit; LPV, lung-protective ventilation.

multicollinearity (variance inflation factor greater than 10). Platelet count, international normalized ratio, total intraoperative opioid, preoperative respiratory rate, and history of cancer were removed, given a lack of use in previous validated cardiac surgery or postoperative pulmonary complication risk score models.[9,10,43] Multiple additional variables were removed via least absolute shrinkage and selection operator (denoted by "−" in Supplemental Digital Content 3, http://links.lww.com/ALN/C28). Through multivariable analyses adjusting for postoperative pulmonary complication risk factors, an intraoperative lung-protective ventilation bundle was independently associated with reduced postoperative pulmonary complications (adjusted odds ratio, 0.56; 95% CI, 0.42–0.75, figs. 4 and 5). Modelling lung-protective ventilation exposure as a treatment, we observed a number needed to expose of 18 (95% CI, 14–33) to prevent one postoperative pulmonary complication.

We observed no associations between a lung-protective ventilation bundle and minimum postoperative PaO_2/FIO_2 while intubated, initial postoperative ventilator duration in hours, length of ICU stay in hours, or length of hospital stay in days (Supplemental Digital Content 4, http://links.lww.com/ALN/C29). We observed similar findings for logarithmically transformed secondary outcomes. Postoperative mortality occurred in 49 cases (1.0%); our study was not adequately powered to analyze independent associations between lung-protective ventilation and mortality.

Among individual pulmonary complications (pneumonia, prolonged ventilation longer than 24 h, reintubation, and PaO_2/FIO_2 less than 100 mmHg postoperatively while intubated), a lung-protective ventilation bundle demonstrated univariate associations across all postoperative pulmonary complication components; after multivariable adjustment, a lung-protective ventilation bundle remained protective against all postoperative pulmonary complication components except for prolonged ventilation longer than 24 h (Supplemental Digital Content 5, http://links.lww.com/ALN/C30, and Supplemental Digital Content 6, http://links.lww.com/ALN/C31).

Sensitivity Analyses

When analyzing each component of the lung-protective ventilation bundle separately, we found that modified
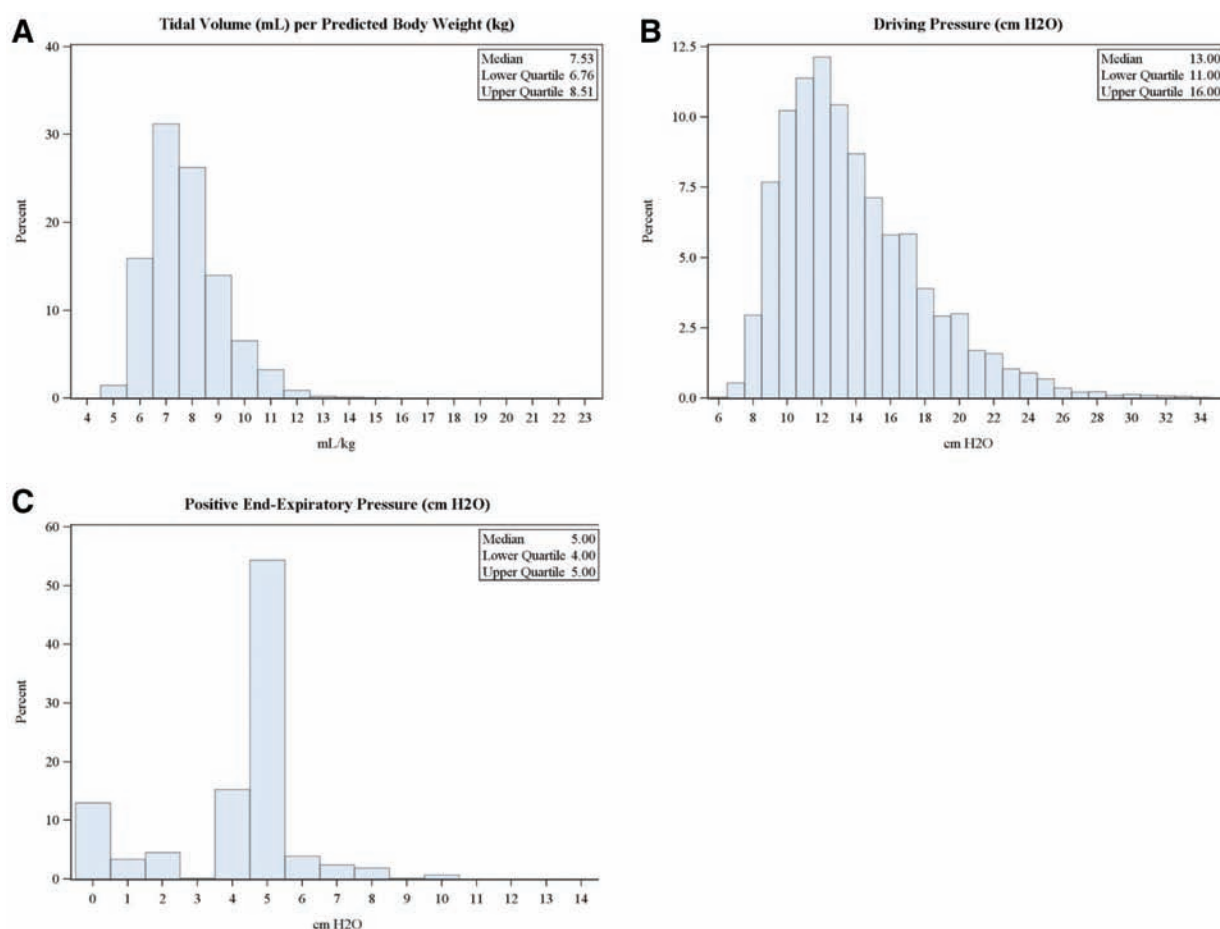
Fig. 2. Frequency distributions of per-case median intraoperative ventilator parameters, including tidal volume per predicted body weight, modified driving pressure, and positive end-expiratory pressure (in *A*, *B*, and *C*, respectively).

driving pressure driving pressure less than 16 cm H_2O was independently associated with reduced postoperative pulmonary complications (adjusted odds ratio, 0.51; 95% CI, 0.39–0.66) whereas V_T below 8 ml/kg predicted body weight and PEEP at or above 5 cm H_2O did not demonstrate significant independent associations (adjusted odds ratios [95% CIs] 0.99 [0.75–1.30] and 1.18 [0.91–1.53], respectively; fig. 4). Furthermore, driving pressure less than 16 cm H_2O was independently associated with improvements in all secondary outcomes.

When analyzing the lung-protective ventilation bundle as partitioned into pre-CPB and post-CPB periods, we observed no collinearity between corresponding pre-CPB and post-CPB variables (variance inflation factors below 10) and thus included all variables into a single model. We found that adherence to the post-CPB lung-protective ventilation bundle was associated with less postoperative pulmonary complications (adjusted odds ratio, 0.53; 95% CI, 0.38–0.74) whereas the pre-CPB lung-protective

ventilation bundle was not associated with postoperative pulmonary complications (adjusted odds ratio, 1.19; 95% CI, 0.84–1.68, Supplemental Digital Content 7, http://links.lww.com/ALN/C32). Similarly, when analyzing the lung-protective ventilation components individually partitioned into pre-CPB and post-CPB periods, we observed no collinearity between corresponding pre-CPB and post-CPB components and thus included all variables into a single model. We observed post–CPB driving pressure less than 16 cm H_2O was associated with lesser likelihood of postoperative pulmonary complication (adjusted odds ratio, 0.57; 95% CI, 0.42–0.78), but neither the pre-CPB driving pressure below 16 cm H_2O (adjusted odds ratio, 0.77; 95% CI, 0.56–1.07) nor V_T below 8 ml/kg predicted body weight nor PEEP at or above 5 cm H_2O pre-CPB and post-CPB components was associated with postoperative pulmonary complications.

Logistic regression models using either least absolute shrinkage and selection of operator restricted to 24
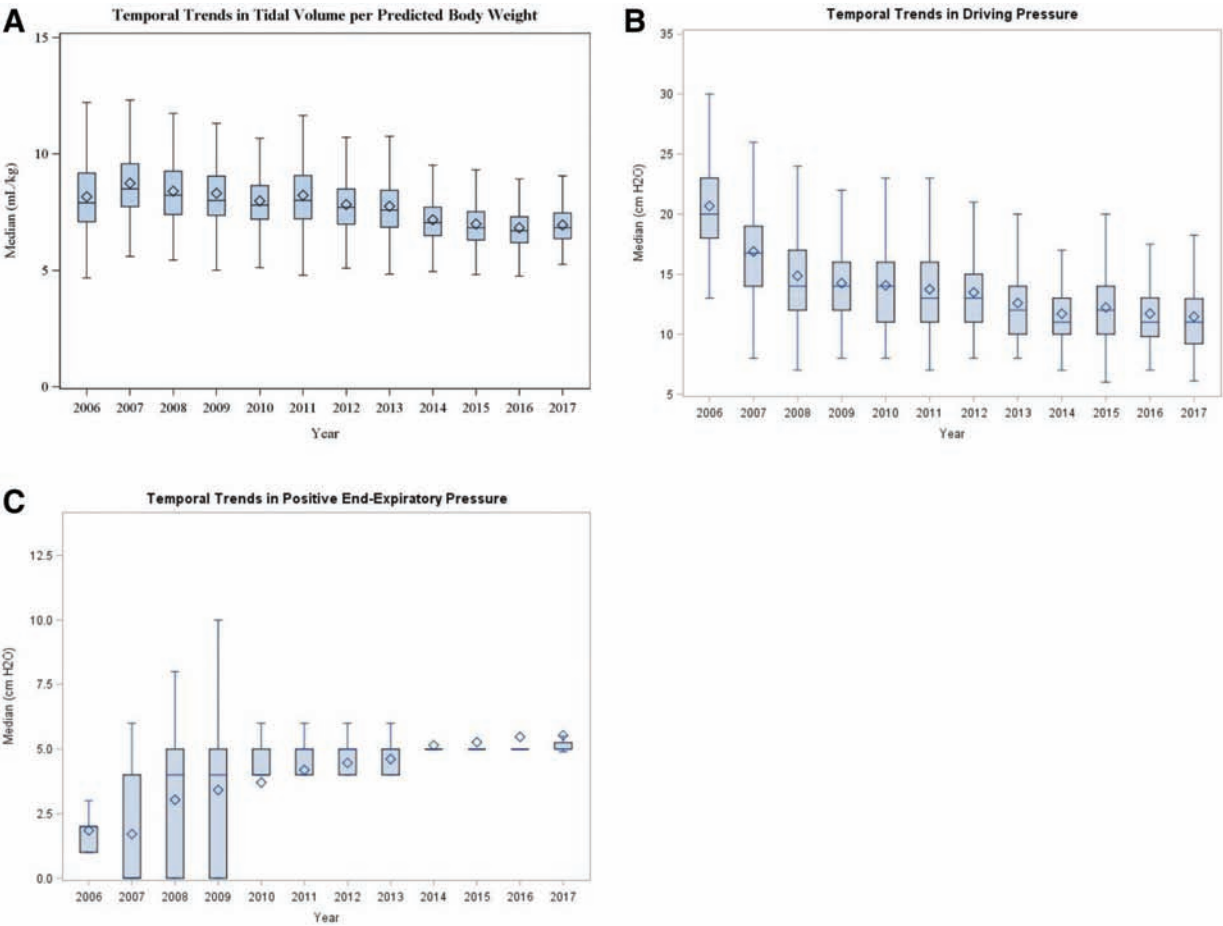
Fig. 3. Temporal trends in intraoperative ventilator strategies, including tidal volume per predicted body weight, modified driving pressure, and positive end-expiratory pressure (in *A*, *B*, and *C*, respectively).
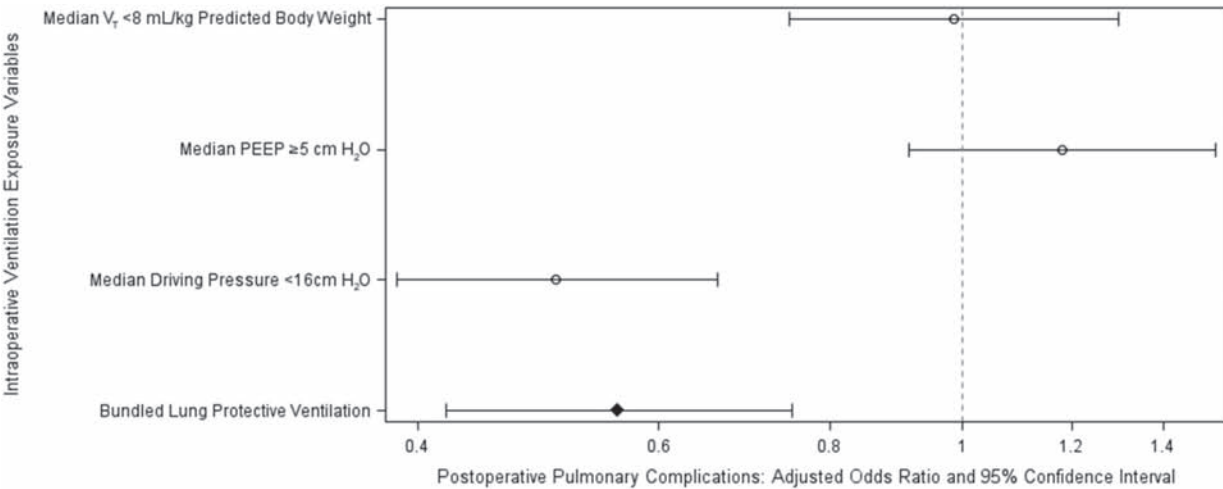

Fig. 4. Independent associations between intraoperative lung protective ventilation strategies and postoperative pulmonary complications.
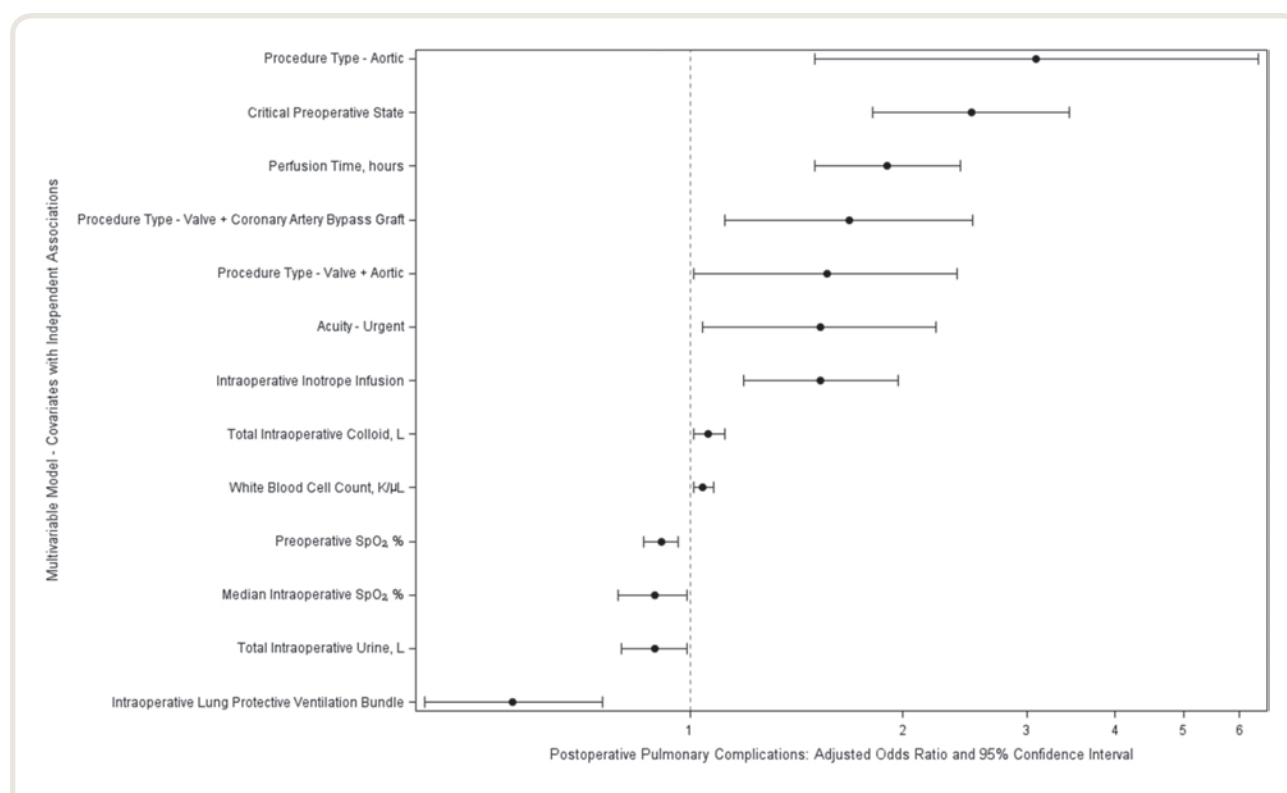
Fig. 5. Significant independent associations between multivariable model components and postoperative pulmonary complications.

covariates, or forward selection of univariate association thresholds ($P < 0.10$) found independent associations between lung-protective ventilation, driving pressure, and postoperative pulmonary complications, but not V_T or PEEP (Supplemental Digital Content 8, http://links.lww.com/ALN/C33, and Supplemental Digital Content 9, http://links.lww.com/ALN/C34). Finally, sensitivity analyses of clinically important subgroups yielded similar independent associations between the lung-protective ventilation bundle and outcomes. The protective association of the lung-protective ventilation bundle was observed in both males and females, in elective but not urgent cases, across all body mass index ranges, only in patients without chronic lung disease, and in patients undergoing valve procedures (Supplemental Digital Content 10, http://links.lww.com/ALN/C35).

Discussion

Using robust, validated observational databases, we report an overall pulmonary complication incidence of 10.9% after cardiac surgery, and identify an intraoperative lung-protective ventilation bundle as independently associated with a clinically and statistically significant reduction in pulmonary complications. Our study builds on existing literature by providing an analysis of the impact of intraoperative ventilation strategies on postoperative outcomes among a generalizable cardiac surgery population. Although unaccounted for in current risk scoring systems, we report that

an intraoperative lung-protective ventilation strategy is independently associated with development of postoperative pulmonary complications. Through a sensitivity analysis evaluating components of the lung-protective ventilation bundle, we importantly note that driving pressure, but not V_T or PEEP, is independently associated with postoperative pulmonary complications.

Compared with previous literature, our findings demonstrate the importance of considering multiple components of lung-protective ventilation when evaluating the impact of mechanical ventilation on outcomes. Notably, we observed that not all components of lung-protective ventilation were independently associated with decreased postoperative pulmonary complications; however, a lung-protective ventilation bundled approach was independently associated with decreased postoperative pulmonary complications. Furthermore, within the lung-protective ventilation bundle studied, we observed driving pressure as the component primarily driving the association with reduced postoperative pulmonary complications, rather than V_T or PEEP. These findings offer insight toward sustaining a trend of expedited recovery from cardiac surgery, a process in which postoperative care teams are increasingly reliant on intraoperative practices—such as lung-protective ventilation—to target reduced postoperative complications and to safely enable rapid de-escalation of care on arrival to the ICU.[46,47]

Our study highlights the importance of driving pressure, and conversely the limitations of V_T and PEEP, as independently associated with postoperative pulmonary complications and secondary outcomes. We offer two hypotheses to explain these findings: (1) increased driving pressure is a marker for noncompliant lungs, assuming such patients are at increased risk of postoperative pulmonary complications and remain unidentified by model covariates; or (2) increased driving pressure reflects direct pulmonary injury via barotrauma as a postoperative pulmonary complication mechanism. Countervailing to a hypothesis that driving pressure serves as a marker for noncompliance, however, was our observation that lower V_T was not independently associated with increased postoperative pulmonary complications, as would be the case for increasingly noncompliant lungs at a given constant driving pressure exposure (controlled covariate). This finding was similarly observed in an analysis performed among 3,562 patients with acute respiratory distress syndrome enrolled across nine randomized trials.[17] Within a surgical population, a recent randomized, controlled trial demonstrated a driving pressure-guided ventilation strategy during one-lung ventilation to be similarly associated with a lower incidence of postoperative pulmonary complications compared with conventional ventilation strategies, during thoracic surgery.[48]

Additionally of note, in a sensitivity analysis analyzing pre-CPB driving pressure and post-CPB driving pressure separately, our observations that (1) pre-CPB and post-CPB variables were not collinear and (2) post-CPB driving pressure but not pre-CPB driving pressure below 16 cm H_2O was independently associated with postoperative pulmonary complications, suggests our driving pressure findings cannot solely be explained as a marker for poor baseline lung function. However, whether this independent association between post-CPB driving pressure below 16 cm H_2O and postoperative pulmonary complications can be explained by a direct lung injury hypothesis, *versus* a marker for varying degrees of CPB-induced pulmonary dysfunction, remains unanswerable based on our data. Other explanations for a lack of collinearity between pre-CPB and post-CPB driving pressure may include nuanced surgery stage-specific ventilation strategies, such as low V_T and low driving pressure during internal mammary artery surgical dissection and/or cannulation before CPB. Finally, although a driving pressure threshold below 16 cm H_2O enabled class balance between cases adherent *versus* nonadherent to an overall lung-protective ventilation bundle, an optimal driving pressure threshold defining lung-protective ventilation remains unclear, and likely varies by clinical context.

Our findings that lower intraoperative driving pressure was associated with improved outcomes suggest an opportunity for improved care through the implementation of an lung-protective ventilation protocol favoring lower driving pressure. Additionally, our observation that intraoperative

driving pressure, but not V_T or PEEP, was independently associated with postoperative pulmonary complications, reflects a potential benefit of individualized ventilation strategies among patients with varying respiratory compliance (ignored with V_T-targeted ventilator management) or varying volume of aerated functional lung (ignored with uniform application of PEEP). However, given the observational nature of this study, our findings require prospective interventional evaluation and validation before large-scale adoption of the technique.

Our 10.9% observed incidence of postoperative pulmonary complications is consistent with previous studies.[1,6] However, this comparison is challenged by varied definitions of a postoperative pulmonary complication, which remain subject to debate. Our postoperative pulmonary complication definition is consistent with international consensus guidelines[35,36] and was derived from clinician-adjudicated data available within the Society of Thoracic Surgeons database or our electronic health record. Nonetheless, other recognized components of postoperative pulmonary complications include (1) atelectasis defined by radiographic evidence,[35] (2) pulmonary aspiration defined by clinical history and radiographic evidence,[35] (3) pleural effusion defined by radiographic evidence,[36] (4) pneumothorax,[35] (5) bronchospasm defined by expiratory wheezing treated with bronchodilators,[36] or (6) aspiration pneumonitis.[36] We determined *a priori* to exclude these additional postoperative pulmonary complication components in our composite outcome on the basis of either unclear clinical significance in a cardiac surgical population, underlying mechanisms likely not amenable to treatment via lung-protective ventilation, or lack of access to component-specific high-fidelity data across all patients in the study cohort.

Study Limitations

Our study has several limitations. First, we were unable to account for all potential mechanisms leading to a composite postoperative pulmonary complication. Mechanisms for pulmonary injury after cardiac surgery are multifactorial.[7] In our study, we investigated lung-protective ventilation as a means to reduce ventilator-induced lung injury, leading to postoperative pulmonary complications through mechanisms including volutrauma, barotrauma, and atelectasis, and respectively mitigated by lower V_T, lower driving pressure, and application of PEEP.[8] However, additional postoperative pulmonary complication mechanisms to be targeted by anesthesiologists include (1) pulmonary edema, mitigated by fluid and transfusion management,[49] (2) inadequate respiratory effort, mitigated by monitoring/reversal of neuromuscular blockade[50,51] or rapid-acting, opioid-limiting anesthetic agents,[52] and (3) respiratory infection, mitigated by ventilator associated pneumonia prevention bundles.[53,54] In our study, we successfully accounted for several of these targets as covariates. However, the relative importance of each technique, and the impact of lung-protective

ventilation on the association between such techniques and postoperative pulmonary complications, remains beyond the scope of this study.

In our study, precise times for sternotomy and chest closure were unavailable; however, cases excluded redo-sternotomies with protracted closed chest times. As such, driving pressures were assessed during open-chest conditions for a majority of intraoperative ventilation. Our study adds new data to studies of protective ventilation, previously performed during closed-chest conditions. As this relates to the driving pressures observed, our study may demonstrate comparatively less bias introduced by variable chest wall compliance. Thus, airway driving pressure in this study is likely to more closely reflect actual transpulmonary driving pressure, a determinant of dynamic lung strain.[55] Despite this strength, we caution generalizing our findings to more commonly studied patient populations ventilated under closed-chest conditions. We additionally caution generalizing our driving pressure threshold below 16 cm H_2O as lung-protective ventilation without consideration of clinical context. In previous studies of cardiac surgical populations,[16,37] thresholds for lung-protective ventilation defined by driving pressure (plateau pressure − PEEP) ranged from 8 to 19 cm H_2O. Such variation may be explained by (1) time of measurement (*e.g.*, intraoperative *versus* postoperative), (2) surgical conditions (*e.g.*, closed-chest *versus* open-chest), (3) patient populations and practice patterns varying by year and institution, and (4) covariates used for multivariable adjustment. However, it should be noted that despite such sources of variation influencing driving pressure-based lung-protective ventilation thresholds, independent associations between increased ventilator driving pressures and increased postoperative complications have been consistently observed.

Additional limitations to our study include those inherent to our single-center, observational study design: our conclusions require prospective multicenter validation. Patients receiving a lung-protective ventilation bundle were nonrandom; although multiple covariates associated with the lung-protective ventilation exposure were accounted for via multivariable analyses, unmeasured confounders influencing receiving a lung-protective ventilation bundle and impacting our postoperative pulmonary complication primary outcome was a source of potential bias. As pertaining to our lung-protective ventilation exposure variable, limitations included a lack of formal P_{plat} ventilator data for more accurate characterization of driving pressure. Although differences between ventilator peak inspiratory pressure and P_{plat} may be approximated in specified circumstances, the availability of all data necessary for calculations—and the degree to which confounding factors may bias such calculations (*e.g.*, patient differences in airway resistance, endotracheal tube obstructions from kinking/secretions, and the use of end-inspiratory pressure to approximate inspiratory pause pressure for calculating true P_{plat})—remain beyond the scope of our study.

Consistent with existing literature,[1,28] we represented the intraoperative period using lung-protective ventilation exposure median values—potentially failing to account for brief periods of profoundly injurious ventilation. Finally, although our study goal was to specifically examine relationships between intraoperative ventilation and postoperative pulmonary complications, relationships between postoperative ventilation and postoperative pulmonary complications were not studied.

Conclusions

Despite limitations, our study advances understanding of the relationship between intraoperative lung-protective ventilation and impact on costly, life-threatening postoperative pulmonary complication outcomes. In summary, we describe a 10.9% incidence of postoperative pulmonary complications among adults undergoing cardiac surgery. Importantly, we observed that a bundled lung-protective ventilation strategy was independently associated with a lower likelihood of postoperative pulmonary complications and that this was mostly associated with lower driving pressure. Through robust capture of variables describing intraoperative anesthesia management for cardiac surgery patients, our study provides data which may better inform postoperative pulmonary complication multivariable models in this population. Additionally, our findings offer targets for future prospective trials investigating the impact of specific lung-protective ventilation strategies for improving cardiac surgery outcomes.

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Appendix 1. Postoperative Pulmonary Complications – Data Definitions

Postoperative Pulmonary Complication Component	Data Source	Definition
Prolonged initial postoperative ventilator duration longer than 24 h	STS database	Yes/No Indicate whether the patient had prolonged postoperative pulmonary ventilation longer than 24.0 h.
Pneumonia	STS database	Yes/No Indicate whether the patient had pneumonia according to the Centers for Disease Control and Prevention definition.
Reintubation	STS database	Yes/No Indicate whether the patient was reintubated during the hospital stay after the initial extubation. This may include patients who have been extubated in the OR and require intubation in the postoperative period.
Postoperative PaO_2:FiO_2 below 100 mmHg within 48 hours postoperatively while intubated	Hospital enterprise electronic health record (Epic Systems Corporation, USA)	Yes/No Indicate whether the patient had a postoperative PaO_2:FiO_2 below 100 mmHg within 48 h while intubated: • Intubated determined by ventilator mode • FiO_2 determined by ventilator setting • PaO_2 determined by arterial blood gas analysis

FiO_2, fraction of inspired oxygen; PaO_2, arterial partial pressure of oxygen; STS, Society of Thoracic Surgeons.

ANESTHESIOLOGY REFLECTIONS FROM THE WOOD LIBRARY-MUSEUM

Dr. Rudolph Matas: Surgeon, Author, and an American Pioneer of Spinal Anesthesia
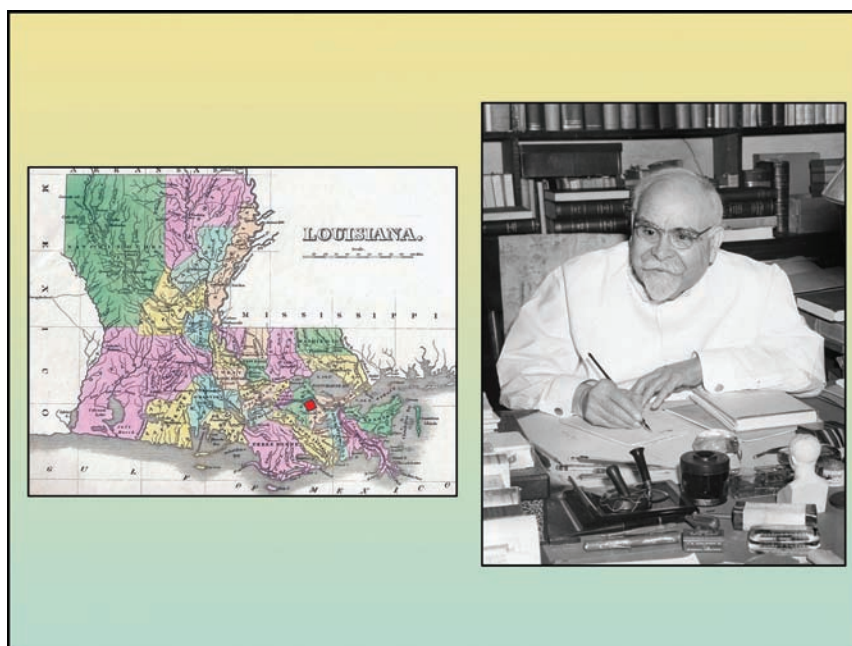
Born in Bonnet Carre, Louisiana (see *red diamond*, *left*), Rudolph Matas (1860 to 1957) was raised in Spain and then Texas before returning to his birth state for eventual medical schooling at the future Tulane University School of Medicine. After earning his M.D. at 19 yr of age, Dr. Matas began transforming himself into "the most learned surgeon" that Dr. Will Mayo had "ever known." Along the way, in 1889, Dr. Matas would also conduct America's first spinal anesthetic. By December of 1940, Matas was completing his eighth year of penning (*right*) a five-volume medical history of Louisiana. (Copyright © the American Society of Anesthesiologists' Wood Library–Museum of Anesthesiology.)

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ANESTHESIOLOGY

Nitrous Oxide Impairs Axon Regeneration after Nervous System Injury in Male Rats

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Methionine is the single carbon donor in mammalian cells and is an essential participant in a diversity of metabolic pathways, including myelin and neurotransmitter synthesis and regulation of DNA transcription. Conversion of serine to glycine provides a methyl group for the synthesis of methionine from homocysteine.[1] The methyl group binds tetrahydrofolate to generate 5,10–methylenetetrahydrofolate, which is reduced to 5–methyltetrahydrofolate by 5,10–methylenetetrahydrofolate reductase. The methyl group is then transferred from 5–methyltetrahydrofolate to cobalamin to produce methylcobalamin, the final methyl group donor for methionine synthesis. Accordingly, methionine synthase requires 5–methyltetrahydrofolate as its single carbon source and is irreversibly inactivated by nitrous oxide with oxidation of its cobalamin cofactor.[1] Parenteral folic acid administered before and after spinal cord injury produces a tenfold or greater dose–dependent improvement in axon regeneration in the adult central nervous system, with peak effects observed at 80 µg/kg folic acid.[2] At higher folic acid doses beneficial effects diminish with no toxicity observed. Eighty percent N_2O in 20% oxygen administered 3 days before spinal cord injury for 4 h, and thereafter every other day for 2 h for 2 weeks, antagonizes the beneficial effects of folic acid on axon regeneration after sharp spinal cord injury.[3] To further resolve the effects of nitrous oxide on axon regeneration after injury to the nervous system,

ABSTRACT

Background: Nitrous oxide can induce neurotoxicity. The authors hypothesized that exposure to nitrous oxide impairs axonal regeneration and functional recovery after central nervous system injury.

Methods: The consequences of single and serial *in vivo* nitrous oxide exposures on axon regeneration in four experimental male rat models of nervous system injury were measured: *in vitro* axon regeneration in cell culture after *in vivo* nitrous oxide administration, *in vivo* axon regeneration after sharp spinal cord injury, *in vivo* axon regeneration after sharp optic nerve injury, and *in vivo* functional recovery after blunt contusion spinal cord injury.

Results: *In vitro* axon regeneration 48 h after a single *in vivo* 70% N_2O exposure is less than half that in the absence of nitrous oxide (mean ± SD, 478 ± 275 um; n = 48) *versus* 210 ± 152 um (n = 48; $P < 0.0001$). A single exposure to 80% N_2O inhibits the beneficial effects of folic acid on *in vivo* axonal regeneration after sharp spinal cord injury (13.4 ± 7.1% regenerating neurons [n = 12] *vs.* 0.6 ± 0.7% regenerating neurons [n = 4], $P = 0.004$). Serial 80% N_2O administration reverses the benefit of folic acid on *in vivo* retinal ganglion cell axon regeneration after sharp optic nerve injury (1277 ± 180 regenerating retinal ganglion cells [n = 7] *vs.* 895 ± 164 regenerating retinal ganglion cells [n = 7], $P = 0.005$). Serial 80% N_2O exposures reverses the benefit of folic acid on *in vivo* functional recovery after blunt spinal cord contusion (estimate for fixed effects ± standard error of the estimate: folic acid 5.60 ± 0.54 [n = 9] *vs.* folic acid + 80% N_2O 5.19 ± 0.62 [n = 7], $P < 0.0001$).

Conclusions: These data indicate that nitrous oxide can impair the ability of central nervous system neurons to regenerate axons after sharp and blunt trauma.

(ANESTHESIOLOGY 2019; 131:1063–76)

EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Nitrous oxide exposure can induce spinal cord neurodegeneration and related neuropathy in humans rendered susceptible by genetic or acquired risk factors
- The effects of nitrous oxide on the regeneration of nervous system following trauma has not been previously reported

What This Article Tells Us That Is New

- In *in vitro* and *in vivo* experimental models of male rats, nitrous oxide exposure impairs folic acid-induced axonal regeneration of dorsal root and retinal ganglion neurons
- The beneficial effects of folic acid on functional recovery following spinal cord contusion in male rats are hindered by co-administration of nitrous oxide
- These experiments suggest that nitrous oxide can interfere with axonal regeneration and functional recovery following central nervous system injury

we hypothesized that single and serial *in vivo* nitrous oxide exposures impair axon regeneration in four experimental models of nervous system injury: *in vitro* axon regeneration in cell culture after *in vivo* nitrous oxide administration, *in vivo* axon regeneration after sharp spinal cord injury, *in vivo* axon regeneration after sharp optic nerve injury, and *in vivo* functional recovery after blunt contusion spinal cord injury.

Materials and Methods

Institutional Review and Animal Care

With the approval of the University of Wisconsin Animal Care and Use Committee (Madison, Wisconsin), and in compliance with published Public Health Service-National Institutes of Health guidelines, all experiments were conducted with 4- to 8-week-old male Sprague–Dawley rats weighing 200 to 250 g (Harlan Laboratories, Inc., USA) that were housed in approved facilities at the University of Wisconsin-Madison (Madison, Wisconsin) staffed by licensed veterinarians. Female rats were conserved to maintain an animal colony sufficient to perform the experimental protocols. Animals were fed Harlan Rodent Diet #8604 (Harlan Laboratories, Inc.), containing B_6 (95 mcg/kg), B_{12} (51.20 mcg/kg), folic acid (2.72 mg/kg), and methionine (0.42% by weight) with unrestricted access other than on the morning of surgery. All experiments were carried out between 8:00 AM and 5:00 PM by investigators blinded to treatment. The animals used in this study were randomized to experimental conditions. Surgical preparation and assessment were randomized between control and treated animals on each experimental day. The number of animals and animal suffering were reduced maximally in all experiments. The percentage of animals that did not survive nitrous oxide exposure, folic acid administration, and surgery to establish the models, and to apply stains to regenerating axons, was less than 5%. Rats did not experience unexpected lethality in the study, and animals were euthanized according to our institutional animal care and use committee guidelines.

Nitrous Oxide Administration

Nitrous oxide in oxygen was administered by placing wire cages housing two to four rats each into a 114 l chamber within a lighted, quiet, negative pressure chemical fume hood. The sealed chamber lid comprised two one-way ports. The ingress port permitted administration of gases, and the egress port permitted pressure equilibration and scavenging of gases. After placing the cages in the exposure chamber, the chamber was brought to 100% oxygen at 6 l/min delivered from a size 200 high-pressure industrial 43 l cylinder (Material Distribution Service, University of Wisconsin, Madison, Wisconsin) yoked to a SurgiVet, Inc. (USA), model #9901544 mixer, and confirmed by a gas analyzer (Ohio 5100 Oxygen Monitor; Ohmeda, Inc., USA) with its transducer placed within the chamber after calibration

to 21% and 100% fraction of inspired oxygen (F_{IO_2}) at the outlet of anesthesia machine. Nitrous oxide was introduced through the ingress aperture to reach the target F_{IO_2} concentration. The F_{IO_2} was continuously monitored and held stable for the duration of each experiment by adjustment of oxygen and nitrous oxide flows. A minimal flow rate of 1 l/min was maintained throughout the exposure interval to assure a slight positive pressure to preclude inward leak of ambient air. The humidity of the atmosphere within the fume hood was 54%, and the temperature was 25°C. Rats were allowed free access to water, feed, bedding, and space to move and interact throughout the exposure interval. Once the exposure interval was complete, the chamber was again flushed to 100% oxygen, and cages housing the animals were removed.

In Vitro Axon Regeneration after *In Vivo* Nitrous Oxide Administration

Protocol. In protocol 1a, control bilateral L4 and L5 dorsal root ganglia were harvested from four rats euthanized with a 10 ul intraperitoneal mixture of pentobarbital sodium 390 mg/ml and phenytoin sodium 50 mg/ml (Beuthanasia Schering-Plough Animal Health, Inc., USA) without antecedent nitrous oxide exposure or sciatic nerve injury (fig. 1; Supplemental Digital Content fig. 1, 1a, http://links.lww.com/ALN/C21). In protocol 1b, bilateral L4 and L5 dorsal root ganglia were harvested from four rats immediately after a single administration of 70% N_2O in 30% oxygen for 2 h and no sciatic nerve injury (Supplemental Digital Content fig. 1, 1b, http://links.lww.com/ALN/C21). In protocol 1c, bilateral L4 and L5 dorsal root ganglia were harvested from four rats after serial administration of 80% N_2O in 20% oxygen for 2 h on each of 3 days before harvest and no sciatic nerve injury (Supplemental Digital Content fig. 1, 1c, http://links.lww.com/ALN/C21). In protocols 1d and 1e, the left sciatic nerve was transected by a sharp scissor through an incision midway between the knee and hip joints with ketamine and xylazine 10:1 0.2–0.3 ml intraperitoneal anesthesia (ketamine [40 to 80 mg/kg; Clipper Distributing Company, LLC, USA] and xylazine [5 to 10 mg/kg; Bimeda-MTC, Animal Health Inc., USA]). The limb wound was closed with Vicryl 4-0 nylon sutures (Ethicon, Inc., USA), and the rats were observed until full recovery from anesthesia. In protocol 1d, L4 and L5 dorsal root ganglia ipsilateral to the left sciatic nerve injury were harvested from eight rats euthanized on day 3 after injury on day 1 (Supplemental Digital Content fig. 1, 1d, http://links.lww.com/ALN/C21). In protocol 1e, the L4 and L5 dorsal root ganglia ipsilateral to the left sciatic nerve injury were harvested from eight rats exposed to 80% N_2O in 20% oxygen for 2 h on each of 3 days before the day 4 sciatic nerve injury, and again on day 6 before euthanasia and harvest on day 7 (Supplemental Digital Content fig. 1, 1e, http://links.lww.com/ALN/C21).
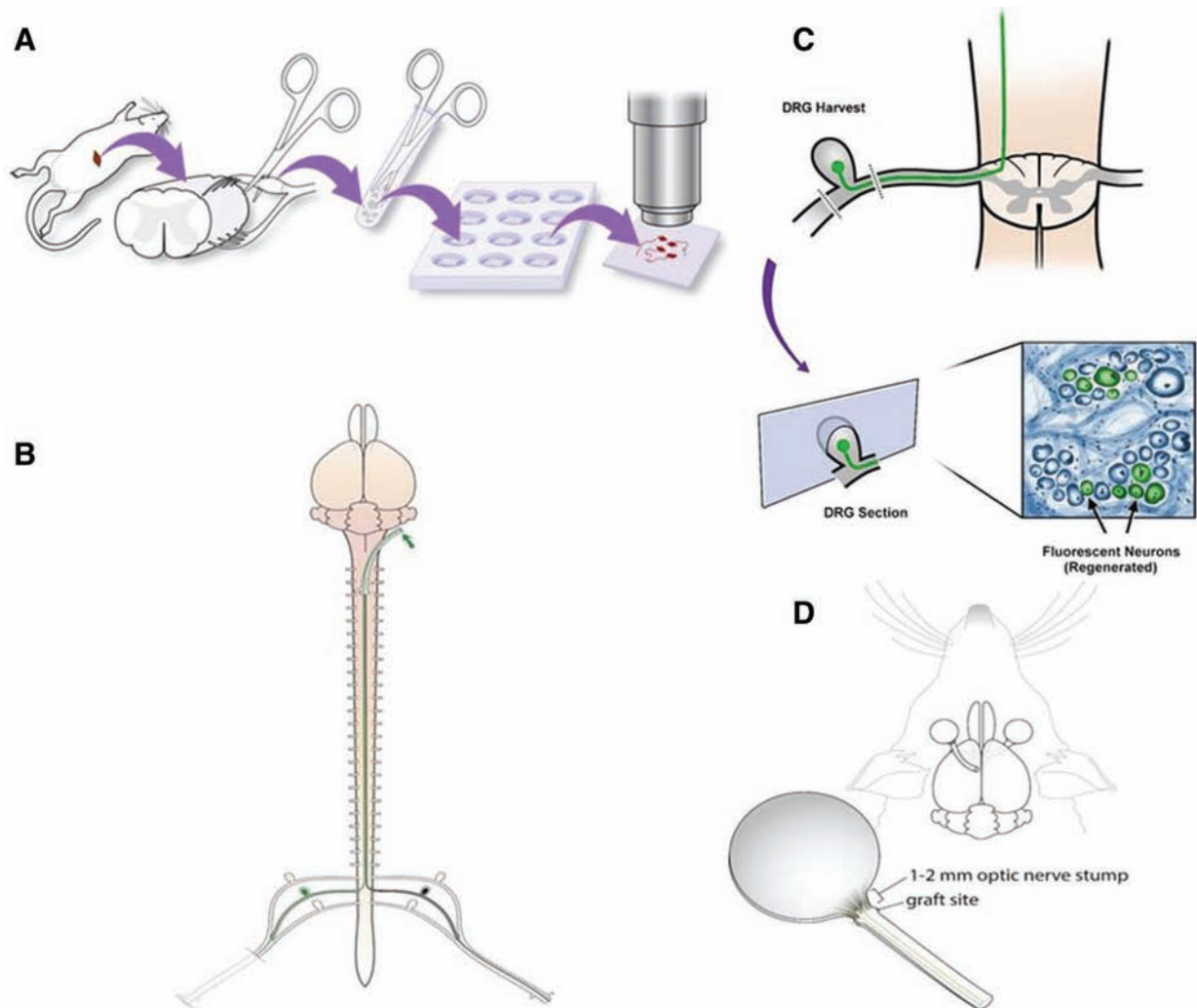
Fig. 1. (*A*) Schematic diagram of *in vitro* axon regeneration in culture after *in vivo* nitrous oxide administration experiments. Axon length and the number of cells with an axon length greater than 300 um were measured under 20× magnification after dorsal root ganglia (DRG) cell harvest, dissociation, culture and immune-staining. (*B*) Schematic diagram of *in vivo* axon regeneration after sharp spinal cord injury experiments. Dorsal root ganglia preparation after bilateral C3 dorsal column lesion and a sciatic nerve graft (*green arrow*). At 2 weeks, fluorescent tracer is applied to the distal graft, and is detected 2 days later in lumbar dorsal root ganglia neuronal axons that have grown into the graft (*green*). The fluorescent tracer is only taken up in regenerated neurons. (*C*) Regenerated fluorescent neurons (*green*) are counted under 20× fluorescent microscope magnification after dorsal root ganglia harvest and sectioning. (*D*) Schematic diagram of *in vivo* axon regeneration after sharp optic nerve injury experiments. Retinal ganglion cells with regenerated axons are counted under 20× fluorescent microscope magnification after retinal extraction and transfer to glass slides.

Dorsal Root Ganglia Cell Harvest, Culture, Immunostaining, and Analysis. All experiments were performed in triplicate, with cells from 16 bilateral L4 and L5 dorsal root ganglia harvested from four rats comprising each of three independent replicate pools for subsequent culture in uninjured protocols 1a, 1b, and 1c, and cells from 16 unilateral L4 and L5 dorsal root ganglia harvested from eight rats comprising each of three independent replicate pools for subsequent culture in injured protocols 1d and 1e.[4] To dissociate the pooled cells, excised ganglia were placed in 4°C media comprising 48.5 ml Roswell Park Memorial Institute media (Hyclone, Thermo Scientific, Inc., USA) and 1.5 ml B27 (GIBCO, Invitrogen, Inc., USA), and mechanically disrupted by a micro–dissecting scissor before washing (fig. 1A). After removal of the media, the dorsal root ganglia fragments were washed twice with Ca^{++} and Mg^{++} free 0.1M phosphate buffered saline (Hyclone). The pooled ganglia from each replicate were added to 2 ml phosphate buffered saline containing 200 µl dispase (Life Technology, USA) and 100 µl collagenase (35 mg/ml; Sigma, LLC, USA), and incubated

at 37°C in 5% CO_2 (Thermo Scientific, Inc., Napco series 8000 Ohforma Sterile Cycle CO2 Incubator) for 10 min after gentle mixing with a Pasteur pipette. Twenty-five microliters of DNAse (Sigma, LLC) were added and the ganglia incubated for an additional 10 min at 37°C.

After dissociation, cellular debris and non-neuronal cells were removed by centrifugation (CS-6 centrifuge, Beckman–Coulter, Inc., USA) at 500 rpm for 5 min in 5 ml Roswell Park Memorial Institute media–fetal bovine serum solution comprising 47.5 ml Roswell Park Memorial Institute media and 2.5 ml fetal bovine serum (Hyclone). The supernatant was aspirated and the cells centrifuged a second time at 500 rpm for 5 min after resuspension in 5 ml fresh Roswell Park Memorial Institute media–fetal bovine serum. After the second aspiration, the cells from each replicate were resuspended by pipette in 1.6 ml Roswell Park Memorial Institute-B-27 media. Thus, 100 µl of media contained the dissociated cells of each of 16 dorsal root ganglia to yield the cells of one dorsal root ganglia for each of 16 wells on two 12-well tissue sterilized culture plastic plates free of pyrogens, DNA, DNAse, RNA, and RNAse (Techno Plastic Products, Pvt Ltd., Switzerland); that is, eight wells were used per plate per replicate. Glass coverslips (Thermo Scientific, Inc.) were used as substrates for cell culture. Coverslips were sterilized in nitric acid for 14 h, stored in 100% ethanol, and dried in a sterile hood before placing one coverslip in each well of the 12-well plates (fig. 1A). The coverslips were then immersed in 100 mg/ml poly D-lysine (Sigma, LLC) and placed in the carbon dioxide incubator for 12 h at 37°C. Five hours before plating the cells for culture, the poly D-lysine was aspirated, and the coverslips were washed three times with phosphate buffered saline and immersed in laminin (L2020 100 mg/ml in phosphate buffered saline; Sigma, LLC) for 4 h. After aspiration of the laminin and three phosphate buffered saline washes, 330 ul of Roswell Park Memorial Institute-B27 media was placed in each well, and 100 ul of the 1.6 ml stock suspended cell suspension was added by pipette to each well at room temperature such that each well and coverslip had a paired duplicate for analysis. The plates comprising wells containing coverslips and cells were placed in the incubator at 37°C, and refed once every 24 h with Roswell Park Memorial Institute-B27 media after gentle aspiration of the preceding media.

Duplicate, paired coverslips were removed for fixation, image acquisition and scoring at 5, 10, 13, 17, 24, 36, and 48 h for protocols 1a, 1c, 1d, and 1e, and at 13, 24, and 48 h for protocol 1b. After aspiration of the media, the cells were washed three times with Roswell Park Memorial Institute-27 media, and fixed in 250 ul 4% paraformaldehyde (Sigma, LLC) at 4°C for 30 min. The paraformaldehyde was then removed and the coverslips rinsed twice with phosphate buffered saline with 0.01% Triton X-100 (Acros Organics, Inc. USA). The cells were blocked with a solution comprising 5 ml of 10× phosphate buffered saline, 1 ml 10% Triton X, 2.5 ml 5% fetal bovine serum, and water. Dorsal root ganglia

neurons were stained with Sigma T-8660 mouse monoclonal anti-bIII tubulin antibody (Sigma, LLC) diluted to 1:1,000 with blocking solution, by adding 500 ul of the mixture to each well followed by incubation at room temperature for 30 min. After removal of the anti-bIII tubulin antibody and three washes in phosphate buffered saline with 0.01% Triton X-100, Alexa Fluor donkey anti-mouse secondary IgG antibody (Alexa Fluor 594; Invitrogen, Inc., USA) 0.2 mg/ml was diluted 1:1,000 in blocking solution, placed on the cells, and incubated at room temperature for 30 min in the dark, before three further washes in phosphate buffered saline with 0.01% Triton X-100. The coverslips were then removed from the wells, rinsed twice with distilled water, and mounted cell side down on glass slides (Thermo Scientific, Inc.) with 10× polyvinyl alcohol in phosphate buffered glycerol with 0.1% sodium azide (Fluoromount G, Electron Microscopy Sciences, USA) one drop per cover slip, dried for 10 min, and frozen at −80°C (Thermo Scientific Forma 916, USA) thereafter.

Quantitative analyses of the primary outcomes of axon length and the percentage of cells with axons greater than 300 um were performed by identification of the longest axon from each cell for measurement. Images were acquired using Axiovision software (Zeiss, LLC, USA) for an Axioscope 20 fluorescent microscope (Zeiss, LLC) at 20× magnification by two observers blinded to treatment and time interval. Each coverslip was divided into four quadrants, and four images containing at least one complete cell each were selected at random in each quadrant by tracking the longest axon away from each cell body of each image to ensure that the entire length was measurable. When at least one cell was found in an image, its axon was measured. A score of 0 indicates that a cell has been found, but that it has no axon. Multiple cells found within a single image were counted separately. Rarely were more than five cells identified within an image. The percentage of cells greater than 300 um was calculated with a denominator of all cells counted per coverslip/time interval/replicate experiment divided into the number of cells with an axon greater than 300 um on the same coverslip. For all values, the measurements of the two blinded observers were averaged to a single value for each quadrant, and the average axon lengths and percentage cells greater than 300 um were calculated for each of the three replicates. ImageJ Software (http://imagej.nih.gov/ij/; accessed July 27, 2018) was used to calibrate the measurement scale using the scale image taken at 20× magnification, and to archive the photomicrographs.

In Vivo Axon Regeneration after Sharp Spinal Cord Injury

Protocol. In protocol 2a, 14 control rats received no folic acid or nitrous oxide before or after spinal cord surgery and injury (fig. 1B; Supplemental Digital Content fig. 2, 2a, http://links.lww.com/ALN/C22). In protocol 2b, 12 rats received 80 µg/kg intraperitoneal folic acid each day for 3 days before the day of surgery and injury, and for 13 days after surgery. After weighing, preservative-free folic acid

(5mg/ml; APP Pharmaceuticals, Inc., USA) was diluted with double-distilled water to 0.125 mg/ml and injected intraperitoneally in 20 ul volume between 9:00 and 11:00 AM. No nitrous oxide was administered (Supplemental Digital Content fig. 2, 2b, http://links.lww.com/ALN/C22). In protocol 2c, five rats received a single administration of 80% N$_2$O in 20% oxygen for 4h immediately before injury. No folic acid was administered (Supplemental Digital Content fig. 2, 2c, http://links.lww.com/ALN/C22). In protocol 2d, four rats received a single administration of 80% N$_2$O for 4h immediately before surgery, and 80 µg/kg intraperitoneal folic acid each day for three days before surgery, and for 13 days after surgery (Supplemental Digital Content fig. 2, 2d, http://links.lww.com/ALN/C22). In protocol 2e, nine rats received 40% N$_2$O in 60% oxygen for 4h immediately before surgery, and then for 2h every other day after surgery for 13 days. No folic acid was administered (Supplemental Digital Content fig. 2, 2e, http://links.lww.com/ALN/C22). In protocol 2f, seven rats received 40% N$_2$O in 60% oxygen for 4h each day for 3 days before surgery and every other day after surgery for 13 days, and 80 µg/kg intraperitoneal folic acid each day for 3 days before surgery and for 13 days after surgery (Supplemental Digital Content fig. 2, 2f, http://links.lww.com/ALN/C22). In protocol 2g, 16 rats received 80% N$_2$O in 20% oxygen for 4h immediately before surgery and then for 2h every other day after surgery for 13 days. No folic acid was administered (Supplemental Digital Content fig. 2, 2g, http://links.lww.com/ALN/C22). In protocol 2h, 16 rats received 80% N$_2$O in 20% oxygen for 4h each day for 3 days before surgery and then for 2h every other day after surgery for 13 days, and 80 µg/kg intraperitoneal folic acid each day for 3 days before surgery and for 13 days after surgery (Supplemental Digital Content fig. 2, 2h, http://links.lww.com/ALN/C22).

Surgery. To quantify regeneration of spinal axons into a peripheral nerve graft transferred to the cervical spinal cord, adult male Sprague-Dawley rats were anesthetized with ketamine and xylazine, the cervical spinal cord was exposed through a C3 laminectomy, and the dura was opened as previously described[2,3,5] (fig. 1B). With operating microscope guidance, a 0.5-mm-deep injury was made in both posterior columns with a sharp jeweler's forceps, thereby severing primary somatosensory axons ascending the spinal cord. A 1.5-cm autologous sciatic nerve graft harvested from the left hind limb was then implanted at the cervical spinal cord injury site with the distal terminus of the graft sewn to the distal site of spinal cord injury using two to three 10-0 nylon sutures to the dura. The opposite, proximal end of the sciatic nerve graft was tagged with a loosely tied 4-0 silk suture, and freely laid in the subcutaneous space. The wound was closed with 4-0 nylon sutures, and the rats were observed until full recovery from anesthesia. Buprenorphine 0.05 to 0.1 mg/kg (Midwest Veterinary Supply, Inc., USA) was administered subcutaneously as needed for pain.

On the 14th day after injury, the sciatic nerve graft was exposed under ketamine and xylazine anesthesia, sharply cut close to the free end, and a surgical gelfoam 5 mm by 5 mm (Surgicel Johnson-Johnson Ethicon-SARL, LLC, Switzerland) soaked in 5 µl of the preservative-free tracer Fluorogold (Fluorochrome, LLC, USA) was placed to cover the free end of the nerve graft. The fluorescent tracer solely enters axons extending from the dorsal root ganglia that have ascended from the spinal cord injury site into the graft and undergoes rapid retrograde transport to the soma of neurons residing in the dorsal root ganglia. Forty-eight hours later the animals were euthanized.

Dorsal Root Ganglia Section Handling and Analysis. The primary outcome of the percentage of neurons in the dorsal root ganglia that have taken up and transported Fluorogold to their somata provides the index of axon regeneration after injury. After euthanasia, L5 dorsal root ganglia ipsilateral to the sciatic nerve donor site were removed, fixed in 4% paraformaldehyde (Sigma, LLC) at 4°C overnight, and placed the next day in 30% sucrose at room temperature for 3 h incubation (Thermo Scientific D-Sucrose BP220-1, Thermo Scientific, Inc.; fig. 1C). The dorsal root ganglia were then placed in a tissue embedding mold (Polysciences, Inc., USA) with optimum cutting temperature tissue embedding media (10.24% polyvninyl alcohol, 4.25% polyethylene glycerol, 85.50% nonreactive ingredients; Tissue-Tek, Fisher Scientific, Inc., USA), quick-frozen, and placed in a −20°C freezer for 1 to 3 weeks before sectioning. Subsequently, the embedded tissue was cut into 10-um sections with a cryostat (Leica 3050-S, Leica, LLC, Germany) to yield 24 to 35 sections per microscope glass slide (Fisher Scientific, Inc.) stored in a −80°C freezer (Thermo Forma, Fisher Scientific, Inc.) for up to 1 week before scoring. For scoring, the sections were thawed and examined by fluorescent microscopy using an Axioscope 20 fluorescent microscope (Zeiss, LLC, USA) at 20× magnification by two independent observers blinded to treatment and time interval, and the percentage of neurons in the dorsal root ganglia averaged to a single score. The method of Abercombie, in which only cells with visible nucleoli are counted, was used to determine the normative number of neurons observed in rat dorsal root ganglia (*i.e.,* 3,040 on the side ipsilateral to the sciatic nerve injury).[6]

In Vivo Axon Regeneration after Sharp Optic Nerve Injury

Protocol. In protocol 3a, seven control rats received no folic acid or nitrous oxide before or after optic nerve surgery (fig. 1D; Supplemental Digital Content fig. 3, 3a, http://links.lww.com/ALN/C23). In protocol 3b, seven rats received intraperitoneal injections of 80 µg/kg folic acid 3 days before surgery, and then every day until 14 days after surgery (Supplemental Digital Content fig. 3, 3b, http://links.lww.com/ALN/C23). After weighing, preservative-free folic acid (5 mg/ml; APP Pharmaceuticals, LLC, USA) was diluted with double-distilled water to 0.125 mg/

ml and injected intraperitoneally in 20 ul volume between 9:00 and 11:00 AM. In protocol 3c, eight rats received 80% N_2O in 20% oxygen for 4 h 3 days before surgery, and for 2 h on days 2 and 1 before surgery, and then for 2 h every other day thereafter until 14 days after surgery (Supplemental Digital Content fig. 3, 3c, http://links.lww.com/ALN/C23). In protocol 3d, seven rats received 80% N_2O in 20% oxygen for 4 h 3 days before surgery, and for 2 h on days 2 and 1 before surgery, and then for 2 h every other day thereafter until 14 days after surgery together with intraperitoneal injections of 80 μg/kg folic acid 3 days before surgery, and then every day until 14 days after surgery (Supplemental Digital Content fig. 3, 3d, http://links.lww.com/ALN/C23).

Surgery. Using ketamine and xylazine anesthesia, the optic nerve was exposed through a midline incision and lateral orbital approach superior to the globe, and cut with scissors within 3 mm of the globe.[7,8] One end of an autologous 1.5-cm sciatic nerve graft was attached to the optic nerve stump using two to three 10-0 interrupted nylon sutures, with the distal end of the graft approximated to the optic nerve stump, and the proximal end allowed to lie freely under the skin (fig. 1D). The wound was closed with 4-0 nylon sutures and the rats were observed until full recovery from anesthesia. On the 60th postoperative day, the sciatic nerve graft was exposed under 10:1 ketamine and xylazine anesthesia, trimmed to expose a fresh surface, and a gelfoam saturated in 5 μl of the retrograde preservative-free tracer Fluorogold tracer was applied to the graft at a distance of 1.5 cm from the globe. The wound was closed with 4-0 nylon sutures, and the rats were observed until full recovery from anesthesia. Forty-eight hours later, the animals were anesthetized with ketamine and xylazine, and euthanized by perfusion through the heart with 4% paraformaldehyde.

Retina Handling and Analysis. Each globe was dissected free with forceps and scissors and placed in 4% paraformaldehyde overnight. The following day each globe was transferred to 30% sucrose for 3 h. The cornea was then opened, the lens removed, and the retina extracted through four 0.2-cm incisions that were made along the periphery of each globe. The retinas were then transferred to microscopic glass slides, covered with a coverslip (Thermo Fisher, Inc.), and frozen at −80°C. After thawing, the primary outcome of all retinal ganglion stained cells were counted at 20× under fluorescent microscopy by two independent observers blinded to treatment, and their results per retina were averaged to a single score.

In Vivo Functional Recovery after Blunt Contusion Spinal Cord Injury

Protocol. In protocol 4a, nine rats received no folic acid or nitrous oxide before or after surgery and injury (Supplemental Digital Content fig. 4, 4a, http://links.lww.com/ALN/C24). In protocol 4b, nine rats received daily intraperitoneal folic acid 80 μg/kg in saline beginning 3 days

before surgery and injury and continuing daily for 17 days after surgery and injury (Supplemental Digital Content fig. 4, 4b, http://links.lww.com/ALN/C24). After weighing, preservative-free folic acid (5 mg/ml, APP Pharmaceuticals) was diluted with double-distilled water to 0.125 mg/ml and injected intraperitoneally in 20 ul volume between 9:00 and 11:00 AM. In protocol 4c, eight rats received 80% N_2O in 20% oxygen for 4 h on 3 days before surgery and injury, for 2 h on days 2 and 1 day before surgery and injury, and on every other day for 17 days after surgery and injury (Supplemental Digital Content fig. 4, 4c, http://links.lww.com/ALN/C24). In protocol 4d, seven rats received daily intraperitoneal folic acid 80 μg/kg in saline beginning 3 days before surgery and continuing daily for 17 days, and 80% N_2O in 20% oxygen for 4 h on day 3 before surgery, for 2 h on days 2 and 1 day before surgery, and then every other day for 17 days after surgery (Supplemental Digital Content fig. 4, 4d, http://links.lww.com/ALN/C24).

Surgery. Using ketamine and xylazine anesthesia, the thoracic spinal cord of adult male Sprague–Dawley rats was exposed *via* laminectomy at T9 under aseptic conditions, and a 12.5-gm/cm injury was created using a New York University impactor as previously described.[9,10] The wound was closed with 4-0 nylon sutures and the rats were observed until full recovery from anesthesia. Buprenorphine 0.05 to 0.1 mg/kg was administered subcutaneously as needed for pain.

Behavior Testing. Rats were videotaped for 4 min while ambulating in an open-field environment at baseline 3 days before injury, and weekly intervals thereafter for a period of 5 weeks. Ambulatory function was scored by two blinded and independent observers using the primary outcome of the Basso, Beattie, Bresnahan rating scale, which assigns points for the frequency of occurrence of specific features of normal posture and locomotion, and their results were averaged.[9,10]

Temperature Stability During *In Vivo* Nitrous Oxide Exposure

To test the stability of body temperature during nitrous oxide exposure, hourly rectal temperature measurements were recorded more than a 6-h period of exposure to 80% N_2O in 20% oxygen in six adult male Sprague-Dawley rats.

Statistical Analysis

Data analysis for all protocols was performed using SAS version 9.2 (SAS Institute, Inc., USA). Hypothesis testing was two-sided throughout.

Table 1 provides the number of measured cells used to compare dorsal root ganglia axon length in culture. For statistical analysis of *in vitro* axon regeneration data (protocol 1), pairwise comparisons of axon length at 24 and 48 h among the five groups (*i.e.*, 1: no nitrous oxide [uninjured]; 2: single 70% N_2O exposure [uninjured]; 3: serial 80% N_2O exposures [uninjured]; 4: no nitrous oxide [injured]; and 5: serial 80% N_2O exposures [injured]) were performed using Wilcoxon

Table 1. Dorsal Root Ganglion Axon Length (um) in Culture

Group	No. of Hours	No. of Measured Cells	Mean ± SD	Percent Axons > 300 um
No N_2O (uninjured)	5	87	31 ± 55	1%
	10	76	25 ± 28	0%
	13	143	122 ± 117	10%
	17	155	151 ± 121	12%
	24	155	214 ± 172	31%
	36	53	329 ± 229	49%
	48	46	478 ± 275	76%
Single 70% N_2O (uninjured)	13	135	36 ± 42	0%
	24	105	101 ± 112	6%
	48	192	210 ± 152	24%
Serial 80% N_2O (uninjured)	5	48	29 ± 34	0%
	10	88	36 ± 58	1%
	13	147	50 ± 86	3%
	17	211	64 ± 83	2%
	24	138	100 ± 114	7%
	36	187	107 ± 106	8%
	48	125	115 ± 112	8%
No N_2O (injured)	5	79	68 ± 76	4%
	10	108	187 ± 175	20%
	13	64	234 ± 227	34%
	17	56	390 ± 302	59%
	24	55	501 ± 358	65%
	36	63	522 ± 347	63%
	48	41	825 ± 470	83%
Serial 80% N_2O (injured)	5	118	46 ± 45	0%
	10	150	184 ± 147	22%
	13	166	214 ± 179	26%
	17	74	235 ± 153	28%
	24	154	290 ± 175	47%
	36	165	332 ± 194	47%
	48	114	270 ± 172	39%

N_2O, nitrous oxide.

Table 2. Dorsal Root Ganglion Axon Length in Culture Pairwise Comparison *P* Values

	Axon Length (um)		Percent Axons > 300 um	
Pairwise Group Comparisons	24 h	48 h	24 h	48 h
Uninjured no N_2O *vs.* uninjured 70% N_2O	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Uninjured no N_2O *vs.* uninjured 80% N_2O	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Uninjured no N_2O *vs.* injured no N_2O	0.003	0.0002	0.0008	0.597
Uninjured 70% N_2O *vs.* uninjured 80% N_2O	0.445	< 0.0001	0.796	< 0.0001
Uninjured 80% N_2O *vs.* injured 80% N_2O	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Injured no N_2O *vs.* injured 80% N_2O	0.068	< 0.0001	0.246	< 0.0001

N_2O, nitrous oxide.

rank sum test. The proportions of axon lengths greater than 300 um were tested by Fisher exact test. To account for multiple testing, a Bonferroni correction was applied with equally divided overall 10% type I errors among the 24 pre-specified comparisons, and a *P* value less than 0.0004 was adopted for all comparisons. Table 2 provides two-sided P values for pairwise comparisons between the five groups.

Table 3 provides the number of animals used to compare the percentage of regenerating dorsal root ganglia neurons after sharp spinal cord injury. For statistical analysis of *in vivo* spinal cord regeneration data (protocol 2), Wilcoxon rank sum tests were performed to compare the percent of regenerating cells. Primary pairwise comparisons comprised: (1) control *versus* folic acid alone; (2) folic acid alone *versus* folic acid plus a single exposure to 80% N_2O; (3) folic acid alone *versus* folic acid plus serial exposures to 40% N_2O; and (4) folic acid alone *versus* folic acid plus serial exposure to 80% N_2O. Secondary pairwise comparisons included: (1) control *versus* single 80%

Table 3. Percentage of Regenerating Dorsal Root Ganglion Neurons after Sharp Spinal Cord Injury

Group	n	Mean ± SD	Minimum	Maximum
Control	14	1.1 ± 0.9	0	2.6
FA	12	13.4 ± 7.1	2.5	23
Single 80% N_2O	5	0.7 ± 0.5	0.2	1.3
Serial 40% N_2O	9	1.3 ± 1.0	0.1	2.9
Serial 80% N_2O	16	0.5 ± 0.8	0	2.4
FA and single 80% N_2O	4	0.6 ± 0.7	0	1.3
FA and serial 40% N_2O	7	0.4 ± 0.5	0	1.3
FA and serial 80% N_2O	16	1.6 ± 1.2	0	3.9

n, number of animals; FA, folic acid 80 µg/kg intraperitoneally; N_2O, nitrous oxide.

Table 4. Number of Regenerating Retinal Ganglion Cells after Sharp Optic Nerve Injury

Group	n	Mean ± SD	Minimum	Maximum
Control	7	895 ± 164	684	1140
FA	7	1277 ± 180	952	1450
FA + 80% N_2O	7	819 ± 155	596	1016

n, number of animals; FA, folic acid 80 µg/kg intraperitoneally; N_2O, nitrous oxide.

N_2O exposure; (2) control *versus* serial 40% N_2O exposure; and (3) control *versus* serial 80% N_2O exposure. A Bonferroni correction for pairwise comparisons was used to test for differences between the groups with a significance level of $P < 0.05$.

Table 4 provides the number of animals used to compare the regenerating retinal ganglion cells after sharp optic nerve injury. For statistical analysis of *in vivo* optic nerve regeneration data (protocol 3), Wilcoxon rank sum tests were performed to compare the number of cells that take up dye in each experimental condition. Pairwise comparisons included: (1) control *versus* folic acid alone and (2) folic acid *versus* folic acid plus nitrous oxide groups. A Bonferroni correction for pairwise comparisons was used to test for differences between the groups with a significance level of $P < 0.05$.

Table 5 provides the number of animals used to compare the spinal cord contusion score estimates for fixed effects. For statistical analysis of functional recovery after contusion spinal cord injury data (protocol 4), a linear mixed model with random intercept and random time effects with the recovery score as the outcome was fitted. Fixed effects in the linear model were: (1) Group (control, folic acid, nitrous oxide, and folic acid plus nitrous oxide); (2) Time (0, 3, 7, 14, 21, 28, and 35 days); and a Group/Time interaction term. A first-order autoregressive matrix was used to model the correlation of the recovery score within the same animal. A significance level of $P < 0.05$ was adopted for all comparisons.

For statistical analysis of temperature during nitrous oxide exposure data, a linear regression model for longitudinal data with a compound-symmetry, intraindividual correlation structure was used to assess temperature constancy more than time. A significance level of $P < 0.05$ was adopted.

No statistical power calculation was conducted before the study. The sample size was based on our previously published experience with the experimental protocols and conduct.[2–4] No outliers were excluded from analysis.

Results

Table 1 provides summary values of experiments conducted to measure *in vitro* dorsal root ganglia axon regeneration at specified hourly intervals after *in vivo* nitrous oxide administration, including the number of longest axons measured per coverslip (n), the mean and SD of the longest axons (um), and the percent of axons greater than 300 um in length. Table 2 provides two-sided P values for pairwise comparisons between the five groups. In the uninjured groups (uninj), axon regeneration is double or greater at 24 h and 48 h in the absence of nitrous oxide when compared with either a single 70% N_2O exposure or to serial 80% N_2O exposures ($P < 0.0001$; fig. 2A–C). At 24 h, no difference in inhibition of axonal regeneration in the uninjured groups were observed between a single 70% N_2O exposure and serial 80% N_2O exposures ($P = 0.445$). At 48 h, dorsal root ganglia axon regeneration was halved in the uninjured rats after serial 80% N_2O exposures compared with uninjured rats with a single 70% N_2O exposure.

Table 5. Spinal Cord Contusion Score Estimates for Fixed Effects

Variable	n	Estimate	SEE	DF	t Value	P Value (Pr > ltl)
Control	9	4.53	0.54	194	8.40	< 0.0001
FA	9	5.60	0.54	194	10.40	< 0.0001
N_2O	8	5.33	0.58	194	9.14	< 0.0001
FA + 80% N_2O	7	5.19	0.62	197	8.38	< 0.0001
Time		1.72	0.20	177	8.82	< 0.0001
Time*FA		0.79	0.28	177	2.87	0.005
Time*80% N_2O		0.08	0.30	157	0.27	0.786
Time*FA + 80% N_2O		0.12	0.31	154	0.39	0.696

*indicates the interaction between two variables. DF, degrees of freedom; FA, folic acid 80 µg/kg intraperitoneally; n, number of animals; N_2O, nitrous oxide; (Pr > ltl), the P values for the effect of the classification variable on the response; SEE, standard error of the estimate.
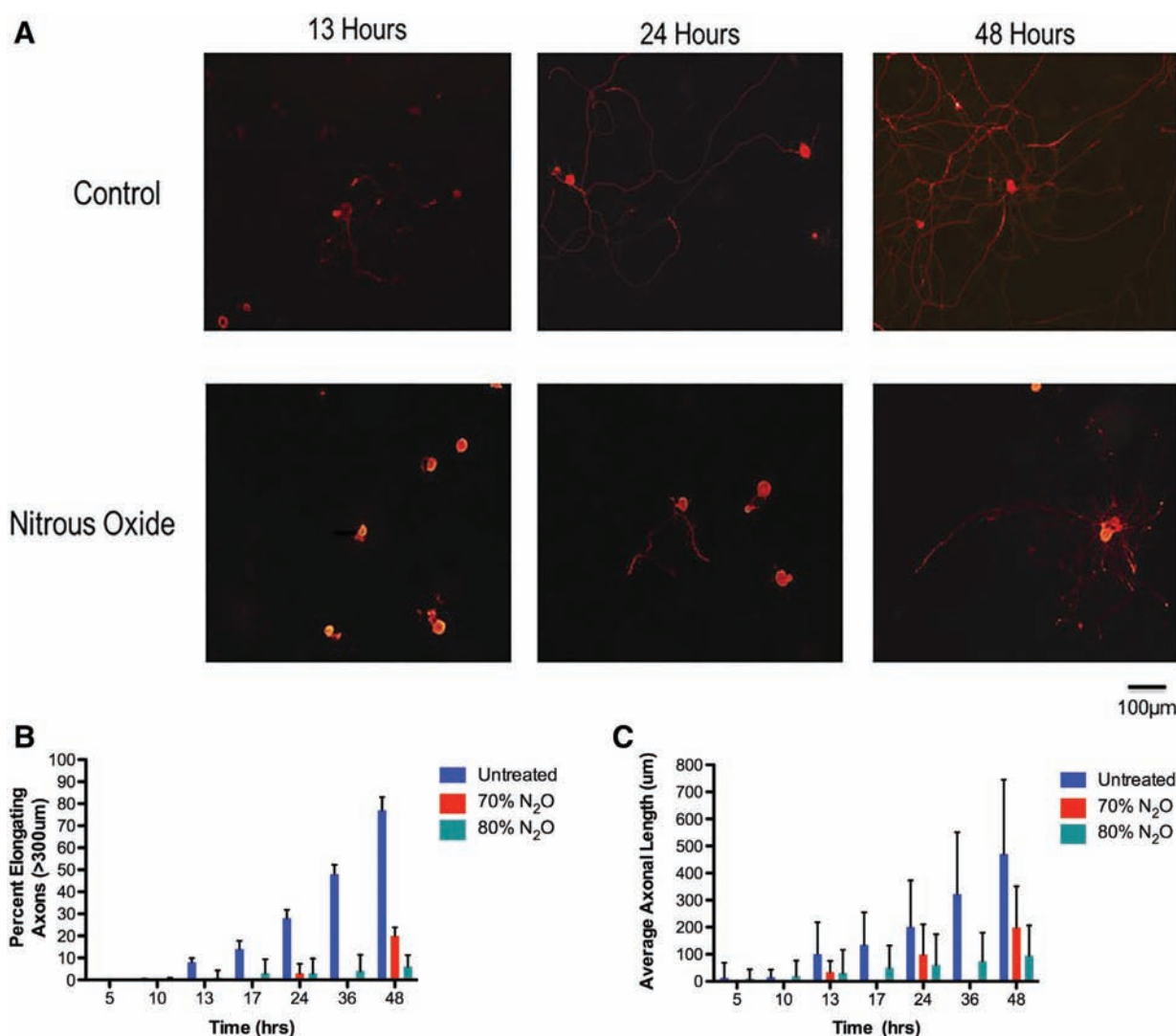
Fig. 2. Representative micrographic images of *in vitro* axon regeneration after *in vivo* nitrous oxide administration (20×). (*A*) Axon growth (*red*) is florid at 48 h in dorsal root ganglia neurons from control animals not exposed to nitrous oxide, but attenuated in dorsal root ganglia neurons from animals exposed to 80% N_2O in 20% oxygen for 2 h on each of 3 days before the day 4 sciatic nerve injury, and again on day 6 before euthanasia and harvest on day 7 (protocol 1e). Nitrous oxide impairs the percentage of elongating axons greater than 300 um (*B*), and the average axonal length (*C*) in animals exposed to a single dose of 70% N_2O (*red*; protocol 1b), and in animals exposed to 80% N_2O in 20% oxygen for 2 h on each of 3 days before the day 4 sciatic nerve injury, and again on day 6 before euthanasia and harvest on day 7 (*green*; protocol 1e) at 24 and 48 h after culture compared with animals not exposed to nitrous oxide (*blue*). Table 1 provides the number of measured cells used to compare dorsal root ganglia ganglion axon length in culture.

In the absence of nitrous oxide, axon regeneration is greater in the injured group compared with the uninjured group ($P = 0.003$) at 24 h and 48 h. In the presence of serial 80% N_2O exposures, axon regeneration is greater in the injured group compared with the uninjured group ($P < 0.0001$). At 24 h no differences in axonal regeneration in the injured groups were observed between no nitrous oxide exposure and serial 80% N_2O exposures ($P = 0.068$), but at 48 h dorsal root ganglia axon regeneration was markedly lower in the serial 80% N_2O exposure group ($P < 0.0001$). These

findings are replicated when the percentage of axons with lengths greater 300 um are tested in all comparisons. The sole exception is between the injured *versus* uninjured group with no nitrous oxide exposure at 48 h, by which time regeneration in the uninjured group is not significantly different from the injured group ($P = 0.597$).

Table 3 provides summary descriptive statistics (*i.e.*, mean, SD, minimum and maximum) for the percentage of dorsal root ganglia neuron axons regenerating after sharp spinal cord injury. Significantly greater spinal cord axon

regeneration was observed in the serial folic acid administration group compared with the control (*i.e.*, no folic acid) group (Z statistic = 4.25, P < 0.001). A single 80% N_2O exposure fully reverses the beneficial effect of serial folic acid administration on axon regeneration compared with serial folic acid administration alone (Z statistic = −2.85, P = 0.004; figs. 3 and 4). No significant difference in axon regeneration percentages were observed in a comparison between the control group with no serial folic acid administration to the single 80% N_2O exposure with serial folic acid administration group (Z statistic = −0.79, P = 0.430). Serial 40% N_2O and serial 80% N_2O exposure reverses the beneficial effect of serial folic acid on axon regeneration (Z statistic = −3.52, P < 0.001, and Z statistic = 4.20, P < 0.001, respectively). No significant difference in axon regeneration percentages were observed in a comparison between the control group with no serial folic acid administration to the serial 40% N_2O exposure group (Z statistic = 0.28,

P = 0.777). However, serial 80% N_2O exposure halves axon regeneration compared with control no serial folic acid administration conditions (Z statistic = 2.24, P = 0.025).

Table 4 provides summary statistics (*i.e.*, mean, SD, minimum, and maximum) for the number of retinal ganglion cells taking up dye after optic nerve injury. Significantly greater retinal ganglion cell axon regeneration was observed in the serial folic acid administration group compared with the control group (Z statistic = 2.81, P = 0.005; figs. 5 and 6). When 80% N_2O was administered together with folic acid, retinal ganglion cell axon regeneration was significantly inhibited (Z statistic = 2.81, P = 0.005) compared with serial folic acid administration alone.

Table 5 provides spinal cord contusion score estimates for fixed effects. Table 6 provides pairwise comparisons of spinal cord contusion Basso, Beattie, Bresnahan scores at each time interval with contrast estimates based on the fitted mixed model. The highest Basso, Beattie, Bresnahan scores are observed in the serial folic acid administration alone group (fig. 7). The mixed model demonstrates a significant difference in the overall Basso, Beattie, Bresnahan score trajectories between the four groups (type III test for fixed effects yields chi-square = 332.51, P < 0.0001). The Basso, Beattie, Bresnahan score trajectories change significantly more than time (type III test for fixed effects yields chi-square = 330.46, P < 0.0001), and the patterns of change
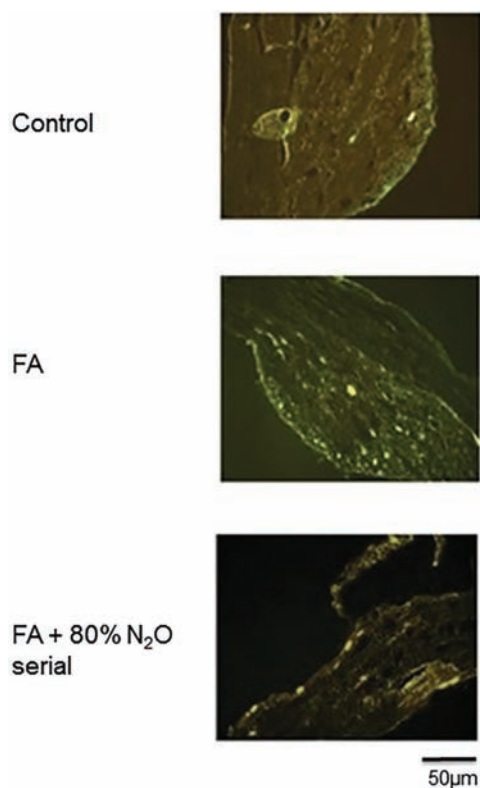

Fig. 3. Representative micrographic images of *in vivo* axon regeneration after sharp spinal cord injury with *in vivo* folic acid ([FA] 80 µg/kg intraperitoneally) and nitrous oxide administration (20×). Folic acid markedly increases the number of dorsal root ganglia neurons that regenerate axons after spinal cord injury and take up a Fluorgold marker for retrograde transport to their cell bodies (*bright green ovals and circles*). Exposure to 80% N_2O for 3 days before injury and every other day thereafter (protocol 2h) reverses the benefit of serial folic acid administration.
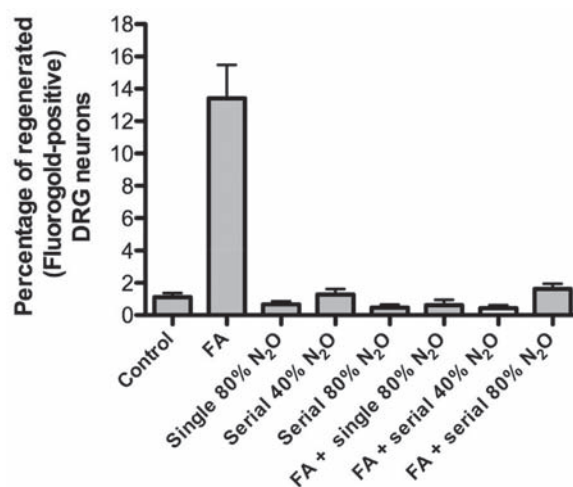

Fig. 4. Folic acid (80 µg/kg intraperitoneally) markedly increases the percentage of regenerated (*i.e.*, Fluorogold-positive) dorsal root ganglia (DRG) neurons (protocol 2b) compared with no folic acid (FA) control (protocol 2a), single 80% N_2O exposure (protocol 2c), serial 40% N_2O exposure (protocol 2e), and serial 80% N_2O exposure (protocol 2g). A single exposure to 80% N_2O (protocol 2d), serial exposure to 40% N_2O (protocol 2f), and serial exposure to 80% N_2O (protocol 2h) reverses the benefit of serial folic acid administration. Table 3 provides the number of animals used to compare the percentage of regenerating dorsal root ganglia neurons after sharp spinal cord injury.
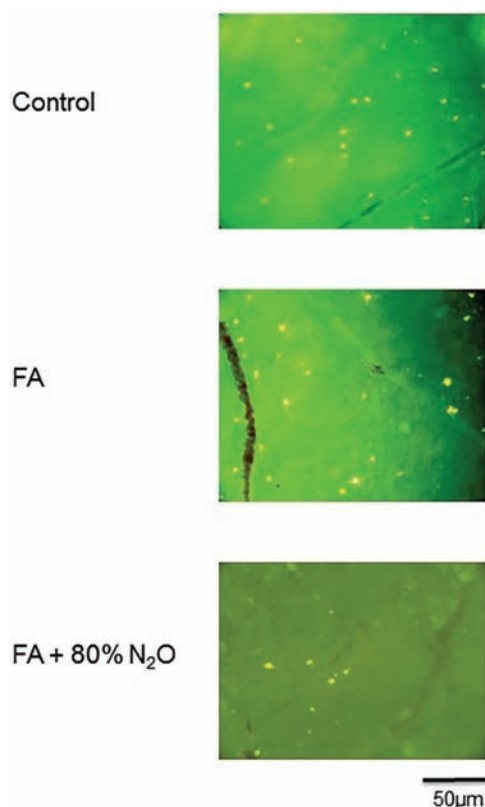
Fig. 5. Representative micrographic images of *in vivo* axon regeneration after sharp optic nerve injury with *in vivo* folic acid ([FA] 80 µg/kg intraperitoneally) and nitrous oxide administration (20×). Folic acid (80 µg/kg intraperitoneally) markedly increases the number of retinal ganglion cells that regenerate axons after optic nerve injury (*bright green circles*). Exposure to 80% N_2O for 3 days before injury and every other day thereafter (protocol 3d) reverses the benefit of serial folic acid administration.
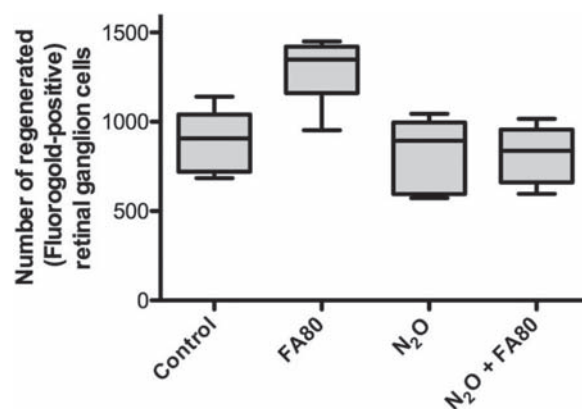

Fig. 6. Folic acid ([FA] 80 µg/kg intraperitoneally) markedly increases the number of (Fluorogold-positive) retinal ganglion cells that regenerate axons (protocol 3b) compared with no folic acid control retinal ganglion cells (protocol 3a), and to serial 80% N_2O exposure retinal ganglion cells (protocol 3c). Serial exposure to 80% N_2O (protocol 3d) reverses the benefit of serial folic acid administration. Table 4 provides the number of animals used to compare the regenerating retinal ganglion cells after sharp optic nerve injury.

Discussion

Nitrous oxide administered in clinical concentrations and durations during anesthesia is toxic to the dorsal columns of the spinal cord in the absence of nervous system trauma in humans rendered susceptible by genetic and acquired risk factors.[11–30] The effects of nitrous oxide on regeneration of the dorsal columns of the spinal cord and other tissues of the nervous system after sharp and blunt trauma are presently unknown. We report that nitrous oxide in concentrations and durations encountered during clinical anesthesia impairs regeneration of central nervous system axons after peripheral nerve trauma, sharp dorsal spinal cord trauma, sharp optic nerve trauma, and blunt dorsal spinal cord trauma in rodents.

In experiments comprising *in vitro* dorsal root ganglia axon regeneration after *in vivo* nitrous oxide exposure, axon regeneration is greater in the injured sciatic nerve groups compared with the uninjured sciatic nerve groups in the absence and in the presence of nitrous oxide. *In vivo* nitrous oxide exposure inhibits axon regeneration in both the uninjured and injured sciatic nerve groups, with nearly a fourfold difference in axon regeneration observed at 48 h compared with controls after serial nitrous oxide exposure. Of note, both a single nitrous oxide exposure and serial nitrous oxide exposures halve axon regeneration at 24 h in uninjured groups. At 48 h, axon regeneration after a single nitrous oxide exposure in the uninjured group is less than half that in the absence of nitrous oxide, and axon regeneration after serial nitrous oxide exposures in the uninjured group is less than a quarter that in the absence of nitrous oxide. These data indicate that the deleterious effects of

are significantly different between the four groups (type III test for fixed effects yields chi–square = 10.15, *P* = 0.020). Results in table 6 indicate that Basso, Beattie, Bresnahan scores in the serial folic acid administration alone group are significantly greater than in the control group at each time interval during the experiment. Basso, Beattie, Bresnahan scores in the serial folic acid administration group are significantly greater than those in the serial 80% N_2O exposure group, and greater than those in the serial folic acid administration and serial 80% N_2O exposure group from week 2 on. No significant differences in Basso, Beattie, Bresnahan scores were observed between the serial 80% N_2O exposure alone, and serial folic acid administration and serial 80% N_2O exposure groups at any time interval in the experiment.

Based on the regression model used, no significant changes in rectal temperature were observed at hourly intervals during 6 h of 80% N_2O exposure (Supplemental Digital Content fig. 5, http://links.lww.com/ALN/C25).

Table 6. Spinal Cord Contusion Score Pairwise Comparisons at Weekly Time Intervals with Contrast Estimates Based on the Fitted Mixed Model

Comparison	Numerator *DF*	Denominator *DF*	*F* Value	*P* Value (Pr > F)
FA *vs.* Control (day −3)	1	208	4.90	0.028
FA *vs.* Control (day 7)	1	187	10.26	0.002
FA *vs.* Control (day 14)	1	48.5	28.17	< 0.0001
FA *vs.* Control (day 21)	1	24.7	37.79	< 0.0001
FA *vs.* Control (day 28)	1	28.8	33.86	< 0.0001
FA *vs.* Control (day 35)	1	39.6	28.16	< 0.0001
FA *vs.* 80% N_2O + FA (day −3)	1	208	1.10	0.295
FA *vs.* 80% N_2O + FA (day 7)	1	187	3.00	0.085
FA *vs.* 80% N_2O + FA (day 14)	1	48.2	10.02	0.003
FA *vs.* 80% N_2O + FA (day 21)	1	26.4	13.99	0.001
FA *vs.* 80% N_2O + FA (day 28)	1	30.5	13.11	0.001
FA *vs.* 80% N_2O + FA (day 35)	1	39.9	11.45	0.002
80% N_2O *vs.* FA (day −3)	1	208	0.83	0.363
80% N_2O *vs.* FA (day 7)	1	186	2.63	0.107
80% N_2O *vs.* FA (day 14)	1	47.3	10.00	0.003
80% N_2O *vs.* FA (day 21)	1	25.4	15.03	0.001
80% N_2O *vs.* FA (day 28)	1	29.1	14.69	0.001
80% N_2O *vs.* FA (day 35)	1	38.4	13.14	0.001
80% N_2O *vs.* N_2O + FA (day −3)	1	208	0.03	0.872
80% N_2O *vs.* N_2O + FA (day 7)	1	186	0.02	0.880
80% N_2O *vs.* N_2O + FA (day 14)	1	47.2	0.01	0.924
80% N_2O *vs.* N_2O + FA (day 21)	1	26.7	0.00	0.984
80% N_2O *vs.* N_2O + FA (day 28)	1	30.4	0.00	0.977
80% N_2O *vs.* N_2O + FA (day 35)	1	38.9	0.00	0.955

DF, degrees of freedom; FA, folic acid 80 μg/kg intraperitoneally; N_2O, nitrous oxide; (Pr > F), the *P* values for the effect of the classification variable on the response.

nitrous oxide on folate–mediated axonal regeneration are neuron specific and do not depend on glial, vascular, or nervous system trophic elements for expression.
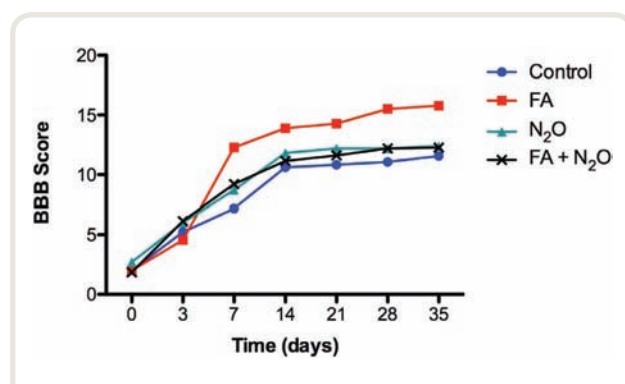

Fig. 7. Functional recovery after blunt contusion spinal cord injury (Basso, Beattie, Bresnahan [BBB]) locomotor scores. Ambulation was scored at baseline 3 days before injury, and at days 7, 14, 21, 28, and 35 after injury. Folic acid ([FA] 80 μg/kg intraperitoneally) improves functional performance scores from day 7 to 35 (protocol 4b; *red squares*) compared with scores from no folic acid control animals (protocol 4a; *blue circles*), and to scores from serial 80% N_2O exposure animals (protocol 4c; *green triangles*). Serial exposure to 80% N_2O (protocol 4d) reverses the benefit of serial folic acid administration (*black x's*). Table 5 provides the number of animals used to compare the spinal cord contusion score estimates for fixed effects.

In experiments comprising *in vivo* dorsal root ganglia axon regeneration after nitrous oxide exposure and sharp spinal cord injury, markedly increased axonal regeneration is observed in the folic acid–treated group compared with the control group as we have previously reported.[2] A single exposure to 80% N_2O is sufficient to fully inhibit the beneficial effects of folic acid on axonal regeneration, as are serial 40% N_2O and 80% N_2O exposures.

In experiments comprising *in vivo* retinal ganglion cell axon regeneration after optic nerve injury and nitrous oxide exposure, serial 80% N_2O administration reverses the regenerative benefits of folic acid. These data indicate that the deleterious effects of nitrous oxide on folate-mediated axonal regeneration are not confined to neurons in the dorsal root ganglia, and that cranial nerves share susceptibility.

In experiments comprising *in vivo* scores of behavioral recovery after direct spinal cord contusion, the marked beneficial effects of folic acid are reversed by coadministration of serial 80% N_2O exposure. These data indicate that the deleterious effects of nitrous oxide on folate-mediated axonal regeneration are not confined to sharp spinal cord injury and have adverse functional consequences in the intact organism in addition to negative histologic outcomes.

The present experiments were not configured to establish a mechanism of neuronal nitrous oxide toxicity. However, reversal of the specific beneficial effects of folic acid with single and serial nitrous oxide exposures, coupled with the well-established property of nitrous oxide to

inactivate methionine synthesis by oxidation of its cobalamin cofactor, points to impaired single carbon metabolism and consequent cellular methyl deficiency as a cause of the observed adverse effects. An important limitation of these data is that we did not examine the effects of nitrous oxide on female rats under identical experimental conditions. To our knowledge, the effects of folic acid and nitrous oxide, the interaction between nitrous oxide and cobalamin, and the capacity for axonal regeneration after injury are shared between the sexes. Future experiments are required to be certain. Although the biophysical interaction between nitrous oxide and cobalamin is identical between species, the time course for nitrous oxide inactivation of methionine synthase is species dependent for reasons yet to be identified. In rats exposed to nitrous oxide, the half-time of hepatic methionine synthase inactivation is 5 min.[31] Recovery takes 3 to 44 days because the vitamin B12 cofactor is irreversibly oxidized and covalently bound to the enzyme. Discontinuation of nitrous oxide does not immediately restore methionine synthase activity since new enzyme must be synthesized to recover from nitrous oxide damage. In humans, the half-life of inactivation in biopsied liver cells is about 45 min.[31] The half-lives of nitrous oxide inactivation and methionine synthase recovery in any tissue of the nervous system *in vivo* are not known in any species. Whether deficiency of methyl substituents necessary for synthesis of neurotransmitters, myelin, DNA and histone methylation and other essential reactions, or accumulation of homocysteine to toxic levels accounts for the pathophysiology alone or in concert is unresolved.

Beyond inter-sex and inter-species differences, a further limitation of the present report is that the data we report are those of first impression, and must therefore await replication by others, and specific clinical correlation before the present results can be safely interpreted. Xenon shares *N*-methyl-D-aspartate glutamate receptor antagonism with nitrous oxide, but has no known effects on cobalamin, methionine synthase and single carbon metabolism. While our experiments comprise specific negative controls, a parallel series of experiments that incorporate equipotent positive control xenon protocols is an attractive next step to begin to discern underlying mechanisms of nitrous oxide neurotoxicity. Future investigations may also comprise direct measures of single carbon pathway enzyme activities, and substrate and product levels in relevant tissues, together with direct measures of nucleic acid, histone and protein methylation.

Surprisingly, nothing is known in any species at present about the effects of nitrous oxide or any other inhaled anesthetic agent on regeneration of nervous system tissues or any other tissue after accidental injury, or after coincidental injury during surgery. Similarly, very little is known about the effects of folic acid, cobalamin, or any other vitamin on regeneration of nervous system tissues after accidental injury, or after coincidental injury during surgery.[2,3] Accordingly, nothing is known about differential susceptibility and

resilience to nitrous oxide toxicity, and differential capacity to respond to folate, of different brain regions. These gaps are remarkable in view of the well-recognized deleterious effects of nitrous oxide on nervous tissue that has not been traumatized during clinical anesthesia, and further in view of the substantial proportion of patients who are folate and cobalamin deficient at the extremes of age, and who are surgical candidates.[32,33] The observations reported here suggest that axonal regeneration depends on functional methionine synthase activity, and on the availability of its methyl-donating product methionine for cellular reactions that require methyl additions. When optimal regeneration is desired after neuronal injury, or when surgery itself imperils the nervous system, avoidance of nitrous oxide may be preferred until its comparative safety has been established.

Research Support

Competing Interests

The authors declare no competing interests.

Correspondence

Address correspondence to Dr. Hogan: Department of Anesthesiology, University of Wisconsin School of Medicine and Public Health, B6/319 Clinical Sciences Center, 600 Highland Avenue, Madison, Wisconsin 53792. khogan@wisc.edu. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

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ANESTHESIOLOGY

Early Postnatal Exposure to Isoflurane Disrupts Oligodendrocyte Development and Myelin Formation in the Mouse Hippocampus

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Oligodendrocyte proliferation and maturation are prerequisites for myelination in the central nervous system. Disruption of these processes can lead to long-term impairment of neural function.
- Laboratory models demonstrate a variety of effects of anesthetics on the immature brain, but the consequences of early life anesthesia exposure on oligodendrocyte development have not been previously reported.

What This Article Tells Us That Is New

- Exposure of 7-day-old mouse pups to isoflurane (1.5%, 4 h) results in lasting impairments of oligodendrocyte proliferation and differentiation.
- These effects lead to defects in myelinations and are associated with cognitive dysfunction.
- The underlying molecular mechanisms involve the isoflurane-induced activation of the mammalian target of rapamycin pathway and a related decrease in DNA methylation in oligodendrocyte progenitors.

ABSTRACT

Background: Early postnatal exposure to general anesthetics may interfere with brain development. We tested the hypothesis that isoflurane causes a lasting disruption in myelin development *via* actions on the mammalian target of rapamycin pathway.

Methods: Mice were exposed to 1.5% isoflurane for 4 h at postnatal day 7. The mammalian target of rapamycin inhibitor, rapamycin, or the promyelination drug, clemastine, were administered on days 21 to 35. Mice underwent Y-maze and novel object position recognition tests (n = 12 per group) on days 56 to 62 or were euthanized for either immunohistochemistry (n = 8 per group) or Western blotting (n = 8 per group) at day 35 or were euthanized for electron microscopy at day 63.

Results: Isoflurane exposure increased the percentage of phospho-S6–positive oligodendrocytes in fimbria of hippocampus from 22 ± 7% to 51 ± 6% ($P < 0.0001$). In Y-maze testing, isoflurane-exposed mice did not discriminate normally between old and novel arms, spending equal time in both (50 ± 5% old:50 ± 5% novel; $P = 0.999$), indicating impaired spatial learning. Treatment with clemastine restored discrimination, as evidenced by increased time spent in the novel arm (43 ± 6% old:57 ± 6% novel; $P < 0.001$), and rapamycin had a similar effect (44 ± 8% old:56 ± 8% novel; $P < 0.001$). Electron microscopy shows a reduction in myelin thickness as measured by an increase in g-ratio from 0.76 ± 0.06 for controls to 0.79 ± 0.06 for the isoflurane group ($P < 0.001$). Isoflurane exposure followed by rapamycin treatment resulted in a g-ratio (0.75 ± 0.05) that did not differ significantly from the control value ($P = 0.426$). Immunohistochemistry and Western blotting show that isoflurane acts on oligodendrocyte precursor cells to inhibit both proliferation and differentiation. DNA methylation and expression of a DNA methyl transferase 1 are reduced in oligodendrocyte precursor cells after isoflurane treatment. Effects of isoflurane on oligodendrocyte precursor cells were abolished by treatment with rapamycin.

Conclusions: Early postnatal exposure to isoflurane in mice causes lasting disruptions of oligodendrocyte development in the hippocampus *via* actions on the mammalian target of rapamycin pathway.

(*ANESTHESIOLOGY* 2019; 131:1077–91)

Modern general anesthesia allows the safe performance of several hundred million surgical procedures annually.[1] However, there is growing concern that some vulnerable categories of patients, particularly young children, geriatric patients, and individuals with underlying brain disorders, may be at risk of lasting cognitive dysfunction.[2–4] While conclusive evidence of anesthetic neurotoxicity has not been established in human studies, some animal studies have shown that exposure to general anesthetics in early development causes impaired neurocognitive performance[5–8] and that the peak period of behavioral and cognitive vulnerability to general anesthetics in rodents occurs in early postnatal life.[9–11] Based on these studies, the U.S. Food and Drug Administration issued a warning that

lengthy or repeated exposure to general anesthetics and sedative drugs from the third trimester of prenatal development through the first 3 yr of life may cause lasting impairment in the cognitive function.[12] The molecular and cellular mechanisms underlying this phenomenon remain poorly understood.

Most studies investigating anesthetic neurotoxicity have focused on neuronal development.[7,13] However, brain function is also dependent on the myelin-forming oligodendrocytes, which undergo critical developmental events during the putative window of vulnerability. Myelination involves proliferation of oligodendrocyte progenitor cells, differentiation of oligodendrocyte progenitor cells into mature oligodendrocytes, and ensheathment of axons. Myelin is critical for neurotransmission in the central nervous system (CNS), and disruptions of myelin function are associated with neurologic and psychiatric disorders.[14,15] In this study, we test the hypothesis that early postnatal exposure to isoflurane affects oligodendrocyte development and myelin formation in the hippocampus in an *in vivo* mouse model. To establish the extent to which isoflurane-induced deficits can be attributed to impaired myelination, we employed clemastine, an antimuscarinic drug approved for multiple sclerosis therapy, which promotes oligodendrocyte differentiation and myelination and reverses the phenotype of several murine disease models involving demyelelination.[16,17]

Exposure to general anesthetics has been shown to impact molecular signaling pathways implicated in the dynamic maintenance of cellular homeostasis and development.[13] Recently, mammalian target of rapamycin (mTOR) signaling has emerged as a critical integrator of activity of nerve cells and synaptic inputs that in turn affect many cellular metabolic processes.[18] Studies have implicated mTOR signaling in neurodevelopmental and neuropsychiatric disorders.[19] We previously showed that isoflurane disrupts development of newborn hippocampal neurons and synaptic formation *via* activation of the mTOR pathway.[7,20] In this study, we further investigate the role of mTOR in isoflurane-induced neurotoxicity in mouse oligodendrocyte development and myelination using mTOR activity markers and rapamycin, a mTOR pathway inhibitor. We explored the effects on axon–oligodendrocyte precursor synapses, which are thought to be critical for turning oligodendrocyte development to match neuronal activity.[21–23] Activity in the mTOR pathway mediates DNA methylation in neurons[24] and cancer cells,[25] and it has been reported that DNA methylation is a well-recognized epigenetic modification that regulates oligodendrocyte development and is necessary for efficient myelin formation.[26–28] Thus, the effect of general anesthetics and mTOR activation on DNA methylation level in oligodendrocytes has also been investigated.

Materials and Methods

Animal Paradigm and Experimental Timeline

A total of 120 (61 male and 59 female) immature C57BL/6 mice (body weight = 4.4 ± 0.9 g at postnatal day 7) were used in this study. Eighty-four (44 male and 40 female) of them were randomly selected for the rapamycin experiment and 36 (17 male and 19 female) for the clemastine experiment. Sex was not factored into the research design as a biologic variable. Both sexes were equally represented in all experiments. All study protocols involving mice were approved by the Animal Care and Use Committee at Johns Hopkins University (Baltimore, Maryland) and conducted in accordance with National Institutes of Health (Bethesda, Maryland) guidelines for care and use of animals. Experimental procedures followed the modified protocols from a previously published journal.[7]

At postnatal day 7, animals were exposed to isoflurane or room air for 4 h. From postnatal days 21 to 35, half of the isoflurane-exposed mice were injected intraperitoneally bidaily with rapamycin (n = 28 per group) or fed daily with clemastine through gastric gavage (n = 12 per group). The other half were injected with vehicle of rapamycin or fed with vehicle of clemastine.

For the rapamycin experiment, a subset of mice from each group was euthanized at postnatal day 35 for immunohistochemistry (n = 8 per group) or Western blotting (n = 8 per group). The remaining mice underwent behavioral testing for spatial learning and memory functions between postnatal days 56 and 62 (n = 12 for each group). After behavior tests, two mice from each group were processed for electron microscopy at postnatal day 63. Only behavior tests were conducted for the clemastine feeding experiment (n = 12 for each group; fig. 1A).

Isoflurane Exposure

At postnatal day 7, two thirds of the mice were evenly distributed across littermate groups and were randomly selected for isoflurane exposure. The other one third of the mice stayed in room air as a naive control. Volatile anesthesia exposure was accomplished using a Supera (USA) tabletop portable nonrebreathing anesthesia machine. Three percent isoflurane mixed in 100% oxygen was initially delivered in a closed chamber for 3 to 5 min, and after loss of righting reflex, animals were transferred to the specially designed plastic tubes. A heating pad (36.5°C) was placed underneath the exposure setup. The mice were exposed to 1.5% isoflurane carried in 100% oxygen for 4 h. A calibrated flowmeter was used to deliver oxygen at a flow rate of 5 l/min, and an agent-specific vaporizer was used to deliver isoflurane. During isoflurane exposure, mice were monitored for change in physiologic state using the noninvasive MouseOx plus instrument (STARR Life Sciences, USA). A collar clip connected to the instrument was placed on the neck and
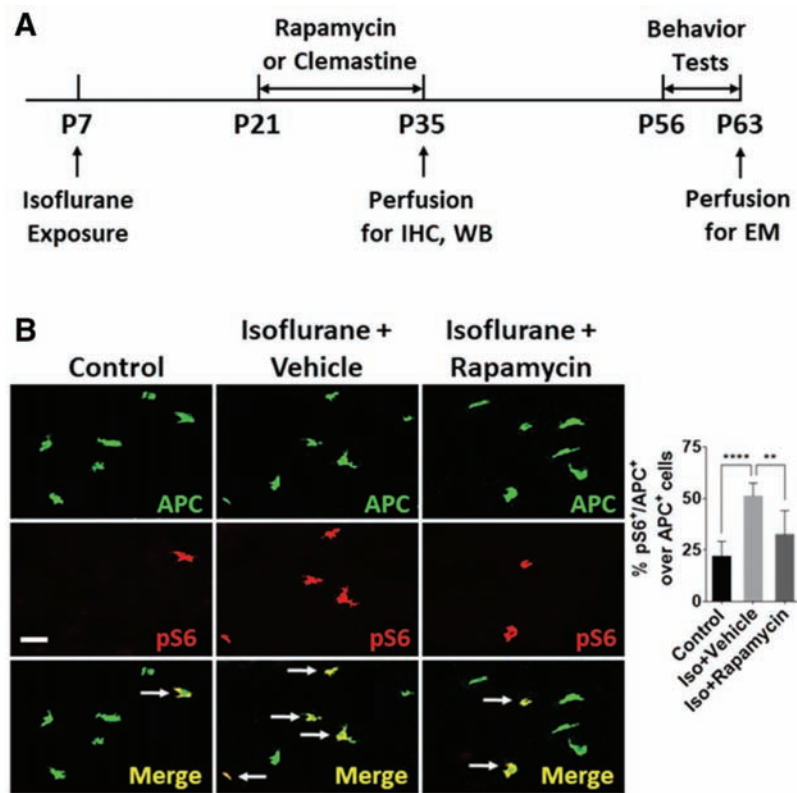
Fig. 1. (*A*) Experimental timeline. A total of 120 mice (84 for rapamycin injection and 36 for clemastine feeding experiments) were used in this study. At postnatal day 7, two thirds of the mice were exposed to isoflurane (Iso) carried for 4 h, and the other one third of the animals remained in room air as naive controls. From postnatal days 21 to 35, isoflurane-exposed mice were injected intraperitoneally with rapamycin or vehicle at 48 h intervals, or daily fed with clemastine or vehicle. Mice were euthanized at postnatal day 35 for immunohistochemistry and Western blotting, or at postnatal day 63 for electron microscopy. The novel objective position recognition test and Y-maze test were performed during postnatal days 57 to 62. (*B*) Effect of early isoflurane exposure on mammalian target of rapamycin pathway activity in oligodendrocytes of hippocampus fimbria. Coronal brain sections from control, isoflurane exposure, and isoflurane plus rapamycin groups were immunostained with adenomatous polyposis coli (APC; *green*) and phospho-S6 (pS6; *red*) antibodies. Arrows indicate phospho-S6–positive and adenomatous polyposis coli–positive double-labeled cells (*yellow*) in merged images. *Scale bar* = 10 µm. The histogram shows quantitative results. In isoflurane-exposed mice, the ratio of phospho-S6–positive/adenomatous polyposis coli–positive over adenomatous polyposis coli–positive cells is dramatically increased compared to control, and this increase is reversed with rapamycin treatment. n = 8 for each group; one-way ANOVA; **$P < 0.01$; ****$P < 0.0001$. *Error bars*: SD. EM, electron microscopy; IHC, immunohistochemistry; WB, western blot.

a temperature probe placed on the skin of the abdomen. Ten-minute readings with 1-h intervals were taken. Data were collected at four time-points and averaged for each case. The skin temperature (34.1 ± 0.8°C), pulse distention (168.9 ± 36.6 µm), heart rate (376.8 ± 94.1 beats/min), breath rate (77.4 ± 35.8 breaths/min), and oxygen saturation (99.3 ± 0.3%) were recorded. After the isoflurane exposure, mice were returned to their mothers together with their littermates upon regaining righting reflex. All animals (100%) survived the isoflurane exposure.[7]

Rapamycin Injection

A total of 84 mice were equally divided into three groups: (1) naive control, (2) isoflurane exposure plus vehicle, and (3) isoflurane plus rapamycin injection. From postnatal days 21

to 35, half of the isoflurane-exposed mice (group 3; n = 28 per group) were injected intraperitoneally with 0.2% rapamycin dissolved in vehicle solution and the other half with vehicle only (group 2; n = 28 per group). Vehicle consisted of 5% Tween 80 (Sigma-Aldrich, USA), 10% polyethylene glycol 400 (Sigma-Aldrich), and 8% ethanol in saline. Mice received 100 µl rapamycin or vehicle for each injection at 48-h intervals from postnatal days 21 to 35.[7]

Clemastine Feeding

In this experiment, 36 animals were also equally divided into three groups as above. Clemastine (Tocris Bioscience, United Kingdom) was dissolved in dimethylsulfoxide (Sigma-Aldrich) at 10 mg/ml followed by further dilution in double distilled water into 1 mg/ml. From postnatal days

21 to 35, half of the isoflurane exposed mice (n = 12 for each group) were fed clemastine (10 mg/kg) daily *via* gastric gavage using plastic feeding tubes (gauge 22; Instech, USA), and the other half (n = 12 for each group) were fed same volume of 10% dimethylsulfoxide as vehicle.[16,17]

Behavior Tests

The novel object position recognition test and Y-maze test were performed at the last week of the survival period (postnatal days 56 to 62).[7] Experimenters were blinded to condition when behavioral tests were carried out and quantified.

1. Novel object position recognition test: The test was assessed in a 27.5 cm × 27.5 cm × 25 cm opaque chamber. During the pretest day (day 1), each mouse was habituated to the chamber and allowed to explore two identical objects (glass bottles, 2.7 cm diameter, 12 cm height, and colored paper inside) for 15 min. The mouse was then returned to its home cage for a retention period of 24 h. On the test day (day 2), the mouse was reintroduced to the chamber and presented with one object that stayed in the same position (old position) while the other object was moved to a new position (novel position). A 5-min period of movement and interaction with the objects was recorded with a video camera that was mounted above the chamber, and exploratory behavior was measured by a blinded observer. Exploratory behavior was defined as touching the object with snouts. The numbers of exploratory contacts with the novel object and with the old object were respectively recorded, and the ratios over the total exploratory contact numbers were calculated.
2. Y-maze test: In the pretest phase (day 1), mice explored and habituated in the start arm (no visual cue) and one out of two possible choice arms with overt visual cue (old arm) for 15 min. This was followed by the recognition phase (day 2) 24 h later, in which the animals could move freely in the three arms and choose between the two choice arms (old arm and novel arm) after being released from the start arm. The timed trials (5 min) were video recorded as well as graded by an observer blinded to the conditions for exploration time in each choice arm, and the percentages over total exploratory time were calculated.

Immunohistochemistry

During postnatal days 30 to 35, 5-bromo-2'-deoxyuridine (Abcam, United Kingdom) was injected intraperitoneally at 50 mg/kg daily in animals randomly selected from three groups (n = 8 for each group). At postnatal day 35, mice were perfused with 40 ml 4% paraformaldehyde in phosphate buffered saline. Brains were removed and postfixed at 4°C overnight, followed by 30% sucrose in phosphate buffered saline at 4°C for 48 h. The brains were coronally sectioned in 40-μm thickness using a freezing microtome. For each brain, 72 sections containing fimbria were collected

in a 24-well tissue culture plate, and they were divided into 12 wells in a rotating order (six sections per well). Seven wells of sections were immunostained for (1) phospho-S6 and adenomatous polyposis coli, (2) 5-bromo-2'-deoxyuridine and neural/glial antigen 2, (3) adenomatous polyposis coli and platelet-derived growth factor receptor alpha, (4) vesicular glutamate transporter 1 and neural/glial antigen 2, (5) myelin basic protein, (6) DNA methyltransferase 1 and Olig2 (oligodendrocyte transcription factor marker), and (7) 5-methylcytosine and adenomatous polyposis coli. For 5-bromo-2'-deoxyuridine staining, sections were pretreated with 2 Normal HCl to denature DNA (37°C; 45 min), and with 2 × 15 min borate buffer (pH 8.5) to neutralize the HCl. After 3 × 10 min phosphate buffered saline washing, sections were blocked in 10% normal goat serum and 0.1% Triton X-100 (Sigma-Aldrich) for 60 min, followed by primary antibody incubation at 4°C overnight. Primary antibodies used in this study were rabbit anti–phospho-S6 (1:1,000; Cell Signaling, USA), mouse anti–5-bromo-2'-deoxyuridine (1:200; Abcam), rabbit anti–neural/glial antigen 2 (1:200; Millipore, USA), mouse anti–adenomatous polyposis coli (1:2,000; Millipore), mouse anti–myelin basic protein (1:500; Santa Cruz Biotechnology, USA), rabbit anti–platelet-derived growth factor receptor alpha (1:500; Lifespan Bio, USA), mouse anti–vesicular glutamate transporter 1 (1;200; Abcam), mouse anti–DNA methyltransferase 1 (1:100; Santa Cruz Biotechnology,), rabbit anti-Olig2 (1:2,000; Abcam), and rabbit anti–5-methylcytosine (1:2,500; Abcam). After 3 × 10-min washes in phosphate buffered saline, sections were incubated with secondary antibodies for 2 h: Alexa 488–conjugated goat anti-rabbit IgG (1:300; Invitrogen, USA) mixed with Cy3-conjugated goat anti–mouse IgG (1:600; Jackson ImmunoResearch Labs, USA), or Alexa 488–goat anti-mouse IgG (1:300; Invitrogen) mixed with Cy3-conjugated goat anti-rabbit IgG (1:600; Jackson ImmunoResearch Labs). After 3 × 10-min phosphate buffered saline washes, sections were mounted onto slides, air-dried, and cover-slipped.[21]

Cell Counting and Immunofluoresce Intensity Analysis in Fimbria

The sections were observed and imaged using a Leica 4000 confocal microscope (Germany). All single- or double-immunolabeled cells within hippocampal fimbria area were counted using ImageJ with cell counter plugin (National Institutes of Health). The criteria for counting mTOR active oligodendrocytes required a cell to have both phospho-S6–positive (in red channel) and adenomatous polyposis coli–positive (in green channel) cytoplasm, and a merged image of double-labeled cells appeared yellow (fig. 1B). Proliferating oligodendrocyte progenitor cells and 5-methylcytosine–positive oligodendrocytes were counted for cells that have 5-bromo-2'-deoxyuridine–positive or 5-methylcytosine positive nuclei and neural/glial antigen 2–positive or adenomatous polyposis coli-positive cytoplasm.

However, both DNA methyltransferase 1 and Olig2 reactivity were seen in nuclei. Identification of excitatory axon–oligodendrocyte progenitor cell synapses involved vesicular glutamate transporter 1–positive terminal boutons closely apposing on the surface of neural/glial antigen 2–positive oligodendrocyte progenitor cells. For adenomatous polyposis coli and platelet-derived growth factor receptor alpha double-stained sections, almost no double-labeled cells were seen, which means these two markers label cells in different oligodendrocyte development stages without overlapping.

Images containing fimbria were taken at 20× magnification in red (Cy3), green (Alexa 488), and merged channels. All single- (Cy3+ or Alexa 488+) and double-labeled cells in fimbria were counted. Images were opened and initialized in ImageJ. The fimbria area was outlined using the "Freehand" tool. "Plugins," "Analysis," and "Cell Counter" tools were selected, and each labeled cell inside was clicked, with which each counted cell was marked, preventing the same cell from being counted twice. The numbers of counted cells were automatically recorded. The ratio of a specific marker labeled oligodendrocytes (such as yellow-colored phospho-S6–positive/ adenomatous polyposis coli–positive cells over all green adenomatous polyposis coli–positive cells in fig. 1B) was calculated. For each case, numbers from 12 fimbria images (six sections, both sides) were averaged. There was almost no double-staining for adenomatous polyposis coli and platelet-derived growth factor receptor alpha. We then used the ratio of adenomatous polyposis coli–positive over platelet-derived growth factor receptor alpha–positive cells to evaluate the maturation of oligodendrocyte lineage cells. For axon–oligodendrocyte progenitor cells synapse, five neural/glial antigen 2–positive cells from each image (60 cells for each case) were randomly selected, and photos were taken in a higher magnification (40×). All vesicular glutamate transporter 1–positive terminal boutons apposing on each selected cell were counted with ImageJ, and average numbers were calculated.

The fluorescence intensity of myelin basic protein immunoreactivity in fimbria was also quantitatively analyzed using ImageJ. Photos of the fimbria area from immunostained sections were taken at 20× magnification. Identical photo exposure was set for all groups. The image was opened with ImageJ, and an outline of fimbria was drawn with "Freehand" tool. The "set measurements" was selected from the analyze menu, and "integrated density" was activated. A region in lateral ventricle was selected as background. The final myelin basic protein intensity of fimbria area equals measured density minus background.

Western Blotting

Eight animals from each group were quickly perfused with cold saline on day 35. From the medial aspect of the hemisphere, the hippocampus was exposed and separated from brain tissue. Fimbria located in the ventrolateral side of the hippocampus were easily identified by their bright white color under dissection microscope, and then removed with fine forceps. Fimbria tissue was lysed in the lysis buffer, homogenized with a bullet bender (Next Advance, USA), and centrifuged. The supernatant was taken and stored in −80°C. The next day, samples were prepared with 1:1 denaturing sample buffer (Bio-Rad, USA), boiled for 5 min, and run on 4 to 12% Bis-Tris Protein Gels (Invitrogen) in running buffer (Invitrogen) with 150 volts for about 1h. The proteins were transferred to nitrocellulose blotting membranes (Invitrogen). Blots were probed with anti–neural/glial antigen 2 (1:200; Millipore), anti-NK2 homeobox 2 (1:200; Abcam), anti-myelin basic protein (1:500; Santa Cruz Biotechnology), anti-DNA methyltransferase 1 (1:100; Santa Cruz Biotechnology), and anti–β-actin antibodies (1:1,000; Cell Signaling Technology). The membranes with the primary antibodies were stored in 4°C overnight. After incubation in secondary antibodies (1:2,000; Cell Signaling Technology) for 1h, blots were visualized using an ECL Western blotting substrate kit (Pierce Biotechnology, USA). Images were acquired using the ChemiDoc imaging system (Bio-Rad) and were quantitated with ImageJ. First, the images were opened using "File>Open." The rectangles around all lanes (each lane includes bands for detected marker and β-actin) were drawn by choosing "Rectangular Selection." Then, proceeding to "Analyze>Gels>Plot Lanes," peaks were generated representing the density of bands, followed by clicking the "Straight Line" tool to enclose the peaks and selecting the "Wand" tool to highlight the peaks. After this, "Analyze>Gels>Label Peaks" was used to get numbers for the peak area (band intensity). The ratios of band density of oligodendrocyte lineage markers over β-actin were calculated.[21]

Electron Microscopy

Two animals from each group were perfused with 2% glutaraldehyde (Electron Microscopy Sciences, USA) plus 2% paraformaldehyde (Electron Microscopy Sciences) in phosphate buffered saline at postnatal day 63 (after behavior tests) and postfixed at 4°C for 1 week. Brains containing fimbria were dissected into small blocks (2 mm × 2 mm × 2 mm). The blocks were placed into 1% osmium tetraoxide (Electron Microscopy Sciences) for 1h, stained in 0.5% uranyl acetate (Electron Microscopy Sciences) overnight, and dehydrated in a series of alcohols followed by propylene oxide for 3h. After being infiltrated with a 1:1 mixture of propylene oxide and EMBed-812 embedding resin (Electron Microscopy Sciences) for 3h, the blocks were embedded with the same resin in the plastic templates at 60°C overnight.[21] Parasagittal semithin sections (1 μm) were cut and stained with 1% Toluidine blue (Sigma-Aldrich) for preliminary light microscopy observation. Then, 90-nm ultrathin sections were cut, picked up on Forvar-coated slotted grids (Electron Microscopy Sciences), and stained with 0.5% uranyl acetate and 0.5% lead citrate (Electron

Microscopy Sciences). Thin sections were observed and imaged with a Hitachi 7600 transmission electron microscope (Chiyoda, Japan). For each case, 10 photos were randomly photographed at 20,000×. The thickness of myelin was quantitatively measured by determining the g-ratio, which was calculated by dividing the diameter of the axon by the diameter of the entire myelinated fiber as previously described. ImageJ was used by first opening ultrastructural images. The scale was set according to the scale bar in the images by selecting "Analyze>Set Scale." The "straight line tool" was selected to measure axonal caliber and diameter of myelinated axons. One hundred axons per group (2 animals, 50 from each) were randomly selected and quantitatively analyzed (n = 100).[16]

Statistical Analysis

The statistics were performed with GraphPad Prism 6 (USA) program. The sample size was based on our previous experience with this design. No *a priori* statistical power calculation was conducted. Normal distribution was verified using the D'Agostino–Pearson test. Data for immunohistochemistry, Western blotting, and electron microscopy were analyzed using one-way ANOVA. The factor of variable was comparisons among groups (control *vs.* isoflurane plus vehicle *vs.* isoflurane plus rapamycin). The behavior tests were analyzed with two-way ANOVA. For this analysis, the second factor was the animal's choice between old *versus* novel positions (or arms), and only the values for this variable in each individual group were compared. The Tukey *post hoc* test was employed for intergroup comparisons. The two-tailed test was set according to convention. The criterion for significant difference was set *a priori* at $P < 0.05$. In this study, all results were expressed as mean ± SD. The sample size "n" represents the number of animals for each group. The only exception is g-ratio analysis with electron microscopy, in which "n" indicates the number of randomly selected axons from two mice per group (n = 100). This analysis method is extensively applied for g-ratio study.[16] Because all animals survived tests, there were no missing data in this study. No exclusions for outliers were made in this study. In some experiments, the sample size was increased in response to peer review.

Results

Effect of Early Isoflurane Exposure on mTOR Activity in Oligodendrocytes in Hippocampal Fimbria

All experiments compared the three groups as follows: naive control, isoflurane exposure plus vehicle, and isoflurane exposure plus treatment (rapamycin or clemastine). We first assayed for activity in the mTOR pathway in oligodendrocytes by double-labeling for phospho-S6, a reliable reporter of activity in this pathway,[29] and adenomatous polyposis coli, a standard marker for oligodendrocyte. In the control group, 22 ± 7% adenomatous polyposis coli–positive

oligodendrocytes in fimbria were also immunolabeled for phospho-S6. There was a profound increase in the percentage of phospho-S6–positive/ adenomatous polyposis coli–positive cells over adenomatous polyposis coli–positive cells to 51 ± 6% in isoflurane exposure mice ($P < 0.0001$). However, this increase of phospho-S6–positive/adenomatous polyposis coli–positive cells over adenomatous polyposis coli–positive cells was prevented by treatment with rapamycin (32 ± 12%; $P = 0.001$). These data indicate early exposure of a general anesthetic agent causing a lasting increase in the activity of the mTOR signaling pathway in the oligodendrocytes of hippocampus white matter, and rapamycin attenuates this increase.

Effect of Isoflurane Exposure on Spatial Learning

Next, we asked whether isoflurane exposure impairs spatial learning and memory behaviors using the novel objective position recognition test (fig. 2A) and Y-maze test (fig. 2B), and whether treatment with rapamycin or clemastine restores these functions in our exposure paradigm.

1. Rapamycin study: In the novel object position recognition test, control animals made 58 ± 8% contacts with the object that had been repositioned as compared to 42 ± 8% contacts with the unchanged object ($P < 0.0001$). The isoflurane-exposed mice made essentially equal contacts at both objects (53 ± 6% *vs.* 47 ± 6% times; $P = 0.398$). Rapamycin treatment restored the performance to near control levels (55 ± 8% *vs.* 45 ± 8% times; $P = 0.016$; fig. 2C). In the Y-maze test, control animals exhibited a higher percentage of exploration time in novel arm (58 ± 5% exploration time) compared to the old one (42 ± 5% exploration time; $P < 0.0001$). Isoflurane-exposed animals without rapamycin treatment had equal exploration times in both arms (50 ± 5% *vs.* 50 ± 5% duration; $P = 0.999$), and rapamycin treatment restored performance in this task (56 ± 8% *vs.* 44 ± 8% duration; $P < 0.001$; fig. 2D).

2. Clemastine study: In the novel object recognition test, control animals made more contacts with the object in the novel position (57 ± 8% *vs.* 43 ± 8% times; $P = 0.007$), but isoflurane exposed animals exhibited no exploration preference (51 ± 10% *vs.* 49 ± 10% times; $P = 0.998$). Clemastine treatment increased the difference near the control cases (56 ± 7% *vs.* 44 ± 7% times; $P = 0.028$; fig. 2E). Similarly, in the Y-maze test, unlike controls (58 ± 6% *vs.* 42 ± 6% duration; $P < 0.0001$), isoflurane-exposed mice spent identical time in both old and novel arms (51 ± 7% *vs.* 49 ± 7% duration; $P = 0.999$), and this effect of isoflurane was reversed by clemastine treatment (57 ± 6% *vs.* 43 ± 6% duration; $P < 0.001$; fig. 2F).
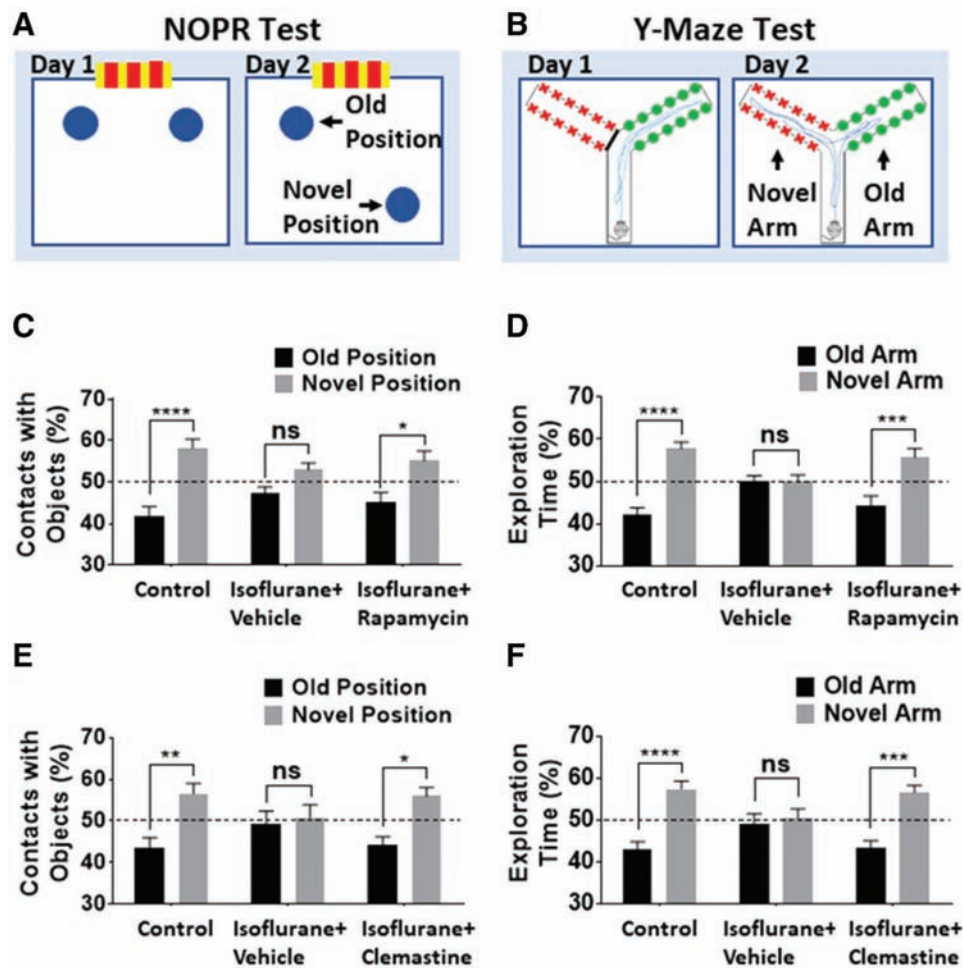
Fig. 2. Isoflurane impairs cognitive functions *via* mammalian target of rapamycin activity. (*A*) Novel object recognition test (NOPR). On day 1, mice were allowed to explore two identical objects in an opaque chamber. On day 2, one object was moved to a novel position. Exploratory behavior was defined as the number of object-contacting with snouts. (*B*) Y-maze test. On day 1, mice habituated in the start arm and one choice arm. On day 2, the animal could choose between two arms. Exploration time in both arms was respectively recorded. (*C*) Novel object recognition test for rapamycin study. Control animals made more contact with the object in the novel position than that in the old position. Isoflurane-exposed mice have identical contacts for both positions. Rapamycin treatment restores performance to near control levels. (*D*) Y-maze study. Control animals stayed in the novel arm for longer time than in the old arm. Isoflurane-exposed animals stayed in both arms for same time. Rapamycin treatment reversed this ratio to near control. (*E*) Novel object recognition test for clemastine study. Control animals spent more time exploring the object in the novel position, but isoflurane-exposed animals exhibited no exploration preference. Clemastine treatment increased difference near the level of control animals. (*F*) Y-maze test. Similarly, isoflurane mice spent identical time for both arms, but this effect of isoflurane was reversed by feeding clemastine. The statistics were two-way ANOVA. n = 12 for each group; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$; ns, no significance. *Error bars*: SD.

Effects of Isoflurane on Oligodendrocyte Development

In order to measure the proliferation of oligodendrocyte progenitor cells, the brain tissue was immunolabeled with antibodies against 5-bromo-2'-deoxyuridine and an oligodendrocyte progenitor cells marker, neural/glial antigen 2. We examined proliferating oligodendrocyte progenitor cells by counting 5-bromo-2'-deoxyuridine and neural/glial antigen 2 double-labeled cells in fimbria. We counted $49 \pm 14\%$ neural/glial antigen 2–positive oligodendrocyte progenitor cells in controls, and $27 \pm 9\%$ neural/glial antigen 2–positive cells in isoflurane-exposed animals were 5-bromo-2'-deoxyuridine–positive ($P = 0.001$). This ratio number increased to $47 \pm 7\%$ ($P = 0.003$) in the isoflurane plus rapamycin injection group (fig. 3A). Interestingly, we found that neural/glial antigen 2 expression from Western blot in the isoflurane exposure group ($83 \pm 20\%$ intensity over β-actin) is slightly lower than the control ($87 \pm 15\%$; $P = 0.882$) and rapamycin treatment groups ($89 \pm 17\%$; $P = 0.819$), but there is no statistical difference
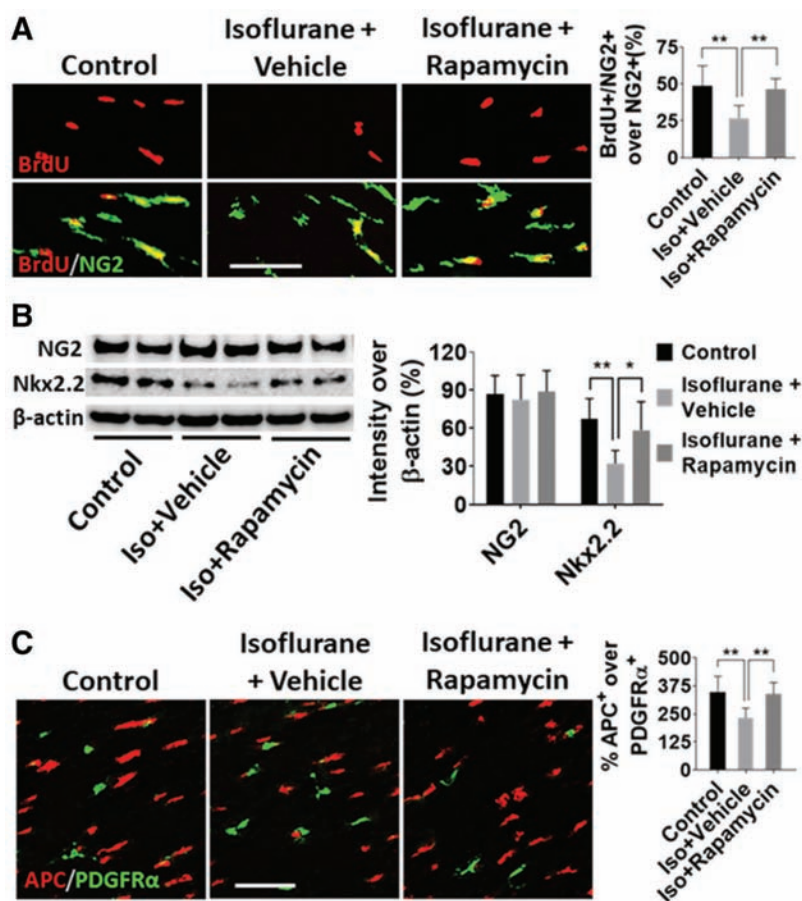
Fig. 3. The effect of isoflurane (Iso) exposure and rapamycin treatment on oligodendrocyte development in fimbria. (*A*) Oligodendrocyte progenitor cell proliferation was detected with 5-bromo-2'-deoxyuridine and neural/glial antigen 2 double-immunolabeling. A reduction in 5-bromo-2'-deoxyuridine–positive/neural/glial antigen 2–positive cells in isoflurane-exposed animals was observed compared to the control. This number was increased in isoflurane plus rapamycin injection group. *Scale bar* = 20 μm. (*B*) Western blot data indicated that the neural/glial antigen 2 level was not altered by isoflurane exposure and rapamycin administration. Expression of NK2 homeobox 2, a transcript factor for oligodendrocyte differentiation, was downregulated by isoflurane, and rapamycin treatment attenuated this effect. (*C*) Oligodendrocyte differentiation was analyzed with lineage tracing using immunohistochemistry. The ratio of adenomatous polyposis coli–positive mature oligodendrocyte number over platelet-derived growth factor receptor alpha–positive oligodendrocyte progenitor cells in isoflurane-exposed mice revealed reduction compared to control, and rapamycin treatment increases the ratio. *Scale bar* = 20 μm. n = 8 for each group; one-way ANOVA; *$P < 0.05$; **$P < 0.01$; ns, no significance. *Error bars*: SD. APC, adenomatous polyposis coli; PDGFR-α, platelet-derived growth factor α.

among groups (fig. 3B). However, expression level of NK2 homeobox 2, a transcription factor that identifies oligodendrocyte differentiation, was downregulated by isoflurane ($67 \pm 16\%$ *vs.* $32 \pm 10\%$ intensity over β-actin; $P = 0.001$), and rapamycin attenuated this effect ($58 \pm 22\%$ intensity over β-actin; $P = 0.015$; fig. 3B). We then performed double-immunolabeling for adenomatous polyposis coli and the oligodendrocyte progenitor cell marker platelet-derived growth factor receptor alpha, and applied oligodendrocyte/oligodendrocyte progenitor cell ratio as a parameter to evaluate the oligodendrocyte differentiation in fimbria.[30] The ratio of the number of adenomatous polyposis coli–positive mature oligodendrocytes over the number of platelet-derived growth factor receptor alpha–labeled

oligodendrocyte progenitor cells in isoflurane-exposed mice ($233 \pm 43\%$) revealed lower than in control ($347 \pm 70\%$ cells; $P = 0.002$), and rapamycin treatment increases this ratio ($338 \pm 52\%$ cells; $P = 0.003$; fig. 3C).

To test the effects of isoflurane on axon–oligodendrocyte progenitor cell synapses, we identified excitatory axon–oligodendrocyte progenitor cell synapses in the fimbria as puncta that were immunopositive for vesicular glutamate transporter 1, which were closely apposed on neural/glial antigen 2–positive cell bodies. We found that the number of axon–oligodendrocyte progenitor cell synapses on each oligodendrocyte progenitor cell in isoflurane exposure (0.8 ± 0.6 vGlut1[+] terminals per oligodendrocyte precursor cell) showed a statistically significant reduction compared to

control (2.6 ± 1.2 terminals per cell; $P = 0.001$), and rapamycin treatment rescued these synapses (2.2 ± 0.7 per cell; $P = 0.008$) from isoflurane exposure (fig. 4).

Effects of Isoflurane Exposure on Myelination

To test for changes in myelination after anesthesia exposure, we measured fluoresce intensity of immunolabeling for the myelin basic protein in fimbria. We found an immunointensity reduction in isoflurane exposure (70 ± 18% intensity over control) compared to control conditions (100 ± 17% control; $P = 0.006$), which were partially restored with rapamycin treatment (92 ± 17% rapamycin treatment; $P = 0.041$; fig. 5A). We then conducted Western blot from fimbria tissue to confirm this finding. The band intensity of myelin basic protein over β-actin showed a statistically significant decrease by isoflurane exposure (110 ± 30% *vs.* 60 ± 19% intensity ratio; $P = 0.002$), and expression of myelin basic protein was restored with rapamycin treatment (100 ± 26% intensity ratio; $P = 0.013$; fig. 5B). For further confirmation, we conducted electron microscopy to test for changes in the thickness of myelin wraps after isoflurane exposure.

The quantitative analysis revealed a statistically significant increase of g-ratio (thinner myelin sheath) in isoflurane-exposed animals than control (0.76 ± 0.06 *vs.* 0.79 ± 0.06 g-ratio; $P < 0.001$), and rapamycin treatment reversed this difference (0.75 ± 0.05 g-ratio; $P < 0.0001$; fig. 5C).

Effects of Isoflurane Exposure on DNA Methylation in Oligodendrocytes

We asked if early exposure to isoflurane has a lasting effect on DNA methylation levels in oligodendrocytes. We observed that 60 ± 15% of Olig2^{+} cells (a transcription factor marker for oligodendrocyte lineage cells) in control conditions were double-labeled with DNA methyltransferase 1 in fimbria as compared to only 42 ± 12% Olig2^{+} cells ($P = 0.027$) in the isoflurane exposure group. Rapamycin treatment after isoflurane exposure increased the ratio of DNA methyltransferase 1[+]/Olig2^{+} over Olig2^{+} cells (62 ± 11% ratio; $P = 0.01$; fig. 6A). Western blots were conducted which confirmed that DNA methyltransferase 1 expression is reduced with isoflurane treatment (25 ± 8% intensity ratio over β-actin) compared to control (58 ± 21% intensity ratio over β-actin; $P < 0.001$)
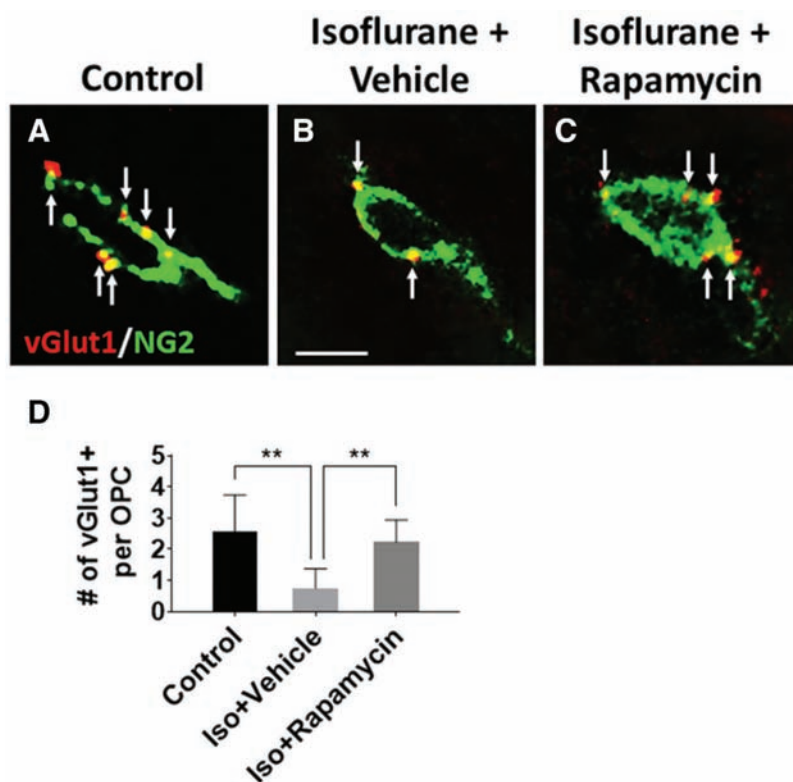
Fig. 4. The effect of isoflurane (Iso) exposure and rapamycin treatment on the numbers of excitatory axon–oligodendrocyte progenitor cell (OPC) synapses in fimbria. The vesicular glutamate transporter 1–positive axon–oligodendrocyte progenitor cell synapses were identified with terminals apposing on neural/glial antigen 2–positive oligodendrocyte progenitor cells (*arrows* indicate these synapses). The number in isoflurane exposure mice was lower than control and rapamycin treatment rescued these axon–oligodendrocyte progenitor cell synapses. *Scale bar* = 5 μm. (*A*) Control. (*B*) Isoflurane exposure plus vehicle group. (*C*) Isoflurane exposure with rapamycin treatment. (*D*) Graph showing quantitative data. n = 8 for each group; one-way ANOVA; **$P < 0.01$. *Error bars*: SD.
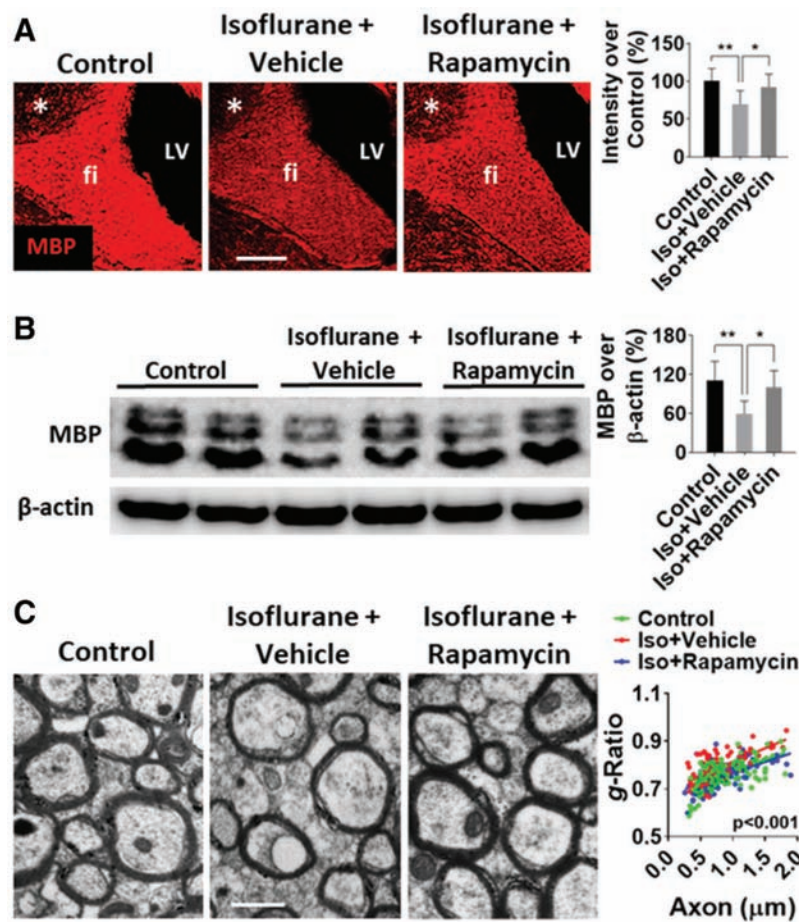
Fig. 5. The effect of isoflurane (Iso) exposure and rapamycin treatment on myelination in fimbria. (*A*) Myelination was quantitatively analyzed with myelin basic protein immunostaining. The myelin basic protein (MBP) intensity in isoflurane-exposed mice was reduced compared to control conditions and is increased with rapamycin treatment. For this analysis, the same exact photo setting was performed for all groups. *Asterisk* in photos: CA3 of hippocampus; fi: fimbria of hippocampus; LV: lateral ventricle. *Scale bar* = 200 μm. (*B*) Western blot data indicated expression of myelin basic protein was decreased by isoflurane exposure and then elevated by rapamycin treatment. (*C*) Electron microscopy analysis was performed in fimbria parasagittal ultrathin sections. The ratio of axonal caliber over diameter of myelinated fiber (g-ratio) was increased in isoflurane-exposed animals (it means decreased myelin thickness) compared to control, and rapamycin treatment reversed this change. *Scale bar* = 0.5 μm. n = 8 for each group in *A* and *B*, and n=100 for each group in *C*. One-way ANOVA; *$P < 0.05$; **$P < 0.01$. *Error bars*: SD.

and a partial recovery results from rapamycin treatment (49 ± 9% ratio; $P = 0.006$; fig. 6B). In order to determine whether changes in DNA methyltransferase 1 levels have functional significance, we assayed levels of 5–methylcytosine, which is a product of DNA methyltransferase 1–mediated DNA methylation, in oligodendrocytes. Isoflurane exposure decreased the ratio of 5–methylcytosine–positive nuclei over adenomatous polyposis coli–labeled oligodendrocytes (33 ± 13% ratio) relative to control (52 ± 13% ratio; $P = 0.006$), and rapamycin treatment reversed this decrease (48 ± 7% ratio; $P = 0.031$; fig. 6C).

Discussion

In this study, we report that early postnatal exposure to isoflurane in mice causes a substantial disruption of oligodendrocyte development and myelination in fimbria of the hippocampus, which is the predominant bundle of efferent axonal fibers from the hippocampus. Proliferation and differentiation of oligodendrocyte progenitor cells are chronically impaired by early isoflurane exposure, as is the formation of synaptic connections between oligodendrocyte progenitor cells and axons. This results in a measurable loss of myelin in the fimbria. Proper connections and communications between hippocampus and the neocortex are critical for performing the cognitive and psychologic functions.[14,31,32] Myelination is essential in establishing connectivity in the growing brain by facilitating rapid and synchronized information transfer across the nervous system. Once thought of as solely a passive insulator, myelin is now understood to be actively involved in the function and
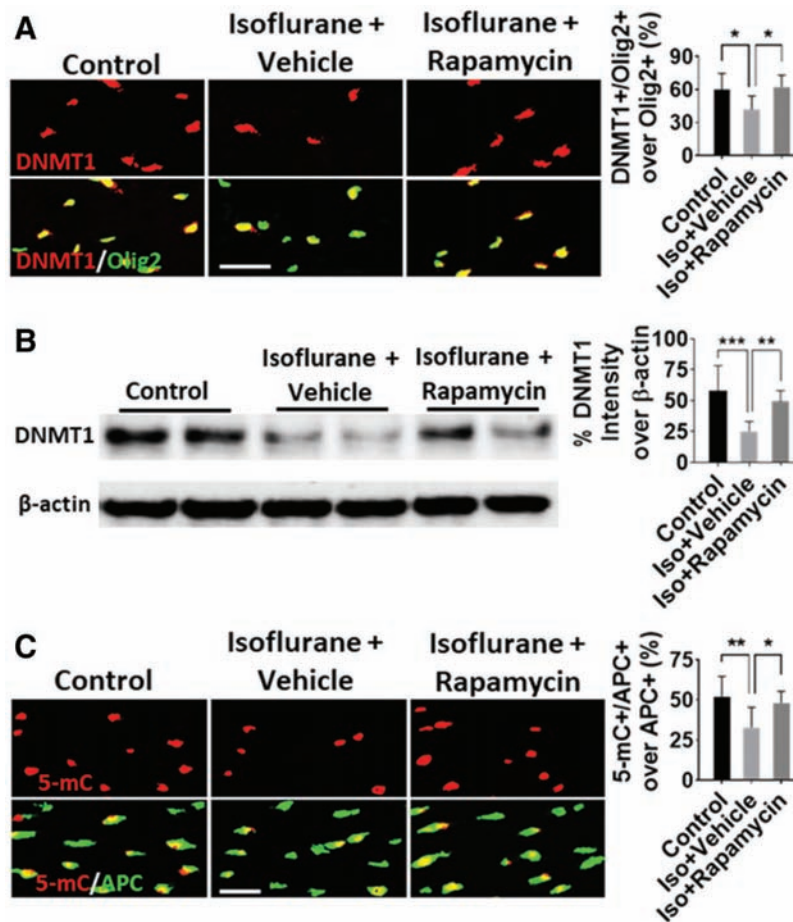
Fig. 6. The effect of isoflurane (Iso) exposure and rapamycin treatment on DNA methylation level in oligodendrocytes. (*A*) DNA methylation level examined with DNA methyltransferase 1 and Olig2 double immunolabeling. The percentage of DNA methyltransferase 1+/Olig2+ over Olig2+ nuclei in isoflurane-exposed mice was lower than control, and rapamycin increased this ratio. *Scale bar* = 25 μm. (*B*) Western blot data revealed isoflurane dramatically decreased the DNA methyltransferase 1 level, and rapamycin increased DNA methyltransferase 1. (*C*) 5-Methylcytosine (5-mC), the product of DNA methylation catalyzed by DNA methyltransferase 1, was detected in nuclei of adenomatous polyposis coli (APC)–positive oligodendrocytes. The ratio of 5-methylcytosine–positive/adenomatous polyposis coli–positive over adenomatous polyposis coli–positive cells showed a statistically significant decrease with isoflurane exposure and rapamycin injection reversed this decrease. *Scale bar* = 25 μm. n = 8 for each group; one-way ANOVA; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. *Error bars*: SD.

development of the CNS.[33] Abnormal myelination of axons disrupts the communications between brain regions, and it has been reported that myelin deficits in the hippocampus cause cognitive and psychologic disorders.[34–36] Previous studies have shown an acute increase in apoptosis of oligodendrocytes, the myelin-forming glial cells, with early exposure to isoflurane,[37,38] but our findings demonstrate a lasting effect of anesthesia on oligodendrocyte proliferation and differentiation that results in a decrease in myelination in the hippocampal fimbria.

The process of oligodendrocyte development occurs based on an intrinsic program that is modulated by neurotransmitters and electrical activity in the CNS.[39–41] Axonal terminals release glutamate as a transmitter not only at axonal terminals but also at discrete sites along axons in white matter.[42] By acting on α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) or *N*-methyl-D-aspartate (NMDA) receptors expressed on oligodendrocyte progenitor cells, glutamate increases the downstream phosphorylation of the cyclic adenosine monophosphate response element–binding protein and release of calcium from intracellular stores,[43] thereby promoting oligodendrocyte progenitor cell proliferation and differentiation. As an agonist of γ-aminobutyric acid type A and glycine receptors,[44] as well as a potential NMDA inhibitor,[45] isoflurane suppresses excitatory neurotransmission.[46] It raises the possibility that a profound direct action on oligodendrocyte progenitor cells, which express both γ-aminobutyric acid and NMDA receptors, is caused by isoflurane during a critical period in development and that this may alter the developmental

program, thus resulting in deficits in myelination. An alternative or complementary explanation may be an indirect effect mediated by the electrochemical synapses that occur between axonal terminals and oligodendrocyte progenitor cells (axon–oligodendrocyte progenitor cell synapses). Formation of glutamatergic axon–oligodendrocyte progenitor cell synapses plays an important role in promoting activity-dependent oligodendrocyte development and maintenance.[21–23] The chronic, lasting effects of isoflurane on neuronal synapses that we have previously shown may translate into reduced activity at axon–oligodendrocyte progenitor cell synapses, thus leading to suppression of oligodendrocyte progenitor cell development. A previous study showed that plasticity of axon–oligodendrocyte progenitor cell synapses is highly dependent on electrical activity.[21] The data in this study further confirm that exposure of isoflurane reduces the number of excitatory (vesicular glutamate transporter 1–positive) axon–oligodendrocyte progenitor cell synapses in hippocampus, which is likely a key mechanism of general anesthetic–induced hypomyelination.

Our previous work has indicated that exposure to isoflurane disrupts the development of hippocampal neurons generated in the early postnatal period by inappropriately increasing activity in the mTOR pathway, and we found that both behavioral and histological changes could be reversed by pharmacologic mTOR inhibition.[7] In the present study, as in neurons, we observe a lasting alteration in the tone of mTOR signaling in oligodendrocytes in the hippocampal fimbria for a protracted period after isoflurane exposure, which appears to be integral to the developmental disruption. We found a substantial improvement in phenotype with rapamycin treatment in this study as well. The mTOR pathway is an intracellular signaling pathway that regulates cellular activities including proliferation, differentiation, apoptosis, metabolism, transmitter release, and other biologic processes.[18] In the past decade, many studies have implicated mTOR signaling in CNS developmental and neuropsychiatric disorders.[19] Two structurally and functionally distinct mTOR-containing complexes have been identified in oligodendrocytes. The first, mTOR complex 1 mTOR, contains the adaptor protein Raptor, which influences myelin basic protein expression *via* an alternative mechanism and is sensitive to the drug rapamycin. The second complex, mTOR complex 2, contains Ritor, and it is thought to control myelin gene expression at the mRNA level and is relatively rapamycin-insensitive.[47]

The mTOR pathway itself plays a complex role in myelination; mTOR activity can either enhance or suppress oligodendrocyte development depending on the context. A study using a mouse line with oligodendrocyte-specific knockdown of mTOR in CNS has provided evidence that mTOR is essential for oligodendrocyte development and myelination.[48] Inhibition of mTOR *via* rapamycin in cultured adult oligodendrocyte progenitor cells or in a mouse model starting at 6 weeks of age results in oligodendrocyte differentiation deficits along with reduced expression of major myelin proteins and mRNAs.[49–51] In contrast, activation of mTOR induced by tuberous sclerosis complex–1 or –2 gene mutations in early oligodendrocyte progenitor cells caused white matter abnormalities, including myelin deficits in CNS.[52–54] A bidirectional action of the phosphoinositide 3-kinase-protein kinase B-mTOR (PI3K-Akt-mTOR) axis in myelination has also been reported in studies of the peripheral nervous system. If tuberous sclerosis complex–1 deletion occurs in early developmental stages in Schwann cells, mTOR hyperactivity arrests the process by which Schwann cells ensheathe axons. If mTOR activity is increased in Schwann cells after they have begun wrapping around axons, there is actually an increase in myelination.[55] Thus, oligodendrocyte development and myelination may be dependent on precise balance and timing in mTOR signaling. Either increasing or decreasing levels of mTOR complex 1 activity interferes with oligodendrocyte differentiation and causes potentially causes hypomyelination.[56]

While we do not yet have a clear picture of how changes in mTOR activity act on oligodendrocytes, our data indicate changes in DNA methylation as a promising direction. Recent work shows developmental anesthetic toxicity may involve epigenetic modulation[57] and that DNA methylation plays an important role in regulating oligodendrocyte progenitor cell proliferation and differentiation.[26–28] Intriguingly, mTOR signaling has been shown to negatively regulate DNA methylation *via* an action on DNA methyltransferase 1,[24,25] suggesting a possible connection between mTOR signaling and oligodendrocyte development. This finding is consistent with our data showing that isoflurane exposure and concomitant increases in mTOR signaling lead to a decrease in DNA methyltransferase 1 expression and DNA methylation in developing oligodendrocytes, and that both of these changes are reversible with rapamycin treatment.

We propose that oligodendrocytes should be further studied both as a potential target in anesthetic neurotoxicity and as a model system in which to further explore the interplay of anesthetics, mTOR signaling, and DNA methylation. Our work is limited by the rodent model, which has well-known confounds related to anesthetic administration in very young, small mice in which physiologic monitoring and control of respiratory function are challenging. In particular, we have chosen to use 100% oxygen as a carrier for isoflurane, which has the beneficial effect of preventing hypoxia in our model system, but which raises the possibility of a combined effect of isoflurane and hyperoxia damage that cannot be fully controlled for in our experimental model. While it is indeed the case that supplemental oxygen is frequently used in pediatric anesthesia practice, it would be ideal to avoid confounds presented by hyperoxia and other physiologic issues *via* studies in large animal models and in cell culture models, and we hope further work in this area will be undertaken in these systems.

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ANESTHESIOLOGY

Intergenerational Effects of Sevoflurane in Young Adult Rats

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Exposure to environmental stressors and endocrine disruptors can induce multigenerational effects resulting in neurobehavioral and other abnormalities in the offspring
- Early-life anesthesia exposure in rodents alters neurocognitive function in their offspring, but whether exposure of adult animals affects offspring has not been previously reported

What This Article Tells Us That Is New

- Repeated exposures of adult rats to sevoflurane (2.1%, three times, 3 h on every second day) induce neurobehavioral abnormalities in the exposed males and in male but not female progeny
- The neurobehavioral abnormalities in male offspring are accompanied by increased methylation and decreased expression of the potassium ion-chloride ion cotransporter Kcc2 gene that regulates neuronal chloride homeostasis, and, thereby, the functional modalities of γ-aminobutyric acid type A receptor–mediated neurotransmission
- Sevoflurane exposure also induces hypermethylation of the Kcc2 gene in both male and female parental germ cells
- These observations suggest that epigenetic reprogramming of parental germ cells is involved in transmitting the adverse effects of sevoflurane exposure of adult rats to their male progeny

ABSTRACT

Background: Sevoflurane administered to neonatal rats induces neurobehavioral abnormalities and epigenetic reprogramming of their germ cells; the latter can pass adverse effects of sevoflurane to future offspring. As germ cells are susceptible to reprogramming by environmental factors across the lifespan, the authors hypothesized that sevoflurane administered to adult rats could induce neurobehavioral abnormalities in future offspring, but not in the exposed rats themselves.

Methods: Sprague-Dawley rats were anesthetized with 2.1% sevoflurane for 3 h every other day between postnatal days 56 and 60. Twenty-five days later, exposed rats and nonexposed controls were mated to produce offspring.

Results: Adult male but not female offspring of exposed parents of either sex exhibited deficiencies in elevated plus maze (mean ± SD, offspring of both exposed parents *vs.* offspring of control parents, 35 ± 12 *vs.* 15 ± 15 s, $P < 0.001$) and prepulse inhibition of acoustic startle (offspring of both exposed parents *vs.* offspring of control parents, 46.504 ± 13.448 *vs.* 25.838 ± 22.866%, $P = 0.009$), and increased methylation and reduced expression of the potassium ion-chloride ion cotransporter KCC2 gene (*Kcc2*) in the hypothalamus. *Kcc2* was also hypermethylated in sperm and ovary of the exposed rats. Surprisingly, exposed male rats also exhibited long-term abnormalities in functioning of the hypothalamic-pituitary-gonadal and -adrenal axes, reduced expression of hypothalamic and hippocampal *Kcc2,* and deficiencies in elevated plus maze (sevoflurane *vs.* control, 40 ± 24 *vs.* 25 ± 12 s, $P = 0.038$) and prepulse inhibition of startle (sevoflurane *vs.* control, 39.905 ± 21.507 *vs.* 29.193 ± 24.263%, $P < 0.050$).

Conclusions: Adult sevoflurane exposure affects brain development in male offspring by epigenetically reprogramming both parental germ cells, while it induces neuroendocrine and behavioral abnormalities only in exposed males. Sex steroids may be required for mediation of the adverse effects of adult sevoflurane in exposed males.

(*ANESTHESIOLOGY* 2019; 131:1092–109)

The U.S. Food and Drug Administration recommends avoiding anesthesia in children under 3 yr old whenever possible, as it may negatively affect their brain development.[1] This recommendation is based largely on an earlier body of work in animals, particularly rodents, showing that neonates are especially susceptible to lasting adverse effects of anesthesia during the first 2 postnatal weeks.[2] In rodents, the first 2 weeks of life are characterized by unique physiologic properties, such as excitatory signaling through γ-aminobutyric acid type A (GABA$_A$) receptors,[3,4] a major substrate for the inhibitory (sedative) effects of γ-aminobutyric acid–ergic (GABAergic) anesthetics in the more mature brain.[5–7] GABA$_A$ receptor signaling is excitatory during early life due to elevated levels of intraneuronal Cl$^-$, maintained by relatively low and high levels of the potassium ion–chloride ion cotransporter KCC2 and sodium ion–potassium ion–chloride ion cotransporter NKCC1, respectively.[3,4] During the second postnatal week in rats, GABA$_A$ receptor signaling gradually becomes inhibitory, primarily due to age-dependent increases in KCC2.[3,4] The magnitude of GABA$_A$ receptor excitatory signaling and the proper timing of its transition from excitatory to inhibitory are key for normal brain development, and impairments in KCC2 and shifts in GABA$_A$ receptor signaling toward excitation have been

linked to neuropsychiatric disorders in both humans and animal models.[8,9] It appears that exposure to GABAergic anesthetics early in life may induce developmental abnormalities at least in part by enhancing the magnitude of $GABA_A$ receptor excitatory signaling at the time of anesthesia, and by disrupting normal developmental increase in *Kcc2* expression.[10–12] In support of this contention, exposure of neonatal rats to GABAergic anesthetics, such as sevoflurane, causes electroencephalography-detectable epileptic seizures and multifold increases in corticosterone secretion at the time of anesthesia.[12] After maturing to adulthood, these anesthetic-exposed rats have downregulated *Kcc2* levels, exacerbated hypothalamic-pituitary-adrenal axis responses to stress, and behavioral abnormalities.[10,11] Inhibition of NKCC1 at the time of anesthesia ameliorates acute seizure activities, as well as many of the lasting developmental effects of GABAergic anesthetics,[11,12] suggesting that anesthetic-exacerbated $GABA_A$ receptor–mediated excitation in neonatal brain is an important initiating step in anesthetic-induced neurobehavioral abnormalities in the exposed animals.

Surprisingly, neonatal parental sevoflurane also impairs *Kcc2* expression in the brains of future male offspring by initiating DNA methylation at the 5′ position of cytosine residues adjacent to guanines in the *Kcc2* gene in parental gamete cells.[10] These findings suggest that neonatal sevoflurane can induce two distinct types of adverse effects in the exposed animals—excitatory $GABA_A$ receptor signaling–dependent effects in the exposed neonatal brain and epigenetic reprogramming of their germ cells, which can pass adverse effects of neonatal parental sevoflurane to future unexposed offspring.[10] Epidemiologic studies and research in animal models suggest that parental germ cells can be susceptible to reprogramming by environmental factors across the lifespan.[13–15] Therefore, here we tested the hypothesis that sevoflurane can affect future offspring even when administered to adult rats. We further hypothesized that the primarily inhibitory $GABA_A$ receptor signaling in the brain of young adult rats[3,16] renders them resistant to the adverse neuroendocrine and behavioral effects of sevoflurane.

Materials and Methods

Animals

All experimental procedures were approved by the University of Florida Institutional Animal Care and Use Committee (Gainesville, Florida). Sprague-Dawley rats were bred at the University of Florida animal care facility. The rats were housed under controlled illumination (12-h light–dark, lights on at 7:00 AM) and temperature (23 to 24°C) with free access to food and water. Within 24 h of delivery, litters were culled to 12 pups. At the age of 21 days, pups were weaned and housed in sex-matched pairs for the remainder of the study.

Treatment Groups

Male and female rats (generation F0) were randomized for treatment and breeding pairs using a randomization plan generator (http://www.randomization.com/), and the investigators were blind to group assignments. The F0 rats underwent anesthesia on postnatal days 56, 58, and 60. The rats were held in a temperature-controlled chamber to maintain body temperature at +37°C with a continuous supply of 30% oxygen in air (1.5 l/min) during anesthesia, which was induced by 6% sevoflurane for 3 min followed by 2.1% sevoflurane for 177 min for anesthesia maintenance (the Sevoflurane group). Gas monitoring was performed using a calibrated Datex side stream analyzer (Datex-Ohmeda, Finland), which sampled from the animal chamber interior. Rats in the F0 control group (Control group) were not subjected to anesthesia. A subset of the rats in the F0 Control and Sevoflurane groups was sacrificed 1 h after the last exposure to sevoflurane (or equivalent timepoint in the Control group) to collect blood and brain tissue samples to measure serum levels of corticosterone and to evaluate expression of KCC2 in the paraventricular nucleus of the hypothalamus (see fig. 1 for schematic of experimental design). The remaining Control and Sevoflurane F0 male and female rats were mated on postnatal day 85 to produce offspring (generation F1). Only rats from different litters were mated. F1 rats were categorized as the offspring of (1) control males and control females; (2) exposed males and control females; (3) control males and exposed females; and (4) exposed males and exposed females. The females were housed individually throughout the entire gestation and postpartum rearing periods.

Sixty-four F0 rats (32 control and 32 sevoflurane-exposed) were used as breeders to produce 122 F1 rats, which were not exposed to sevoflurane anesthesia and were subjected to animal facility rearing only. The F0 rats were sequentially evaluated in the elevated plus maze starting on postnatal day ~125 and prepulse inhibition of the acoustic startle response on postnatal day ~135 (fig. 1). A subset of these animals was sacrificed on postnatal day ~160 to collect trunk blood, brain, and testis and ovarian tissues for further analyses. The rest of these animals were physically restrained for 30 min on postnatal day ~160 to measure corticosterone responses, followed by collection of tissue samples for further analyses. The F1 rats were evaluated in the elevated plus maze starting on postnatal day ~60 and prepulse inhibition of startle on postnatal day ~70 (fig. 1). One half of these animals were sacrificed on postnatal day ~95 to collect trunk blood and brain tissue samples for further analyses, and the rest of these animals were tested for the corticosterone responses to physical restraint for 30 min on postnatal day ~95 before collecting the brain tissue samples.

Behavioral Tests

Assessment of Behavior in the Elevated Plus Maze. The elevated plus maze studies were performed using an elevated plus maze
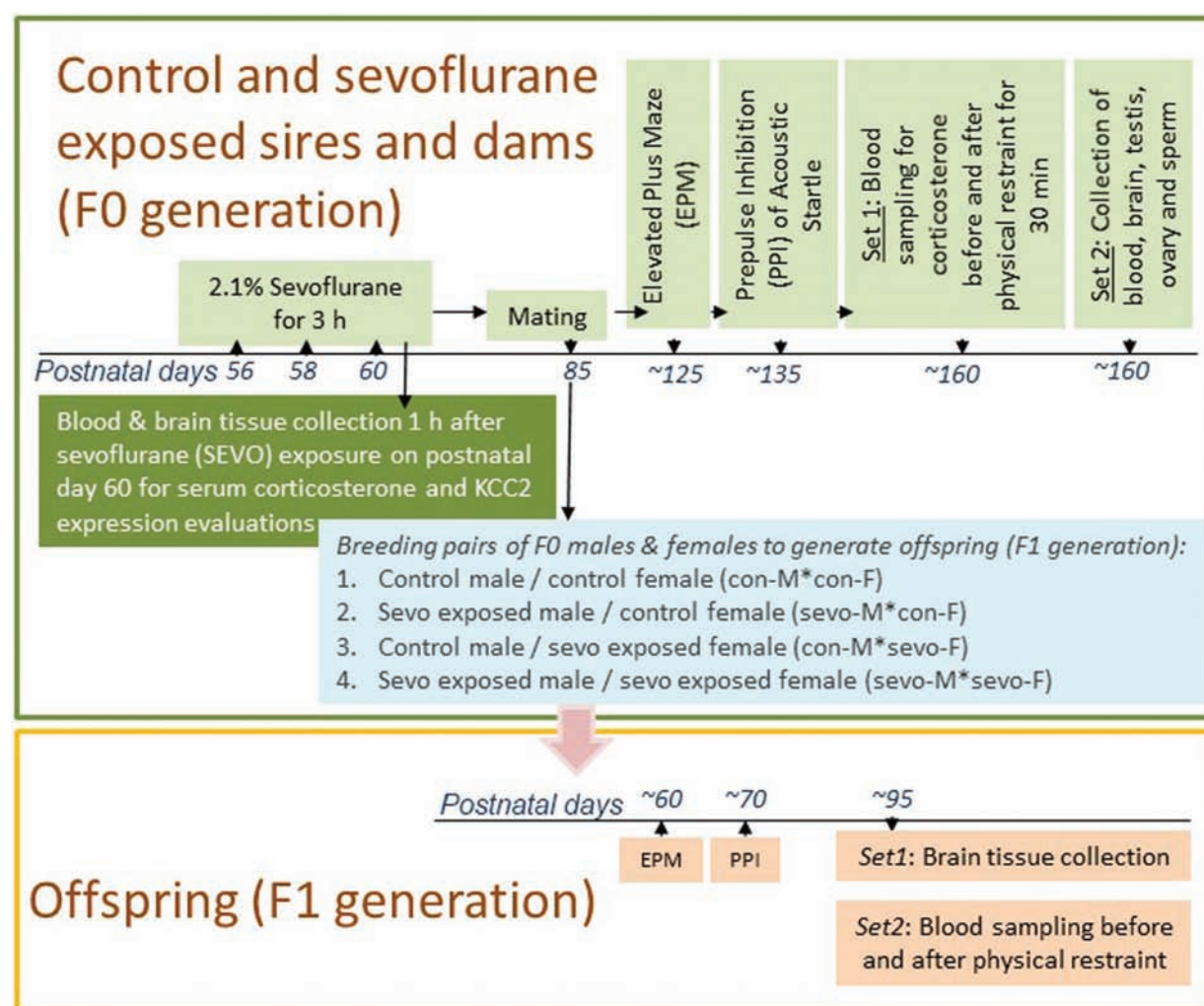
Fig. 1. Study design.

apparatus and BIO-EPM 3C video tracking software (EB Instruments, USA) during the light phase of the dark–light cycle as previously described by our laboratory.[10,12,17] If a fall occurred, the animal was removed from the study (one F0 male rat was removed from the study for this reason).

Assessment of Prepulse Inhibition of Startle. The prepulse inhibition of startle tests were performed using a SR–Lab startle apparatus (San Diego Instruments, USA) as previously described by our laboratory.[10,12,17] The percentage of prepulse inhibition of startle for each prepulse level was calculated using the following formula: % prepulse inhibition of startle = 100 x [(pulse alone) − (prepulse + pulse)]/pulse alone. Data were collected as the highest voltage during the response window amplitude.

Basal and Stress-induced Activity of the Hypothalamic-Pituitary-Adrenal Axis. Blood samples (~300 μl) were collected using the "tail clip" method at rest and 10, 60, and 120 min after the restraint. Physical restraint was administered using

rodent holders (Kent Scientific Corporation, USA). Serum corticosterone was measured using commercial ELISA kits (Cayman Chemical Company, USA) according to the manufacturer's instructions.

Tissue Collection. After decapitation, the trunk blood samples were collected and centrifuged at 4°C, 1,000 g (where g is the relative centrifugal force) for 15 min, and then kept at −80°C for hormone assays. The brain was removed from the skull onto ice pads. The hypothalamus was isolated by making an anterior cut at the level of the optic chiasm, a posterior coronal section anterior to the mammillary bodies, two sagittal cuts parallel to the lateral ventricles, and a dorsal horizontal cut at the level of the anterior commissure, as described previously.[18] The hippocampus was isolated from the respective blocks. All brain tissue samples were placed in vials filled with RNAlater solution (Invitrogen, USA) and then stored at −80°C. Testis tissue was removed and washed with 0.9% saline before weighing. Sperm were isolated from

the caudal epididymis of adult males into phosphate-buffered saline with 1% bovine serum albumin solution at 37°C using a swim-up assay. After settling for 30 min, sperm-containing supernatant was centrifuged for 5 min at 4,000 rpm. Sperm pellets were stored at −80°C. After separation from the adipose tissues, ovaries were stored at −80°C.

Hormonal Measurements. Hormone analyses in serum samples isolated from F0 and F1 rats were performed using commercially available kits according to the manufacturer's instructions. Serum concentrations of follicle stimulating hormone (CSB-E06869r) and luteinizing hormone (CSB-E12654r) were quantified using ELISA kits (Cusabio Technology LLC, USA). Serum testosterone and estradiol concentrations were measured using ELISA kits (582701, Cayman Chemical Company, USA, and ES180S-100, Calbiotech, USA, respectively).

Quantitative mRNA Measurements. Levels of mRNA for *Nkcc1* and *Kcc2* in the hypothalamus and hippocampus, for corticotropin-releasing hormone, estrogen receptor α, estrogen receptor β, aromatase, gonadotropin-releasing hormone in the hypothalamus, and for glucocorticoid receptor in the hippocampus were analyzed *via* reverse transcription–polymerase chain reaction in a StepOnePlus Real-Time polymerase chain reaction System (Applied Biosystems, USA) as previously described by our laboratory.[10] RNA was extracted from the samples using an RNeasy Plus Kit (Qiagen, USA), reverse-transcribed with a high-capacity complementary DNA reverse transcription kit (Bio-Rad Laboratories, USA), and then analyzed *via* reverse transcription–polymerase chain reaction. Taqman probes specific for the genes described at the beginning of this section were obtained from Applied Biosystems: corticotropin-releasing hormone (Rn01462137_m1), glucocorticoid receptor (Rn00561369_m1), estrogen receptor α (Rn01430446_m1), estrogen receptor β (Rn00562610_m1), aromatase (Rn00567222_m1), gonadotropin-releasing hormone (Rn00562754_m1), *Nkcc1* (Rn00582505_m1), and *Kcc2* (Rn00592624_m1). Data were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA (Rn01775763_g1). Gene expression was calculated using the "double delta Ct" method for relative quantification, and data are presented as relative fold change from that of control animals.

Bisulfite Sequencing. Genomic DNA was extracted from the sperm pellet and ovaries of adult F0 rats and from hippocampal and hypothalamic tissues of F1 rats using the DNeasy Blood and Tissue kit (Qiagen, Germany). The sodium bisulfite conversion was performed with EZ DNA Methylation kits (Zymo Research, USA) following the manufacturer's instructions. The primers (*Kcc2*: forward: GATTGTAAGTGTTTTTATTATTGAGTTGTATATT; reverse: AATAAACTTTTCCCCTTTTATACCC) were designed for the bisulfite-converted DNA sequences, using previously published sequences.[10] Polymerase chain reaction amplification was performed with HotStar Taq

(Qiagen, Germany). Amplicons were cloned into pCR4-TOPO vector with the TOPO TA cloning kit for sequencing (Life Technologies, USA). Miniprep was performed on each positive clone using ZR Plasmid Miniprep kit (Zymo Research, USA). Sanger sequencing was done by Genewiz (USA) using M13R primers. The DNA methylation status of all 5′ position of cytosine residues adjacent to guanines sites was analyzed using Benchling Molecular Biology 2.0 Software (Benchling, USA). The bisulfite sequencing analysis of the *Kcc2* DNA was performed only in F1 male progeny of control males and control females and exposed males and exposed females matings.

Immunohistochemistry. Rats were anesthetized with sevoflurane and transcardially perfused with saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were collected and fixed in the 4% paraformaldehyde overnight and then dehydrated in 30% sucrose solution in phosphate-buffered saline at 4°C for 2 days. The brains were cut into 18-μm-thick coronal sections using a cryostat. After blocking with 10% normal goat serum for 1 h at room temperature, the slices were incubated with the primary antibody (rabbit anti-KCC2, 1:500; MilliporeSigma, USA) in 10% normal goat serum at 4°C overnight. After washing with PBS for 3 × 5 min, the slices were exposed to the secondary antibody, Alexa Fluor 549 goat anti-rabbit (Invitrogen, USA). Slides were then washed with phosphate-buffered saline for 3 × 5 min and incubated for 10 min at room temperature with 4′,6-diamidine-2′-phenylindole dihydrochloride (1:1,000; Sigma, USA) in phosphate-buffered saline for 10 min. The sections were mounted and covered with a coverslip after washing with phosphate-buffered saline for 3 × 5 min. A confocal microscope (IX2-DSU spinning disk confocal fluorescent microscope system, Olympus, Japan) was used to capture the fluorescent images. The immunofluorescence intensity of the KCC2-immunoreactive cells was measured using ImageJ software (National Institutes of Health, USA). Three images from the paraventricular nucleus of the hypothalamus of each animal were taken, and the average intensity values were calculated to use for statistical analysis.

Statistical Analysis

Statistical analyses were conducted on raw data using SigmaPlot 14.0 software (Systat Software, Inc., USA), which automatically checks if a data set meets test criteria (Shapiro–Wilk for normality test and Brown–Forsythe for Equal Variance Test). Values are reported as mean ± SD. The primary outcome in this study was the neurobehavioral changes in offspring of rats exposed to sevoflurane in young adulthood. All other outcome measures were the secondary outcomes. One F0 male rat was removed from the elevated plus maze study because of a fall from the maze. Another F0 male rat in the control group was removed from the behavioral studies because of injury while mating with a female rat. Boxplots were used to identify outliers. No outliers were detected that

were in not plausible range of values for the outcome (indicating measurement or recording error). Therefore, all values were maintained in analyses. An independent t test was used to analyze F0 data for serum corticosterone levels and $Kcc2$ expression 1 h after the last exposure to sevoflurane, elevated plus maze, testis weight, hormone levels for testosterone, estradiol, luteinizing hormone, and follicle-stimulating hormone and gene expression for corticotropin-releasing hormone, glucocorticoid receptor, estrogen receptor α, estrogen receptor β, aromatase, gonadotropin-releasing hormone, $Nkcc1$, and $Kcc2$. All comparisons were run as two-tailed tests. To analyze F1 data for elevated plus maze, testis weight, testosterone level, and gene expression for corticotropin-releasing hormone, glucocorticoid receptor, $Nkcc1$, and $Kcc2$, one-way ANOVA was used. A two-way repeated-measures ANOVA was used to analyze the prepulse inhibition of startle data, with the treatment and prepulse intensity as independent variables. A two-way repeated-measures ANOVA was used to analyze changes in serum corticosterone levels at rest and at three time points after the restraint, with experimental groups and time as the independent variables. To assess differences in total corticosterone concentrations, area under the curve with respect to baseline (levels of corticosterone at rest), was calculated and compared across experimental groups using one-way ANOVA. Two-way repeated measures ANOVA with treatment as between-subject factor and the 5′ position of cytosine residues adjacent to guanines site as within-subject factor was used to analyze data on the frequency methylation of 5′ position of cytosine residues adjacent to guanines sites in the $Kcc2$ gene promoter in F0 and F1 tissue samples. An independent t test was used to analyze DNA methylation level at all six 5′ position of cytosine residues adjacent to guanines sites. All multiple pairwise comparisons were done with the Holm–Sidak method. $P \leq 0.05$ was considered statistically significant. Statistical details are presented in text and in figure legends. The sample sizes in this study were based on previous experience with the same experimental techniques.[10,11,17] This study was not designed to detect an anesthetic × sex interaction.

Results

Systemic Abnormalities in Male Offspring of Rats Exposed to Sevoflurane in Young Adulthood

In the elevated plus maze test, there was a statistically significant between-subjects effect of young adult parental exposure to sevoflurane on time spent in open arms (F[3,58] = 8.514, $P < 0.001$; fig. 2A) in F1 males. Specifically, F1 male progeny of sevoflurane exposed fathers and unexposed mothers ($P = 0.040$), unexposed fathers and exposed mothers ($P < 0.001$), and exposed fathers and exposed mothers ($P < 0.001$) spent less time in the open arms compared to F1 male offspring of control fathers and control mothers. Also, there were between-subjects effects of parental exposure to sevoflurane on number of crossings

(F[3,58] = 4.657, $P = 0.006$; fig. 2B) and distance traveled (F[3,58] = 6.288, $P < 0.001$; fig. 2C) during the elevated plus maze test. Only F1 male offspring of both exposed parents made fewer crossings ($P = 0.003$) and traveled shorter distances ($P = 0.001$). All groups of F1 females were similar in respect to time spent in open arms (fig. 2D), number of crossings (fig. 2E), and distance traveled (fig. 2F) during the elevated plus maze test.

There was a statistically significant effect of young adult parental sevoflurane exposure on prepulse inhibition of startle responses in F1 male rats (F[3,174] = 7.590, $P < 0.001$; fig. 2G). Multiple pairwise comparisons indicated that when compared to offspring of unexposed parents, only F1 males of both exposed parents ($P = 0.009$) or those of control fathers and exposed mothers ($P = 0.027$) exhibited reduced prepulse inhibition of startle at prepulse of 3 decibel. In contrast to F1 males, there was no treatment effect on prepulse inhibition of startle in F1 female rats (F[3,168] = 0.584, $P = 0.627$; fig. 2H). The startle amplitudes were similar among all experimental groups of F1 male (F[3,58] = 1.991, $P = 0.125$) and F1 female (F[3,56] = 0.514, $P = 0.674$) rats.

Measurements of serum levels of corticosterone in the postnatal day ~95 F1 male and female rats before and after physical restraint revealed no differences among experimental groups of the same sex (F[3,84] = 0.335, $P = 0.800$, males; fig. 2, I and J; and F[3,84] = 0.142, $P = 0.934$, females; fig. 2, K and L).

Reduction in the Potassium Ion-Chloride Ion Cotransporter KCC2 Gene ($Kcc2$) Expression in Male Offspring of Rats Exposed to Sevoflurane in Young Adulthood

There were similar levels of $Nkcc1$ mRNA in the hypothalamus of all four treatment groups of the postnatal day ~95 F1 male rats (F[3,20] = 0.928, $P = 0.446$; fig. 3A), but there was a between-subjects effect of parental sevoflurane exposure on hypothalamic $Kcc2$ mRNA levels (F[3,20] = 5.636, $P = 0.006$; fig. 3B). Specifically, F1 male progeny of sevoflurane-exposed fathers and mothers had lower levels of $Kcc2$ mRNA when compared to F1 male offspring of control fathers and control mothers ($P = 0.012$). In the hypothalamus of F1 females, there were not between-subjects effects of parental sevoflurane exposure on $Nkcc1$ mRNA levels (F[3,19] = 0.886, $P = 0.466$; fig. 3C) or $Kcc2$ mRNA levels (F[3,20] = 0.738, $P = 0.542$; fig. 3D).

In the hippocampus of F1 males, there were no effects of parental sevoflurane exposure on $Nkcc1$ mRNA levels (F[3,20] = 1.357, $P = 0.284$; fig. 3E), but there was a significant effect on $Kcc2$ mRNA levels (F[3,20] = 41.375, $P < 0.001$; fig. 3F). Only male offspring of both exposed parents ($P < 0.001$) and offspring of exposed fathers and control mothers ($P < 0.001$) had reduced hippocampal $Kcc2$ mRNA levels compared to offspring of two control parents. In the hippocampus of F1 females, there were not significant between-subjects
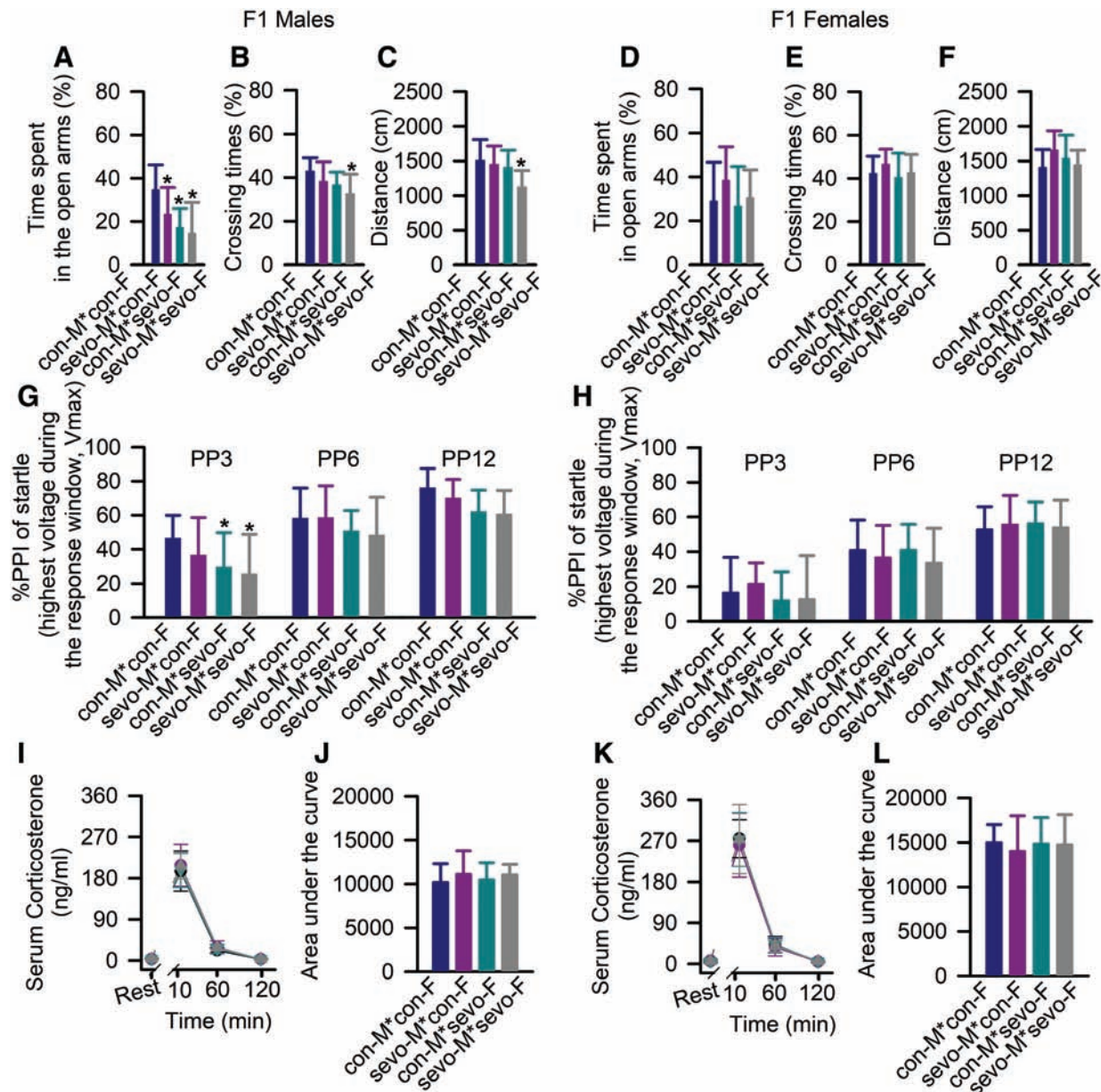
Fig. 2. Systemic second-generation (F1) effects of young adult parental exposure to sevoflurane. (*A–F*) Time spent in open arms of the elevated plus maze, number of crossing the open arms, and distance traveled by male (*A–C*) and female (*D–F*) offspring. F1 rats were categorized as the offspring of (1) control males plus control females (con-M*con-F); (2) exposed males plus control females (sevo-M*con-F); (3) control males plus exposed females (con-M*sevo-F); and (4) exposed males plus exposed females (sevo-M*sevo-F). Data are means ± SD from 15 or 16 rats per group. *$P < 0.05$ *versus* con-M*con-F. (*G* and *H*) Percentage of prepulse inhibition of startle responses at prepulse (PP) intensity of 3, 6, and 12 decibel in male (*G*) and female (*H*) offspring. Data are means ± SD from 14–16 rats per group. *$P < 0.05$ *versus* con-M*con-F. (*I–L*) Plots showing the respective levels of serum corticosterone across each collection point, as well as the total corticosterone responses in male (*I* and *J*) and female (*K* and *L*) offspring. Serum levels of corticosterone at rest were taken as baselines for calculations of the total corticosterone responses. Data are means ± SD from eight rats per group. Multiple pairwise comparisons were done with the Holm–Sidak method. Color coding of experimental groups in *J* and *L* is also applicable to *I* and *K*.

effects of parental neonatal sevoflurane exposure on *Nkcc1* mRNA level (F[3,20] = 0.925, $P = 0.447$; fig. 3G) or *Kcc2* mRNA level (F[3,20] = 0.524, $P = 0.671$; fig. 3H).

Consistent with the observations of normal levels of corticosterone (fig. 2, I and J), levels of corticotropin-releasing hormone mRNA in the hypothalamus (F[3,20] = 0.519,
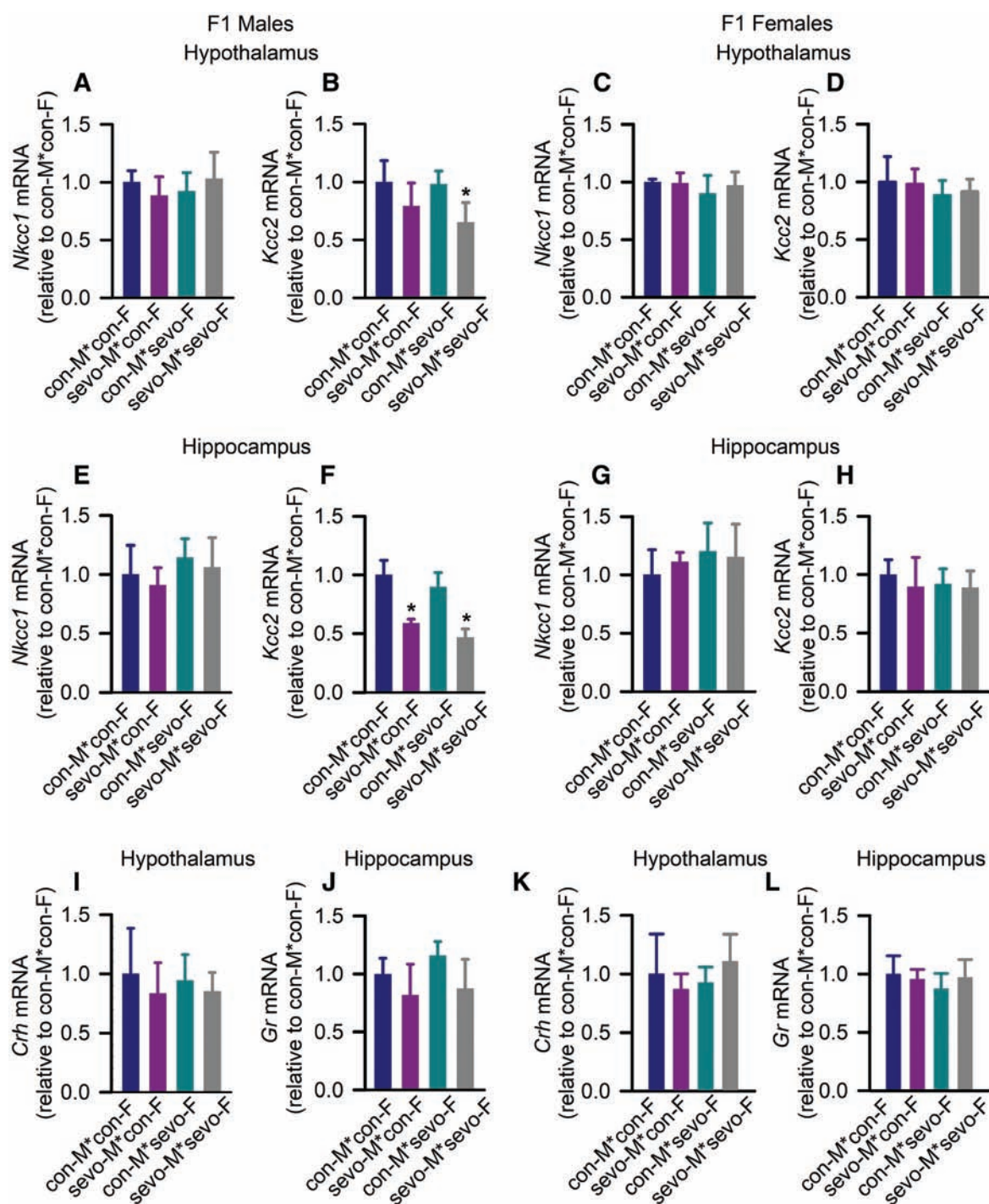
Fig. 3. Molecular second-generation (F1) effects of young adult parental exposure to sevoflurane. (*A–H*) The respective levels of sodium ion-potassium ion-chloride ion cotransporter NKCC1 gene (*Nkcc1*) mRNA and potassium ion-chloride ion cotransporter KCC2 gene (*Kcc2*) mRNA in the hypothalamus of F1 males (*A* and *B*) and F1 females (*C* and *D*) and in the hippocampus of F1 males (*E* and *F*) and F1 females (*G* and *H*). F1 rats were categorized as the offspring of (1) control males plus control females (con-M*con-F); (2) exposed males plus control females (sevo-M*con-F); (3) control males plus exposed females (con-M*sevo-F); and (4) exposed males plus exposed females (sevo-M*sevo-F). Data normalized against control are means ± SD from 6 rats per group (n = 5, female hypothalamic *Nkcc1* in con-M*con-F). *$P < 0.05$ *versus* con-M*con-F. (*I–L*) The levels of corticotropin-releasing hormone gene (Crh) mRNA in the hypothalamus and glucocorticoid receptor gene (Gr) mRNA in the hippocampus in F1 males (*I* and *J*) and F1 females (*K* and *L*). Data normalized against control are means ± SD from 5 or 6 rats per group. Multiple pairwise comparisons were done with the Holm–Sidak method.

$P = 0.674$; fig. 3I) and glucocorticoid receptor in the hippocampus (F[3,19] = 3.293, $P = 0.043$; fig. 3J) of postnatal day ~95 F1 males were not different among all four treatment groups. In addition, there were no significant between-subjects effects of parental sevoflurane exposure on either hypothalamic corticotropin–releasing hormone mRNA (F[3,20] = 1.225, $P = 0.327$; fig. 3K) or hippocampal glucocorticoid receptor mRNA levels (F[3,19] = 0.860, $P = 0.479$; fig. 3L) in F1 female rats.

Elevated DNA Methylation of the Potassium Ion-Chloride Ion Cotransporter KCC2 Gene (*Kcc2*) Expression Promoter Region in Sperm and Ovary of Rats Exposed to Sevoflurane in Young Adulthood and in the Hypothalamus and Hippocampus of Their Offspring

Increased DNA methylation in the *Kcc2* promoter in gamete cells of adult rats exposed to sevoflurane as neonates and in the hypothalamus and hippocampus of their male offspring has recently been described.[10] We therefore sought to determine if similar effects would be observed in response to young adult exposure to sevoflurane. There was a significant effect of treatment (F[1,36] = 30.8, $P < 0.001$) and within-subjects effect of the 5′ position of cytosine residues adjacent to guanines site (F[5,36] = 20.066, $P < 0.001$) on the frequency of 5′ position of cytosine residues adjacent to guanines methylation in the *Kcc2* promoter region in sperm of the postnatal day ~160 F0 rats (fig. 4A). There was also a statistically significant interaction between the 5′ position of cytosine residues adjacent to guanines site and treatment (F[5,36] = 10.036, $P < 0.001$). Pairwise multiple comparison analysis showed that young adult exposure to sevoflurane led to increased methylation in F0 male rats at 5′ position of cytosine residues adjacent to guanines site 1 ($P < 0.001$ *vs.* control) and 5′ position of cytosine residues adjacent to guanines site 2 ($P = 0.006$ *vs.* control). Similarly, there was a significant effect of treatment (F[1,36] = 34.714, $P < 0.001$) and within-subjects effect of 5′ position of cytosine residues adjacent to guanines site (F[5,36] = 42.686, $P < 0.001$) on the frequency of 5′ position of cytosine residues adjacent to guanines site methylation in the *Kcc2* gene promoter in ovarian tissue of F0 rats (fig. 4B). There was also a statistically significant interaction between 5′ position of cytosine residues adjacent to guanines site and treatment (F[5,36] = 12.343, $P < 0.001$). Pairwise multiple comparison analysis showed that young adult exposure to sevoflurane led to increased methylation in F0 female rats at the 5′ position of cytosine residues adjacent to guanines site 1 ($P < 0.001$ *vs.* control) and 5′ position of cytosine residues adjacent to guanines site 2 ($P < 0.001$ *vs.* control).

There was a between–subjects effect of parental treatment on 5′ position of cytosine residues adjacent to guanines site methylation in the promoter of *Kcc2* gene in the hypothalamus of the postnatal day ~95 male offspring of both exposed parents (F[1,36] = 48.286, $P < 0.001$), and a within-subject effect of 5′ position of cytosine residues

adjacent to guanines site (F[5,36] = 42.629, $P < 0.001$; fig. 4C). There was also a statistically significant interaction between 5′ position of cytosine residues adjacent to guanines site and treatment (F[5,36] = 15.886, $P < 0.001$). Pairwise multiple comparison analyses showed that young adult parental exposure to sevoflurane led to increased DNA methylation in the *Kcc2* gene promoter in the hypothalamus of F1 male progeny of both exposed parents at 5′ position of cytosine residues adjacent to guanines site 1 ($P < 0.001$ *vs.* F1 males from the control males and control females group), 5′ position of cytosine residues adjacent to guanines site 2 ($P = 0.002$ *vs.* F1 males from the control males and control females group), and 5′ position of cytosine residues adjacent to guanines site 3 ($P = 0.013$ *vs.* F1 males from the control males and control females group).

There was also a between-subjects effect of parental treatment on 5′ position of cytosine residues adjacent to guanines site methylation in the promoter of *Kcc2* gene in the hippocampus of male offspring of both exposed parents (F[1,36] = 21.740, $P < 0.001$, and within-subject effect of 5′ position of cytosine residues adjacent to guanines site (F[5,36] = 20.852, $P < 0.001$; fig. 4D). There was also a statistically significant interaction between 5′ position of cytosine residues adjacent to guanines site and treatment (F[5,36] = 5.268, $P < 0.001$). Pairwise multiple comparison analyses showed that young adult parental exposure to sevoflurane led to increased DNA methylation in the *Kcc2* gene promoter in the hippocampus of F1 male progeny of both exposed parents at 5′ position of cytosine residues adjacent to guanines site 1 ($P < 0.001$ *vs.* control males and control females), 5′ position of cytosine residues adjacent to guanines site 2 ($P = 0.018$ *vs.* control males and control females), and 5′ position of cytosine residues adjacent to guanines site 3 ($P = 0.018$ *vs.* control males and control females).

Systemic Abnormalities in Male Rats Exposed to Sevoflurane in Young Adulthood

Unexpectedly, we found that more than 2 months after exposure to sevoflurane anesthesia, F0 male rats exhibited behavioral deficiencies. They spent less time in the open arms of the elevated plus maze ($t_{(28)} = 2.18$; $P = 0.038$; fig. 5A), but did not differ from their control counterparts in number of crossings ($t_{(28)} = 1.456$; $P = 0.157$; fig. 5B) or distance traveled ($t_{(28)} = -1.351$; $P = 0.188$; fig. 5C). In F0 females, there was no significant effect of sevoflurane on any of these elevated plus maze parameters (fig. 5, D–F).

As in the elevated plus maze task, there was a significant effect of sevoflurane exposure on prepulse inhibition of startle in adult F0 male rats (F[1,123] = 6.765; $P = 0.010$; fig. 5G), but not in F0 female rats (F[1,60] = 0.049; $P = 0.827$; fig. 5H). Multiple pairwise comparisons indicated that exposure to sevoflurane led to impaired prepulse inhibition of startle responses in F0 male rats at prepulse intensity of 3 decibel ($P = 0.042$ *vs.* control). Startle stimuli
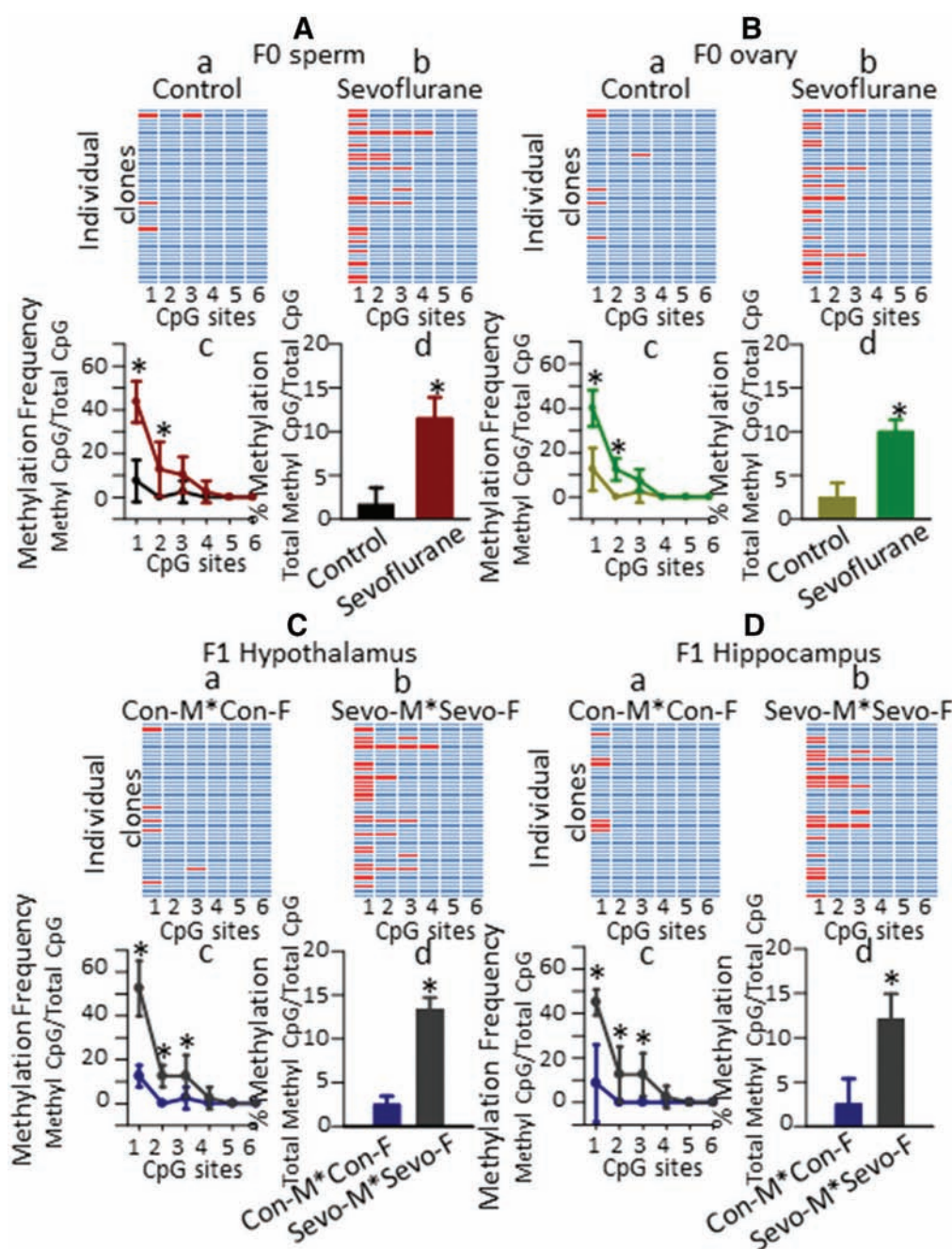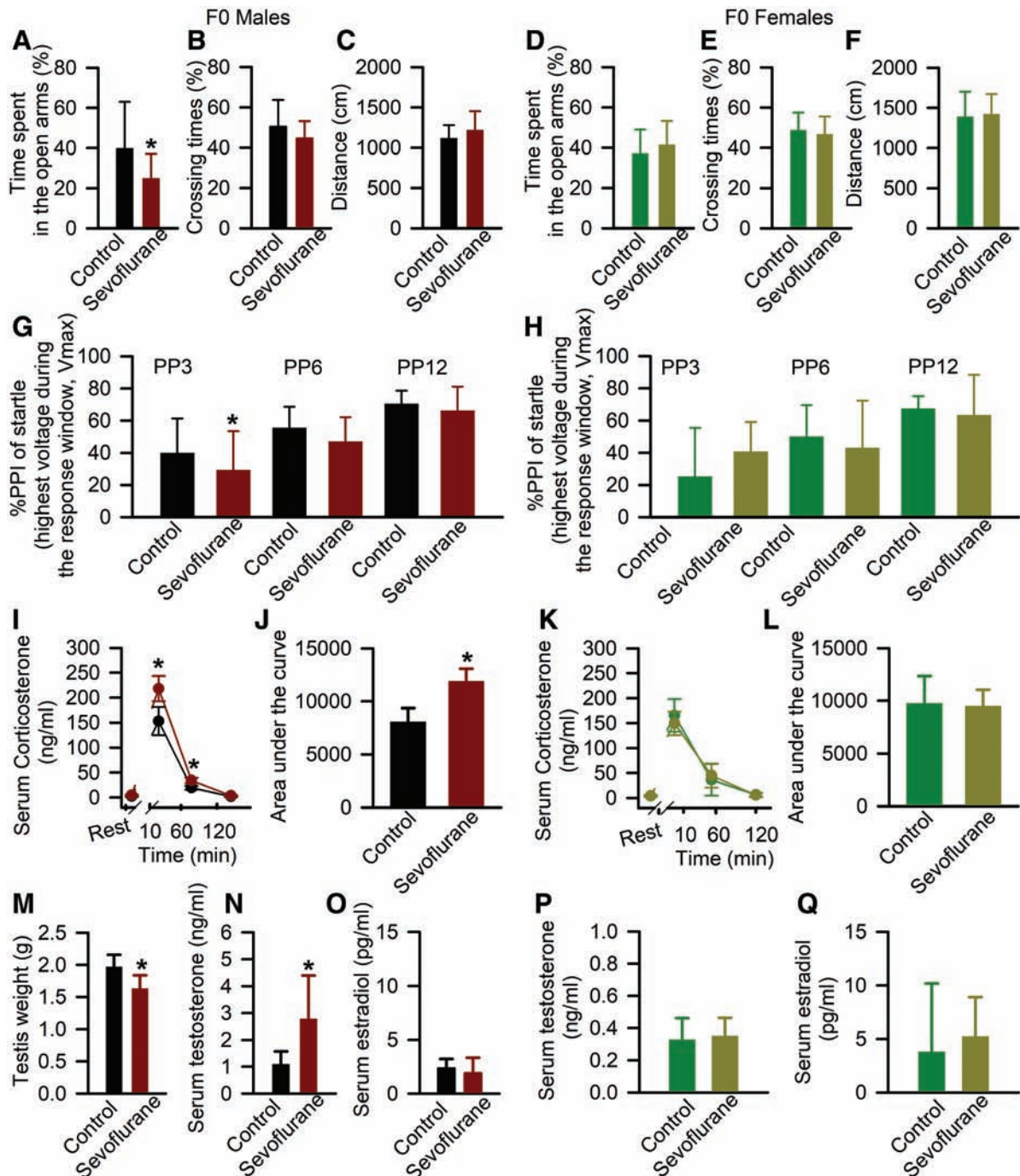
Fig. 4. DNA methylation in the promoter region of potassium ion-chloride ion cotransporter KCC2 gene (*Kcc2*) in parental (F0) sperm and ovary and in hypothalamus and hippocampus of their offspring (F1). (*A, a–d*) Bisulfite sequencing of the 5′ position of cytosine residues adjacent to guanines dinucleotides (CpG sites) in the *Kcc2* gene of 10 clones from four individual sperm DNA samples isolated from control and sevoflurane-exposed F0 male rats. Heat maps show distribution of unmethylated (*blue cells*) and methylated (*red cells*) CpG sites in sperm DNA samples isolated from control (*A, a*) and sevoflurane-exposed (*A, b*) F0 male rats. Histograms showing methylation frequency at each CpG site (*A, c*) and DNA methylation level at all six CpG sites (*A, d*). The results of similar analysis of bisulfite sequencing of CpG dinucleotides in the *Kcc2* gene of 10 clones from four individual ovary DNA samples isolated from sevoflurane-exposed and control F0 female rats shown in *B, a–d*. Color coding of experimental groups in *A, d*, and *B, d*, is also applicable to *A, c*, and *B, c*. (*C* and *D*) Shown are the respective methylation frequency at each CpG site and DNA methylation level at all six CpG sites in the *Kcc2* gene of 10 clones in the hypothalamus (*C, a–d*) and hippocampus (*D, a–d*) of F1 male offspring of control sires and control dams (con-M*con-F) and of sevoflurane-exposed sires and sevoflurane-exposed dams (sevo-M*-sevo-F). Data are means ± SD from 4 rats per group. *P < 0.05 *versus* Control (*A and B*) and *versus* con-M*con-F (*C and D*). Color coding of experimental groups in *C, d*, and *D, d*, is also applicable to *C, c*, and *D, c*. Multiple pairwise comparisons were done with the Holm–Sidak method.

Fig. 5. Systemic first generation (F0) effects of young adult exposure to sevoflurane. The percentage of time spent in open arms of the elevated plus maze, number of crossing the open arms, and distance traveled by male (*A–C*) and female (*D–F*) rats. Data are means ± SD from 15 male and 16 female rats per group. (*G* and *H*) The percentage of prepulse (PP) inhibition of startle responses at PP intensity of 3, 6, and 12 decibel in male (*G*) and female (*H*) rats. Data are means ± SD from 21 males in the Control group, 22 males in the Sevoflurane group, and 16 females per group. *$P < 0.05$ *versus* Control. (*I–L*) The respective levels of serum corticosterone across each collection point, as well as the total corticosterone responses in male (*I* and *J*) and female (*K* and *L*) rats. Serum levels of corticosterone at rest were taken as baselines for calculations of the total corticosterone responses. Data are means ± SD from 8 rats per group. Color coding of experimental groups in *J* and *L* is also applicable to *I* and *K*. (*M–O*) shown are serum levels of testosterone (*M*), estradiol (*N*), and testis weight (*O*) in male rats. (*P* and *Q*) Serum levels of testosterone and estradiol in female rats. Data are means ± SD from 8 rats per group. Data of testis weight are from 16 rats in control group and 13 rats in sevoflurane group. *$P < 0.05$ *versus* Control.

by themselves caused similar responses in the control and sevoflurane groups of F0 male ($t_{(41)} = -0.969$; $P = 0.338$) and F0 female ($t_{(30)} = 1.465$; $P = 0.153$) rats.

Male F0 rats had higher total corticosterone responses to physical restraint on postnatal day ~160 when compared to their control counterparts ($t_{(14)} = -6.209$; $P < 0.001$; fig. 5, I and J). This increase was due to higher levels of corticosterone at 10 min ($P < 0.001$ *vs.* control) and 60 min ($P = 0.036$ *vs.* control) after restraint, as serum levels of corticosterone before the restraint ($P = 0.736$ *vs.* control) and 120 min ($P = 0.787$ *vs.* control) postrestraint were not different in control and sevoflurane-exposed rats (fig. 5I). There was no difference in serum corticosterone levels between control and sevoflurane-exposed F0 female rats (fig. 5, K and L).

During collection of sperm for the DNA methylation studies, we incidentally found that sevoflurane-exposed F0 males had reduced testis weight ($t_{(27)} = 4.494$; $P < 0.001$; fig. 5N) more than 3 months after sevoflurane exposure. Counterintuitively, their serum levels of testosterone were increased ($t_{(14)} = -2.839$; $P = 0.013$; fig. 5M), although their serum levels of estradiol were normal ($t_{(14)} = 0.703$; $P = 0.494$; fig. 5O). F0 female rats in the sevoflurane and control groups were not different with respect to serum levels of testosterone ($P = 0.743$; fig. 5P) or estradiol ($P = 0.600$; fig. 5Q).

Reduction in the Potassium Ion-Chloride Ion Cotransporter KCC2 Expression in Male Rats 1 h after the Last Exposure to Sevoflurane

Our current and recently published findings[10] indicate that rats exposed to sevoflurane in young adulthood and in the early postnatal period develop comparable systemic abnormalities. Given that sevoflurane acts as a stressor in neonatal rats and initiates developmental abnormalities, at least in part, by potentiating excitatory $GABA_A$ receptor signaling,[10–12,17] here we tested whether sevoflurane can induce a stress-like[19–22] reduction in KCC2 expression in young adult rats. To measure acute effects of sevoflurane, rats were exposed to 2.1% sevoflurane anesthesia for 3 h on postnatal day 56, postnatal day 58, and postnatal day 60. Brain tissue and trunk blood samples were collected 1 h after sevoflurane anesthesia on postnatal day 60. Consistent with the stress-like effects of sevoflurane reported in previous work,[10–12,17] the exposed male and female rats had increased serum levels of corticosterone compared to controls ($t_{(6)} = -3.313$; $P = 0.016$, males, fig. 6A; and $t_{(8)} = -3.949$; $P = 0.004$, females; fig. 6B). Despite similar increases in corticosterone levels in sevoflurane-exposed males and females, immunofluorescence evaluations of KCC2 expression in the paraventricular nucleus of the hypothalamus region found reductions in KCC2 level in male ($t_{(6)} = 3.343$; $P = 0.016$), but not female ($t_{(6)} = 0.773$; $P = 0.469$), F0 rats (fig. 6, C–F).

Abnormalities in the Hypothalamic-Pituitary-Gonadal and Hypothalamic-Pituitary-Adrenal Axes in Male Rats Exposed to Sevoflurane in Young Adulthood

Acute KCC2 and long-term systemic effects of young adult sevoflurane in the exposed males only suggest an involvement of sex steroids. To test whether sevoflurane-induced increases in serum testosterone levels involve changes in functioning of the entire hypothalamic-pituitary-gonadal axis, we measured the expression of gonadotropin-releasing hormone in the hypothalamus and serum levels of follicle-stimulating hormone and luteinizing hormone. Consistent with the finding that sevoflurane increased systemic levels of testosterone in postnatal day ~160 F0 male rats, these same rats also had increased hypothalamic levels of gonadotropin-releasing hormone mRNA ($t_{(10)} = -2.519$; $P = 0.030$; fig. 7A) and serum levels of luteinizing hormone ($t_{(14)} = -4.932$; $P < 0.001$; fig. 7B), while serum levels of follicle-stimulating hormone ($t_{(10)} = 1.026$; $P = 0.329$; fig. 7C) were not different from those in F0 control male rats. There were no treatment effects on the hypothalamic levels of gonadotropin-releasing hormone mRNA (fig. 7D) or serum levels of luteinizing hormone (fig. 7E) and follicle-stimulating hormone (fig. 7F) in F0 female rats.

The elevated testosterone may modulate the hypothalamic-pituitary-adrenal axis responses to stress through estrogen receptors after testosterone aromatization to estradiol in the brain. One such mechanism includes modulation by estradiol of the glucocorticoid receptor–mediated negative feedback effect of corticosterone on the hypothalamic-pituitary-adrenal axis activity. Estradiol can both inhibit and enhance the negative feedback effects of glucocorticoids by activating estrogen receptor α and estrogen receptor β, respectively.[23,24] In further support of involvement of testosterone and estradiol in exacerbated hypothalamic-pituitary-adrenal axis responses to stress, we found that the hypothalamic levels of aromatase mRNA were increased in F0 sevoflurane-exposed males ($t_{(10)} = -4.333$; $P = 0.002$; fig. 7G). In addition, the F0 male rats exposed to sevoflurane had increased and decreased hypothalamic levels of estrogen receptor α mRNA ($t_{(10)} = -5.144$; $P < 0.001$; fig. 7H) and estrogen receptor β mRNA ($t_{(10)} = 3.156$; $P = 0.010$; fig. 7I), respectively. Again, consistent with the normal hypothalamic-pituitary-adrenal axis responses to stress in the exposed F0 females, the increase in the hypothalamic aromatase mRNA in sevoflurane-exposed F0 female rats did not achieve statistical significance ($t_{(10)} = -1.878$; $P = 0.090$; fig. 7J). Moreover, in the F0 females, the hypothalamic levels of estrogen receptor α mRNA (fig. 7K) were not different between Sevoflurane and Control groups, while those of estrogen receptor β mRNA were slightly, though significantly increased in the Sevoflurane group ($t_{(10)} = -2.521$; $P = 0.030$; fig. 7L) when compared to the Control group.

In agreement with the exacerbated corticosterone responses to stress in sevoflurane-exposed F0 male rats at postnatal day ~160, these rats had increased hypothalamic
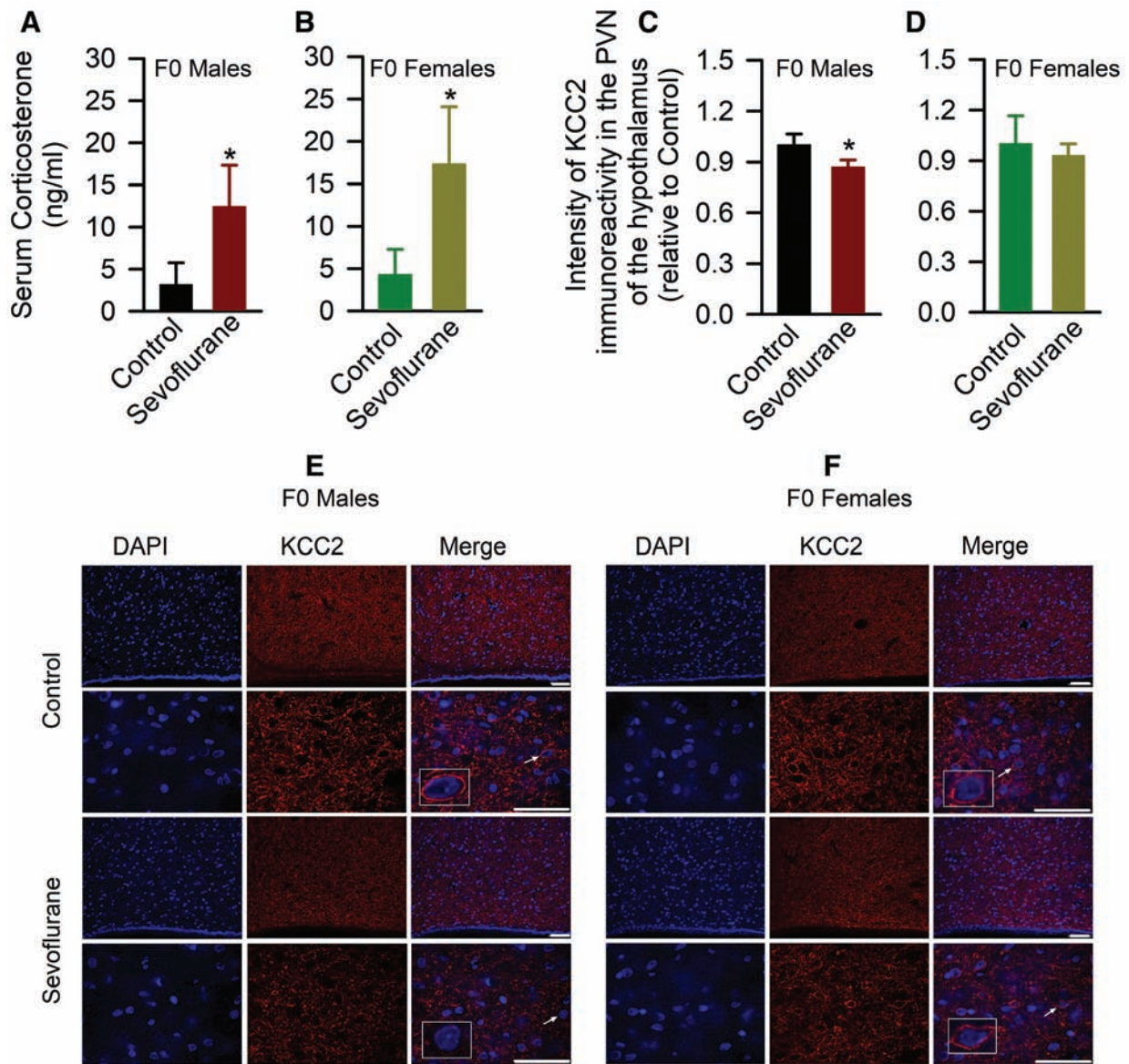
Fig. 6. Acute effects of young adult sevoflurane exposure. (*A* and *B*) The respective levels of serum corticosterone in male (*A*) and female (*B*) rats. Data are means ± SD (n = 4 and n = 5 per treatment group in males and females, respectively). *$P < 0.05$ *versus* Control. (*C–F*) Representative confocal images and quantitative analysis of the potassium ion-chloride ion cotransporter KCC2 immunoreactivity in the paraventricular nucleus (PVN) of the hypothalamus of the control and sevoflurane-exposed male (*C* and *E*) and female (*D* and *F*) rats. (*E*) Representative confocal images of the PVN from the control and sevoflurane-exposed male rats, immunostained for 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI; *left; blue*) and KCC2 (*middle; red*); the merge column shows colocalized images (*right; red* and *blue*). The *arrowheads* indicate the cells shown in the *boxed areas* at higher magnifications. The KCC2 immunoreactivity, located on the periphery of the neurons (*red color*), decreased in the PVN neurons from the sevoflurane-exposed male rats. Similar representative confocal images of the PVN from the control and sevoflurane-exposed female rats shown in (*F*). *Scale bars*, 50 μm. The sevoflurane-exposed males, but not sevoflurane-exposed females, had reduced KCC2 expression (*$P < 0.05$ *vs.* Control; *C* and *E*). Data are means ± SD (n = 4 rats per group).

corticotropin–releasing hormone mRNA levels 2 h after the restraint ($t_{(14)} = -3.181$; $P = 0.007$; fig. 7M), as well as reduced levels of glucocorticoid receptor mRNA in the hippocampus ($t_{(10)} = 2.493$; $P = 0.032$; fig. 7N). Neither hypothalamic

corticotropin–releasing hormone mRNA levels (fig. 7O) nor hippocampal glucocorticoid receptor mRNA levels (fig. 7P) were different in sevoflurane-exposed and control F0 female rats.
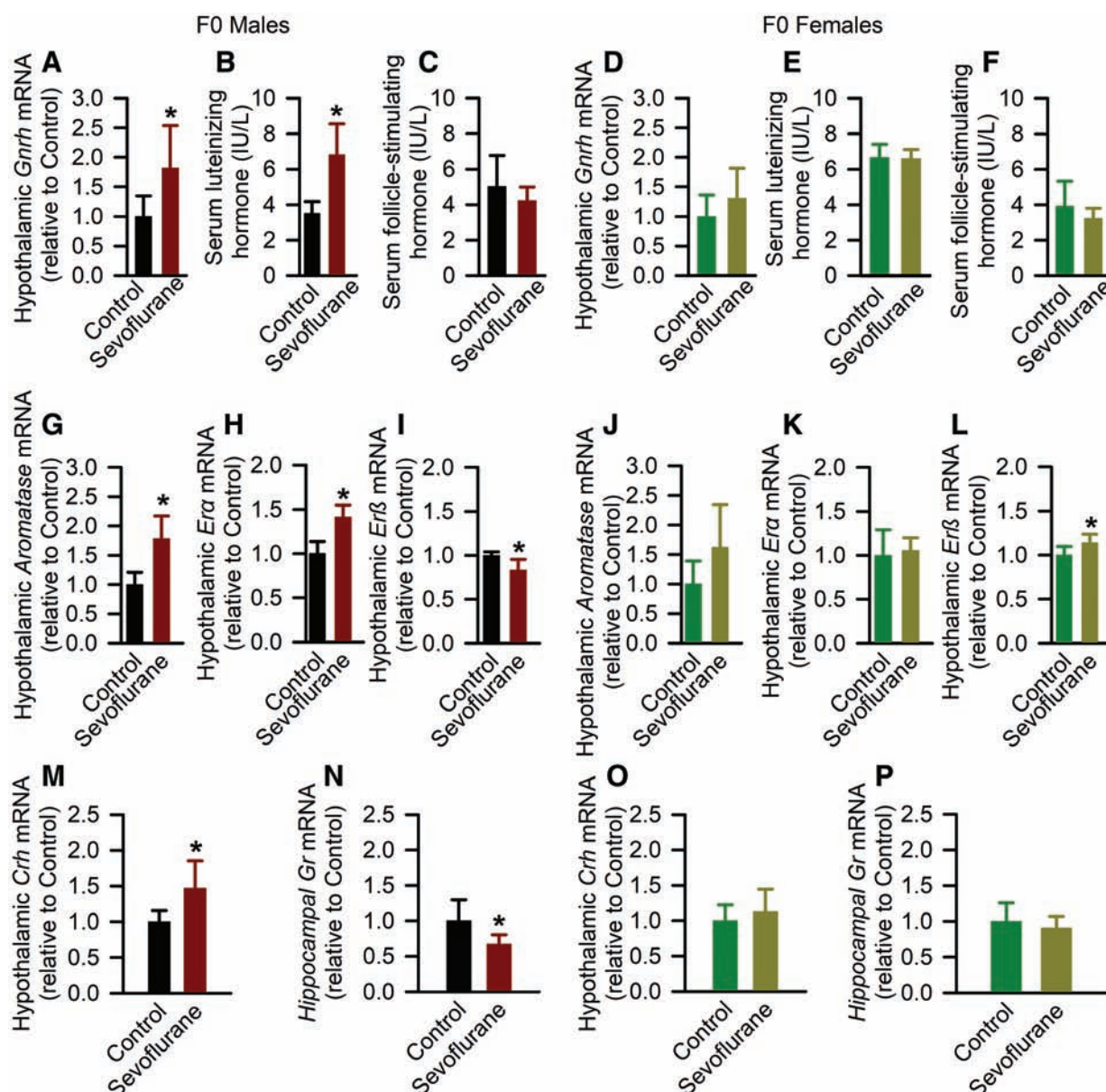
Fig. 7. Molecular first generation (F0) effects of young adult exposure to sevoflurane. (*A–F*) The levels of hypothalamic gonadotropin-releasing hormone (Gnrh) mRNA, serum levels of luteinizing hormone and follicle-stimulating hormone of male (*A–C*) and female (*D–F*) rats. Data are means ± SD from 8 rats per group (n = 6 rats per group in follicle-stimulating hormone). *P < 0.05 *versus* Control. (*G–L*) shown are levels of aromatase mRNA, estrogen receptor α gene (Erα) mRNA, and estrogen receptor β gene (Erβ) mRNA in the hypothalamus of male (*G–I*) and female (*J–L*) rats. Data normalized against control are means ± SD from 6 rats per group. *P < 0.05 *versus* Control. (*M–P*) Shown are the respective levels of hypothalamic corticotropin-releasing hormone gene (Gnrh) mRNA and hippocampal glucocorticoid receptor gene (Gr) mRNA in males (*M* and *N*) and females (*O* and *P*). Data normalized against control are means ± SD from 6 rats per group (n = 8, male corticotropin-releasing hormone; n = 5 in the Control group and n = 4 in the Sevoflurane group in female glucocorticoid receptor). *P < 0.05 *versus* Control.

Finally, considering the controlling roles of GABA$_A$ receptor signaling in hypothalamic gonadotropin–releasing hormone and corticotropin–releasing hormone neuronal activity, it is plausible that sevoflurane initiates enhancements of gonadotropin–releasing hormone and corticotropin–releasing hormone neuronal activity and, hence, the entire reproductive and stress axes, by downregulating *Kα2*

expression and rendering GABA$_A$ receptor signaling less inhibitory or even excitatory. To assess whether exposure of young adult rats to sevoflurane led to persistent alterations in expression of Cl$^-$ transporters, brain hypothalamic and hippocampal tissue samples were collected more than 3 months after exposure (postnatal day ~160). The F0 male rats from the Sevoflurane group had normal *Nkcc1* mRNA
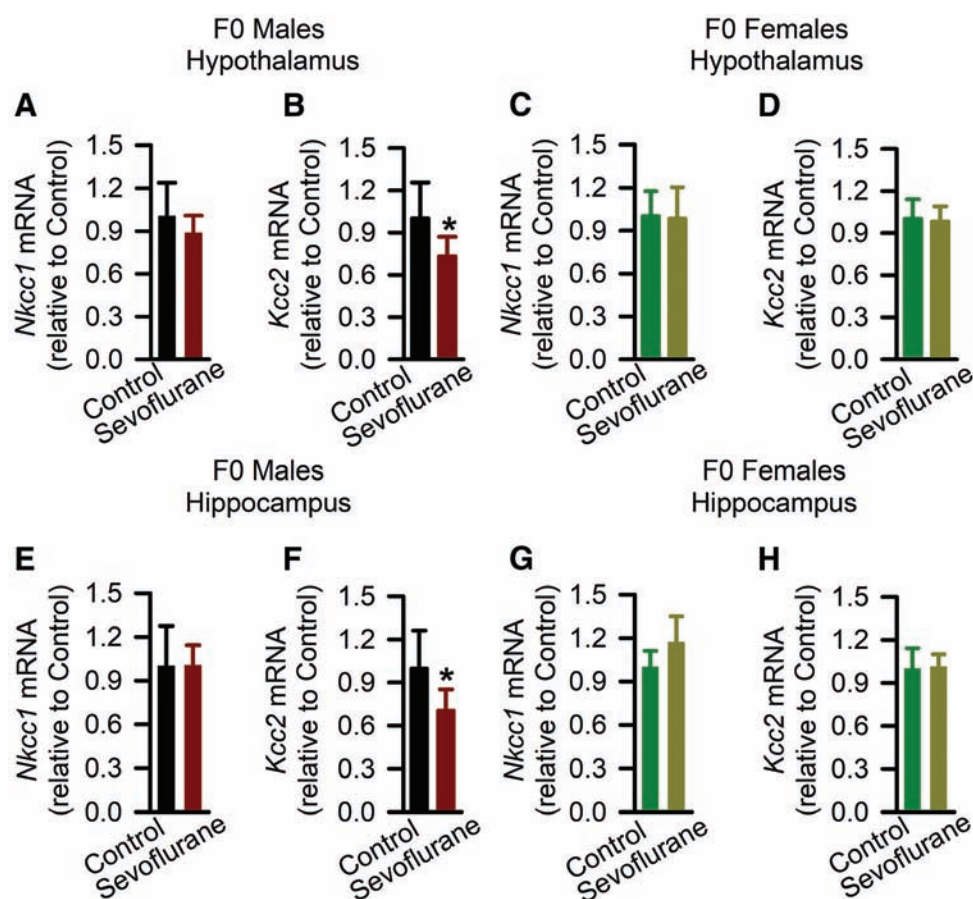
Fig. 8. Effects of sevoflurane exposure on hypothalamic and hippocampal potassium ion-chloride ion cotransporter KCC2 gene (*Kcc2*) and sodium ion-potassium ion-chloride ion cotransporter NKCC1 gene (*Nkcc1*) mRNA in F0 rats. (*A–H*) Shown are the respective levels of *Nkcc1* mRNA and *Kcc2* mRNA in the hypothalamus of F0 males (*A* and *B*) and F0 females (*C* and *D*) and in the hippocampus of F0 males (*E* and *F*) and F0 females (*G* and *H*). Data normalized against control are means ± SD from 6 rats per group (n = 5, female hippocampus in sevoflurane group). *P < 0.05 *versus* Control.

levels ($t_{(10)}$ = 1.065; *P* = 0.312; fig 8A), but decreased *Kcc2* mRNA levels ($t_{(10)}$ = 2.273; *P* = 0.046; fig. 8B) in the hypothalamus. In contrast, the F0 female rats from the Sevoflurane group had unaltered levels of both *Nkcc1* mRNA ($t_{(10)}$ = 0.155; *P* = 0.880; fig. 8C) and *Kcc2* mRNA ($t_{(10)}$ = 1.346; *P* = 0.208; fig. 8D) in the hypothalamus. In the hippocampus of F0 male rats from the Sevoflurane group, *Kcc2* mRNA levels, but not *Nkcc1* mRNA levels, were significantly reduced ($t_{(10)}$ = 2.387, *P* = 0.038, fig. 8, E and F). The hippocampal mRNA levels for *Nkcc1 and Kcc2* were similar in control and sevoflurane-exposed F0 female rats (fig. 8, G and H).

Discussion

The results of these experiments show that sevoflurane administered to young adult rats of either sex can induce abnormalities in their male offspring, as well as in the exposed male rats themselves. The reduction in *Kcc2*

expression in the hypothalamus and hippocampus of the F1 male progeny of the exposed parents, as well as hypermethylation of the *Kcc2* promoter in F0 male and female gamete cells and F1 male hypothalamus and hippocampus, support the involvement of epigenetic mechanisms in transmitting adverse effects of sevoflurane exposure in young adult rats to the next generation. The findings that both the physiology and behavior of sevoflurane-exposed F0 females were normal, while their ovarian *Kcc2* promoter was hypermethylated and male progeny of exposed F0 females exhibited neurobehavioral abnormalities, suggest that sevoflurane affects somatic and germ cells *via* different mechanisms.

Intergenerational Effects of Young Adult Sevoflurane

Human and animal studies provide evidence that exposure to alcohol, endocrine disruptors, and stress can affect future generations.[10,12–15,25–27] Surprisingly, the heritable effects of general anesthetics are poorly studied, despite

the fact that anesthetic agents share molecular mechanisms of action with alcohol and may act as both environmental stressors and endocrine disruptors.[10–12,17,27–29] Anecdotal observations point to the possibility of heritable effects of anesthesia and surgery in humans.[30] Some evidence of heritable effects of general anesthetics comes from clinical surveys indicating that anesthesia care providers may have altered female:male offspring ratios.[31–35] Importantly, sex ratio effects have been linked to alcohol, stress, and endocrine disruptors as well.[36–38] Our recently published[10] and current findings support the possibility that sevoflurane can induce intergenerational effects. Obviously, sevoflurane-induced epigenetic modulation of *Kcc2* in F0 gamete cells and F1 male brain may contribute to the intergenerational effects of young adult sevoflurane exposure, but it is unlikely that sevoflurane-induced changes in *Kcc2* are the only mediating mechanism. Indeed, this study found that male progeny of exposed dams and control sires had normal *Kcc2* mRNA levels in the hypothalamus and hippocampus but exhibited deficiencies in the elevated plus maze and prepulse inhibition of startle behavioral tests. It is possible that *Kcc2* DNA methylation in gamete cells affects other epigenetic mechanisms that program offspring brain development, and/or that multiple genes are independently epigenetically modified by sevoflurane in F0 gamete cells and F1 brain. Furthermore, the epigenetic effects of GABAergic anesthetics may not be limited to DNA methylation, as we and others recently reported experimental evidence for involvement of not only DNA methylation,[10,39] but also histone acetylation,[40,41] in adverse effects of neonatal sevoflurane exposure in rats.

Despite different anesthesia regimens in our recent study of intergenerational effects of neonatal anesthesia[10] and the young adult anesthesia in the current study, similarities in the intergenerational effects of sevoflurane outweighed differences between the two models. These findings are also consistent with those reported by Rodgers *et al.* that 42-day exposure of male rats to stress either throughout puberty or in adulthood leads to similar blunted hypothalamic-pituitary-adrenal axis responses in their progeny.[14] Moreover, repeated exposure of 11-week-old male mice to the anesthetic agent enflurane negatively affected their offspring's behavior.[42] The susceptibility of germ cell maturation to sevoflurane over an extended period of the lifespan, from the early postnatal period[10] through young adulthood (this study), suggests that the gamete cells at different stages of maturation may be susceptible to epigenetic modifications initiated by sevoflurane. It will be important in future studies to analyze epigenetic effects of parental exposure to anesthetics in F0 oocytes, which represent only a minor part of total ovarian tissue that was tested in this study.

Irrespective of whether fathers, mothers, or both parents were exposed to sevoflurane as young adults, only male F1 progeny exhibited epigenetic, gene expression, and systemic abnormalities. Given that *Kcc2* was hypermethylated in both

male and female gamete cells in F0 rats, it is plausible that parental sevoflurane initiates mechanisms leading to disruption of embryonic DNA methylation reprogramming in F1 males, but not in F1 females, resulting in affected F1 males but normal F1 females. Our findings that the exposed but physiologically unaffected dams, similar to exposed and affected sires, pass deleterious effects of sevoflurane to unexposed male offspring raise the possibility that male offspring may be affected even when anesthesia level or duration is not sufficient to induce significant abnormalities in the exposed parents.

Adverse Effects of Young Adult Sevoflurane in the Exposed Rats

The long-term adverse effects of general anesthesia in early childhood and at advanced ages are a widely recognized health-related concern and the subject of extensive clinical and laboratory research.[43] Investigations of such effects in young adults in their prime reproductive age, however, are relatively scarce. Several studies have assessed effects of isoflurane in young adult rats, primarily using young adult rats as comparisons to other age groups. Isoflurane administered to postnatal day 60 rats affected progenitor proliferation and improved spatial memory in one study and had no effect in other one.[44,45] Crosby *et al.* also observed improvement in spatial memory in rats anesthetized with 1.2% isoflurane–70% nitrous oxide at 6 months of age.[46] Aside from the fact that two studies found long-term effects of isoflurane in young adult rats, different isoflurane concentrations and exposure regimens make it difficult to compare the effects across these studies. Such comparison is even more problematic with our current findings in F0 males, given that we tested not only a distinct anesthesia regimen, but also a different anesthetic agent. Clearly, further research is needed to elucidate the full range of long-term effects of young adult sevoflurane anesthesia and mechanisms that mediate such effects.

The GABA$_A$ receptor/testosterone/aromatase/estradiol/KCC2 pathway may be a key mediator of sevoflurane's long-term neuroendocrine effects in F0 male rats. Because many gonadotropin-releasing hormone neurons are excited by GABA$_A$ receptor signaling even under basal conditions,[47,48] sevoflurane may initially stimulate gonadotropin-releasing hormone neuronal activity and, hence, the entire reproductive axis. Male-specific factors may be required for sevoflurane's actions to acutely reduce KCC2 expression, as similar increases in corticosterone levels in sevoflurane-exposed female rats were not accompanied by a reduction in KCC2 expression. The GABA$_A$ receptor/testosterone/aromatase/estradiol/KCC2 pathway in F0 male rats may function as a system with a positive feedback effect leading to a persistently upregulated hypothalamic-pituitary-gonadal axis. Sevoflurane-induced increases in systemic testosterone and in brain aromatase expression may lead to reductions in *Kcc2* expression, and in turn to diminished inhibitory or increased stimulatory control

of gonadotropin-releasing hormone neurons by KCC2/GABA$_A$ receptor signaling. The plausibility of this scenario is supported by literature demonstrating that estradiol increases expression of aromatase and decreases expression of KCC2 in the brain.[49,50] Interestingly, consistent with our findings, Galanopoulou and Moshé found that estradiol reduced KCC2 expression in males only.[50]

The GABA$_A$ receptor/testosterone/aromatase/estradiol/KCC2 pathway is also likely to be involved in dysregulated (exacerbated) hypothalamic-pituitary-adrenal axis responses to stress and stress-dependent behavioral abnormalities. One such mechanism includes modulation by estradiol of the glucocorticoid receptor–mediated negative feedback effect of corticosterone on hypothalamic-pituitary-adrenal axis activity. Estradiol can both inhibit and enhance the negative feedback effects of glucocorticoids by activating estrogen receptor α and estrogen receptor β, respectively.[23,24] In support of this mechanism are our findings that hypothalamic levels of aromatase and estrogen receptor α mRNA were elevated, whereas hypothalamic levels of estrogen receptor β mRNA and hippocampal levels of glucocorticoid receptor mRNA were downregulated. Higher estradiol concentrations are thought to contribute to higher corticosterone at rest or after stress exposure in adult female rats when compared to their male counterparts.[51–53] Hence, our findings of exacerbated corticosterone responses to stress in F0 males, but not F0 females, suggest that exposure to sevoflurane in young adulthood induces a persistent transformation of the male stress response to a more "female-like" form. Upregulated expression of hypothalamic aromatase and resulting elevated levels of estradiol may also explain why in sevoflurane-exposed males, elevated levels of testosterone were accompanied by exacerbated hypothalamic-pituitary-adrenal axis responses to stress, because it was previously shown that gonadectomy of male rats elevated, while androgen replacement blunted the corticosterone response to stress.[54] The persistent downregulation of *Kcc2* expression and resulting GABA$_A$ receptor depolarizing and stimulatory signaling in the hypothalamic paraventricular nucleus are likely to further contribute to dysregulated stress responses and behavioral abnormalities by impairing fundamental mechanisms of hypothalamic-pituitary-adrenal axis functioning; *e.g.,* the neuroactive steroid and GABA$_A$ receptor negative feedback–based mechanism of desensitization to stress.[55] Finally, it is plausible that the negative feedback effect of elevated testosterone contributed to the reduced testis weight in exposed F0 males.

In conclusion, our results demonstrate that parental exposure to sevoflurane in young adulthood epigenetically reprograms germ cells, leading to neurobehavioral abnormalities in adult male progeny. These findings also provide evidence that sevoflurane administered even in young adulthood induces neurobehavioral deficits, profound alterations in the hypothalamic-pituitary-gonadal axis, and dysregulated hypothalamic-pituitary-adrenal axis responses to stress in exposed male rats. Exposed young adult female rats exhibited no long-term physiologic or behavioral abnormalities, but together with the exposed males, passed the adverse effects of sevoflurane exposure to their unexposed male offspring. These differential effects of sevoflurane exposure in males and females could suggest that distinct mechanisms mediate the somatic and germ cell effects of young adult sevoflurane exposure. It is important to note, however, that the current study was not powered to detect sex differences, and hence it will be important in future work to conduct direct comparisons between males and females.

Competing Interests

The authors declare no competing interests.

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ANESTHESIOLOGY

Effect of Polyethylene-glycolated Carboxyhemoglobin on Renal Microcirculation in a Rat Model of Hemorrhagic Shock

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- The optimal fluid for resuscitation of hemorrhagic shock (crystalloid, colloid, or hemoglobin-containing) and mitigation of acute kidney injury is unknown

What This Article Tells Us That Is New

- In a rat model of hemorrhagic shock comparing fluid resuscitation with blood, diluted blood, hydroxyethyl starch, or polyethylene-glycolated carboxyhemoglobin, all fluids restored urine output and creatinine clearance, but only blood and diluted blood improved renal Po_2
- Postresuscitation histologic renal tubular damage was increased compared with nonresuscitated rats but slightly less with blood, diluted blood, and polyethylene-glycolated carboxyhemoglobin compared with hydroxyethyl starch
- Restoration of circulatory hemodynamics and kidney microcirculatory Po_2 was comparable with polyethylene-glycolated carboxyhemoglobin and balanced hydroxyethyl starch solution

ABSTRACT

Background: Primary resuscitation fluid to treat hemorrhagic shock remains controversial. Use of hydroxyethyl starches raised concerns of acute kidney injury. Polyethylene-glycolated carboxyhemoglobin, which has carbon monoxide–releasing molecules and oxygen-carrying properties, was hypothesized to sustain cortical renal microcirculatory Po_2 after hemorrhagic shock and reduce kidney injury.

Methods: Anesthetized and ventilated rats (n = 42) were subjected to pressure-controlled hemorrhagic shock for 1 h. Renal cortical Po_2 was measured in exposed kidneys using a phosphorescence quenching method. Rats were randomly assigned to six groups: polyethylene-glycolated carboxyhemoglobin 320 mg · kg^{-1}, 6% hydroxyethyl starch (130/0.4) in Ringer's acetate, blood retransfusion, diluted blood retransfusion (~4 g · dl^{-1}), nonresuscitated animals, and time control. Nitric oxide and heme oxygenase 1 levels were determined in plasma. Kidney immunohistochemistry (histologic scores of neutrophil gelatinase-associated lipocalin and tumor necrosis factor-α) and tubular histologic damages analyses were performed.

Results: Blood and diluted blood restored renal Po_2 to 51 ± 5 mmHg (mean difference, −18; 95% CI, −26 to −11; $P < 0.0001$) and 47 ± 5 mmHg (mean difference, −23; 95% CI, −31 to −15; $P < 0.0001$), respectively, compared with 29 ± 8 mmHg for hydroxyethyl starch. No differences between polyethylene-glycolated carboxyhemoglobin and hydroxyethyl starch were observed (33 ± 7 mmHg *vs.* 29 ± 8 mmHg; mean difference, −5; 95% CI, −12 to 3; $P = 0.387$), but significantly less volume was administered (4.5 [3.3–6.2] *vs.* 8.5[7.7–11.4] ml; mean rank difference, 11.98; $P = 0.387$). Blood and diluted blood increased the plasma bioavailability of nitric oxide compared with hydroxyethyl starch (mean rank difference, −20.97; $P = 0.004$; and −17.13; $P = 0.029$, respectively). No changes in heme oxygenase 1 levels were observed. Polyethylene-glycolated carboxyhemoglobin limited tubular histologic damages compared with hydroxyethyl starch (mean rank difference, 60.12; $P = 0.0012$) with reduced neutrophil gelatinase-associated lipocalin (mean rank difference, 84.43; $P < 0.0001$) and tumor necrosis factor-α (mean rank difference, 49.67; $P = 0.026$) histologic scores.

Conclusions: Polyethylene-glycolated carboxyhemoglobin resuscitation did not improve renal Po_2 but limited tubular histologic damages and neutrophil gelatinase-associated lipocalin upregulation after hemorrhage compared with hydroxyethyl starch, whereas a lower volume was required to sustain macrocirculation.

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Fluid administration is the first step of rescue therapy during severe hemorrhage or hypovolemic shock before blood products become available.[1] However, aggressive volume fluid resuscitation may lead to secondary severe tissue edema and clinical signs of volume overload, which result in unfavorable outcomes.[2,3] Colloidal solutions are used to sustain intravascular

oncotic pressure and to shorten the circulatory stabilization time while limiting fluid administration. Hydroxyethyl starch solutions remain the most used synthetic colloids for volume replacement. However, no definitive evidence exists for outcome advantage of starches over crystalloids in various settings, and concerns were raised for kidney function. Therefore, the European Medicines Agency (Amsterdam, The Netherlands) restricts the use of hydroxyethyl starch solutions across the European Union.[4,5] Hyperosmotic and hypertonic saline fluids are also considered alternatives, but the fluids produce uncertain outcomes and possible harm.[6] Therefore, crystalloids remain the sole resuscitation fluid for the treatment of hemorrhage and severe hypovolemia before blood product transfusion. Current resuscitation fluids of crystalloids or synthetic colloids possess no oxygen–carrying capacity and may promote inflammation.

Acute kidney injury is one of the most frequent organ failures in perioperative and critically ill patients.[7] The pathophysiology of acute kidney injury is not fully understood to date, but major contributors have been identified, such as renal microcirculatory hypoxemia, tissue inflammation, and fluid overload.[8–10] The early reestablishment of oxygenation and kidney perfusion during hemorrhage is essential to limit the subsequent development of acute kidney injury.[9,10]

Polyethylene-glycolated carboxyhemoglobin (Sanguinate, Prolong Pharmaceuticals, USA) is a novel agent that exhibits a dual action of carbon monoxide release and oxygen carrying and transfer.[11] Carbon monoxide is poisonous at high concentrations because it inhibits cellular respiration and interacts tightly with hemoglobin to limit oxygen delivery, but it also produces beneficial effects at low concentrations. Carbon monoxide's homeostatic effects occur *via* multiple cellular and molecular mechanisms of action, including redox control, antiapoptosis and antiinflammation, modulation of vasoactive responses (vasodilation), and modulation of the innate immune response.[12,13] There is compelling preclinical data on the use of carbon monoxide as a therapeutic agent *via* carbon monoxide–releasing molecules in models of acute lung injury,[14] sepsis,[15] hemorrhagic shock,[16] and ischemia and reperfusion injury.[17] Carbon monoxide mitigated the inflammatory response and ischemic damage in all of these settings.

The present study hypothesized that a molecule with the ability to release carbon monoxide molecules to injured tissues and carry oxygen to hypoxic tissue would sustain kidney function by increasing renal microcirculatory oxygen tension and limiting the damage of ischemia and hypoxia after resuscitation from hemorrhagic shock while providing a low volume of resuscitation fluid. The primary outcome of our study was improvement of cortical renal microcirculatory oxygen tension. Secondary outcomes included the volume of fluid resuscitation, macrohemodynamics, renal immunohistochemistry and histologic damages, acid–base status, and levels of plasma biomarkers of injury. We sought to compare the renal microcirculatory impact of polyethylene-glycolated

carboxyhemoglobin, 6% hydroxyethyl starch balanced in Ringer's acetate, and blood transfusion as resuscitation strategies in a blood pressure–targeted resuscitation model of hemorrhagic shock in the rat.

Materials and Methods

Animals

The Animal Research Committee of the Academic Medical Centre of the University of Amsterdam (Amsterdam, The Netherlands) approved all experiments in this study (DFL 103073). Care and handling of the animals were performed in accordance with the guidelines from the Institutional and Animal Care and Use Committees. This study and the following reporting adhere to the applicable Animal Research: Reporting of In Vivo Experiments guidelines.[18] All experiments were performed on male Wistar albino rats (Charles River, The Netherlands), aged 10 ± 3 weeks with a mean $\pm$ SD body weight of 320 ± 26 g, at 9:30 AM (weekdays) at the Department of Experimental Surgery, University Medical Center, Amsterdam, The Netherlands.

Surgical Preparation

The rats were anesthetized using an intraperitoneal injection of a mixture of 100 mg $\cdot$ kg^{-1} ketamine (Nimatek; Eurovet, The Netherlands), 0.5 mg $\cdot$ kg^{-1} dexmedetomidine (Dexdomitor; Orion Group, Finland), and 0.05 mg $\cdot$ kg^{-1} atropine-sulfate (Centrafarm, The Netherlands). Anesthesia was maintained with 50 mg $\cdot$ kg $\cdot$ h^{-1} ketamine. A tracheotomy was performed. The animals were connected to a ventilator (Babylog 8000, Dräger, The Netherlands) and ventilated with tidal volumes of 6 ml $\cdot$ kg^{-1} with a positive end-expiratory pressure of 3 cm H$_2$O and a fractional inspired oxygen tension of 0.4. A heating pad under the animal allowed the body temperature to be controlled and maintained at 37 ± 0.5°C. The end-tidal carbon dioxide was maintained between 35 and 42 mmHg by adjusting respiratory rate (CapnoMac, Datex-Ohmeda, USA).

The carotid (pressure) and femoral (for blood shedding and samples) arteries and jugular (anesthesia) and femoral (fluid resuscitation) veins were cannulated using polyethylene catheters (outer diameter, 0.9mm; Braun, Germany). Fluid maintenance during surgery was sustained using Ringer's acetate (Baxter, The Netherlands) administration at a rate of 10 ml $\cdot$ kg^{-1} $\cdot$ h^{-1}. The left kidney was exposed without decapsulation and immobilized in a Lucite kidney cup (K. Effenberger, Germany) *via* a 3-cm incision in the left flank. Renal vessels were carefully separated to preserve the nerves and adrenal gland. An ultrasonic flow probe was placed around the left renal artery (type 0.7 RB; Transonic Systems Inc., USA) and connected to a flow meter (T206; Transonic Systems Inc., USA) to continuously measure renal blood flow. The left ureter was isolated, ligated, and cannulated using a polyethylene catheter for urine collection. The animals rested for 30 min after completion of surgery (~1 h).

Experimental Protocol

The animals were randomized according to a unique code that was generated by an internet website (Sealed Envelope Ltd., 2016, simple randomization service; available at: https://www.sealedenvelope.com/simple-randomiser/v1/, accessed September 17, 2016) and placed in sealed envelopes. A technician prepared the resuscitation fluid according to the generated code on the day of the experiment. The investigator performing the experiments was partially blinded to group assignment or treatment. Control and hemorrhage groups received no fluids. Another investigator who was blinded to the type of randomization performed the data analyses.

The animals underwent stabilization period (30 min) and were bled from the left femoral artery catheter at a rate of $1\,ml \cdot min^{-1}$ using a syringe pump (Harvard 33 syringe pump; Harvard Apparatus, USA) until reaching a mean arterial pressure (MAP) of ~30 mmHg. This pressure was maintained for 1 h *via* reinfusing or withdrawing blood. Coagulation of the shed blood was prevented with the addition of 200 IU of heparin in the syringe. The animals were randomized into six groups: (1) polyethylene-glycolated carboxyhemoglobin with a maximum dosage of $320\,mg \cdot kg^{-1}$, (2) 6% hydroxyethyl starch (130/0.4) balanced with Ringer's acetate (Volulyte, Fresenius Kabi, Germany), (3) diluted blood with polyethylene-glycolated carboxyhemoglobin's buffer (to the same hemoglobin content as polyethylene-glycolated carboxyhemoglobin: $4\,g \cdot dl^{-1}$; diluted blood), (4) blood transfusion (blood), (5) nonresuscitated animals, and (6) control, in which surgery was performed without hemorrhage. Fluid resuscitation was divided into two periods of time: a rescue therapy period of 30 min (rescue period, 30 min after resuscitation), in which the fluid tested was administered alone until reaching MAP of 80 mmHg and stopped, and a maintenance period of 30 min (60 min after resuscitation), in which the resuscitated groups all received 6% hydroxyethyl starch. This design allowed comparisons between groups with a similar target (fig. 1).

Measurement of Renal Cortical Microcirculatory Po₂

An optical fiber with a tip diameter of 5 mm was placed ~1 mm above the exposed kidney to measure oxygenation using a phosphorescence lifetime technique described elsewhere.[19] Palladium(II)-meso-tetra(4-carboxyphenyl)-porphyrin at a concentration of $\sim5\,mg \cdot ml^{-1}$ was used as a phosphorescent dye, injected at a dose of $1.5\,ml \cdot kg^{-1}$ and excited at 530 nm. The phosphorescent decay time signal corresponds to the microcirculatory Po₂ in the kidney, and it was continuously recorded and processed using software developed in LabView 2014 (National Instruments, USA) as described previously.[19] The depth of penetration of green light and catchment depth were estimated to be within the renal cortex. We excluded any interference between palladium(II)-meso-tetra(4-carboxyphenyl)-porphyrin and polyethylene-glycolated carboxyhemoglobin (*in vitro*) before the *in vivo* experiment.

Determination of Oxygen-carrying Capacity of Polyethylene-glycolated Carboxyhemoglobin and Spectrophotometric Analyses of Its Derivatives

Because polyethylene-glycolated carboxyhemoglobin is bound with carbon monoxide molecules, and its oxygen-carrying capacity is reduced. The release of carbon monoxide likely increases the oxygen-carrying capacity of the molecule. The oxygen-binding/carrying capacity of polyethylene-glycolated carboxyhemoglobin was determined at 37°C under normal atmospheric conditions using similar previously described methods (Supplemental Digital Content 1, http://links.lww.com/ALN/C41). A second set of experiments determined the oxygen-binding/carrying capacity of the molecule after full saturation of polyethylene-glycolated carboxyhemoglobin in 100% oxygen (for 2 h) to induce carbon monoxide release. However, complete carbon monoxide release was not achieved.

The unknown spectrum was determined spectrophotometrically in UV-visible light ranging from 500 to 650 nm with a 1-nm increment at 37°C (Synergy HTX, BioTek,
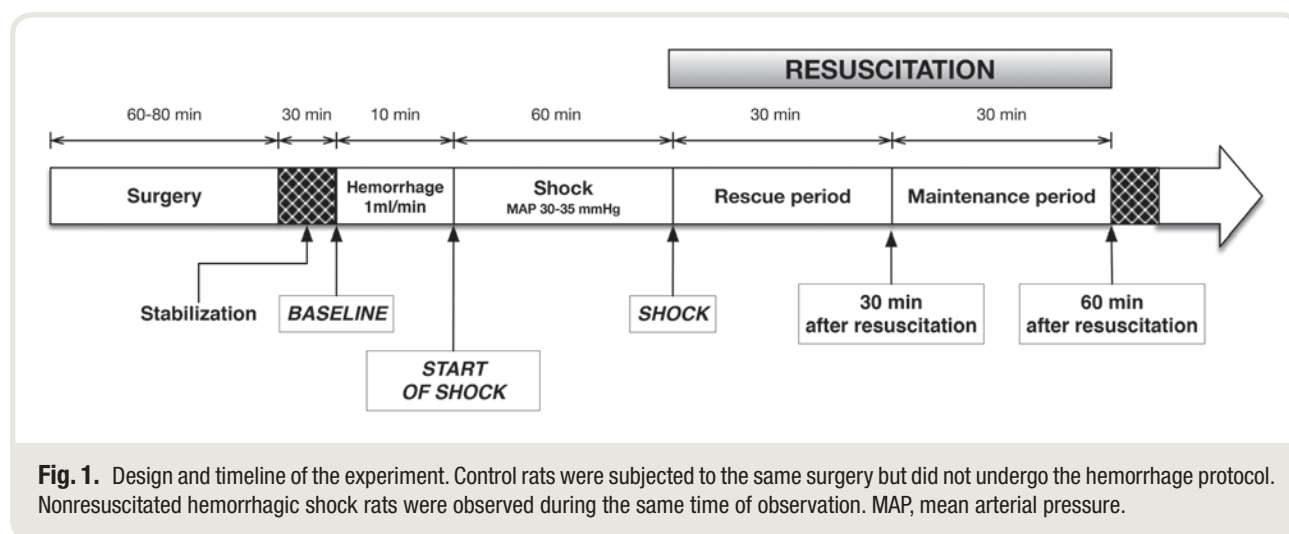
Fig. 1. Design and timeline of the experiment. Control rats were subjected to the same surgery but did not undergo the hemorrhage protocol. Nonresuscitated hemorrhagic shock rats were observed during the same time of observation. MAP, mean arterial pressure.

Germany) to determine the concentration of each derivative (carboxyhemoglobin, oxyhemoglobin, and deoxyhemoglobin) of polyethylene-glycolated carboxyhemoglobin in the plasma. The observed total absorption spectrum of each plasma sample was then compared with the known spectra of the different derivatives of bovine hemoglobin[20] with their absorption values. With the use of the classical least squares' method (mixture of three components with 150 wavelengths, see Supplemental Digital Content 1, http://links.lww.com/ALN/C41), the different concentrations of each component were recovered.

The concentration of polyethylene-glycolated carboxyhemoglobin in the plasma was obtained after the addition of 5 µl of both 2% KCN and 2% $K_3Fe(CN)_6$ to 40 µl of plasma to convert all polyethylene-glycolated carboxyhemoglobin derivatives to hemiglobincyanide. The endpoint absorbance was read at 540 nm against a calibration curve.

Measurements of Arterial Blood Gas and Biochemistry

Arterial blood samples (250 µl) were collected at four time points (fig. 1). Arterial blood gas parameters were determined using a blood gas analyzer (ABL80 Flex, Radiometer, Denmark), and total hemoglobin concentration, hemoglobin oxygen saturation, base excess, and lactate levels were measured. The arterial oxygen content (Cao_2; ml $O_2 \cdot dl^{-1}$) was calculated considering the oxygen-carrying capacity of polyethylene-glycolated carboxyhemoglobin in the plasma: $Cao_2 = (Sao_2 \times Hb_{RBC} \times \gamma_{Hb}) + (S_{polyethylene-glycolated\ carboxyhemoglobin}O_2 \times Hb_{polyethylene-glycolated\ carboxyhemoglobin} \times \gamma_{polyethylene-glycolated\ carboxyhemoglobin}) + Pao_2 \times 0.0031$, where Hb_{RBC} is the hemoglobin concentration in erythrocyte ($g \cdot dl^{-1}$), S is the arterial oxygen saturation of Hb_{RBC} or polyethylene-glycolated carboxyhemoglobin, and γ is the oxygen-carrying capacity of Hb_{RBC} (1.34 ml $O_2 \cdot g^{-1}$ Hb for rat blood) or polyethylene-glycolated carboxyhemoglobin, as previously determined. Renal delivery of oxygen (Do_2; ml $O_2 \cdot min^{-1}$) was determined at the end of the experiment as follows: renal $Do_2 = Cao_2 \times$ renal blood flow.

Plasma creatinine and urine creatinine were measured using an automatic analyzer (c702, Roche Diagnostics, Switzerland). We established a calibration curve for creatinine levels before measurements to correct for a significant interference because of the presence of polyethylene-glycolated carboxyhemoglobin. Plasma osmolarity was determined using the freezing point method with an osmotic pressure meter (OSMOstation OM-6050, Menarini, Benelux, Belgium). The glomerular filtration rate was estimated at the end of the experiment using the measurement of creatinine clearance (Cl_{crea}): $Cl_{crea} = (U_{Crea} \times V)/(time \times P_{Crea})$, where U_{Crea} is the concentration of creatinine in urine ($mmol \cdot l^{-1}$), V is the urine volume (ml) per unit time of urine collection, and P_{Crea} is the concentration of creatinine in plasma ($mmol \cdot l^{-1}$).

Plasma Nitric Oxide and Heme Oxygenase 1 Levels

The samples were placed in the reducing agent vanadium (III) chloride (VCl_3) in 1 mol $\cdot l^{-1}$ HCl at 90°C to reduce the nitrate and nitrite in the plasma samples to nitric oxide. The vanadium (III) chloride reagent converts nitrite, nitrate, and S-nitroso compounds to nitric oxide gas that is guided toward a nitric oxidechemiluminescence signal analyzer (Sievers 280i analyzer, GE Analytical Instruments, USA) to allow the direct detection of nitric oxide.[21] Nitric oxide within the reaction vessel reacts with ozone to generate oxygen and excited-state nitric oxide species, and the decay is associated with the emission of weak near-infrared chemiluminescence. A sensitive photodetector detects this signal and converts it to millivolts. The area under the curve of the detected chemiluminescence (mV · s) represents the amount of nitrix oxide-ozone reactions in real time and thus the amount of bioavailable nitric oxide in the tested samples.

Heme oxygenase 1 constitutes an essential cytoprotective component that ameliorates hemorrhagic shock-induced oxidative tissue injuries.[22] Plasma heme oxygenase 1 levels were measured at the end of the experiment using an enzyme-linked immunosorbent assay (ELISA) kit based on the sandwich assay principle (rat HMOX1/HO-1 ELISA kit [sandwich ELISA], LS-F4085, LSBio LifeSpan BioSciences Inc., USA).

Immunohistochemical Analysis and Histology

The animals were euthanized at the end of the experiment using 40% Euthasol (Produlab Pharma BV, The Netherlands). The kidneys were harvested, fixed in 4% formalin, and embedded in paraffin. Ten tubular areas (n = 10) on each slice per animal were analyzed. A minimum of 40 tubules were examined per group, with n = 6/kidney per animal minimum. The complete description of immunohistochemical analyses and histology are detailed in Supplemental Digital Content 1 (http://links.lww.com/ALN/C41). A histologic score value for neutrophil gelatinase–associated lipocalin and tumor necrosis factor-α was derived for each sample via summing the percentages of cells that stained at each intensity multiplied by the weighted intensity of the staining (histologic score = S Pi [i + 1], where i is the intensity score, and Pi is the corresponding percentage of the cells) under a light microscope at ×400 magnification. Histologic changes (brush border, vacuolar degeneration, cast formation, and invagination) in the cortex were assessed using quantitative measurements of tissue damage.

Statistical Analysis

The values are expressed as means ± SD when normally distributed (Kolmogorov–Smirnov test) or as median [interquartile range] otherwise. Repeated-measures two-way ANOVA (two factors: time as a related within-animal

factor and group as a between-animal factor) and *post hoc* Bonferroni correction test for multiple analyses were used to determine intergroup and/or intragroup differences in hemodynamics, cortical renal Po_2, and biochemical data. We reported the simple main effects of group (type of fluid) compared with the control and to the hydroxyethyl starch groups at each time point when a significant interaction was observed between time and group, and the simple main effect of time *versus* baseline within the same group. Ordinary one-way ANOVA with Bonferroni correction was used for the analyses of the oxygen-binding capacity of polyethylene-glycolated carboxyhemoglobin, renal oxygen delivery, ELISA heme oxygenase 1, and volume of blood withdrawn. Analyses of creatinine clearance, total fluid volume, plasma nitric oxide, histologic damages to the kidney, and tumor necrosis factor-α and neutrophil gelatinase–associated lipocalin histologic scores were performed using a Kruskal–Wallis test with Dunn correction test because of their non-Gaussian distribution. Outliers were evaluated, but no action was necessary. Statistical analyses were performed using GraphPad Prism version 7.0a for Mac (GraphPad Software, USA). The overall significance level for each hypothesis was 0.05. Adjusted *P* values are reported throughout the manuscript in *post hoc* tests.

We assumed that the microcirculatory Po_2 in the kidney would be of 26 ± 3 mmHg (means ± SD) at 30 min after resuscitation of hemorrhagic shock using 6% balanced hydroxyethyl starch based on previous experiments lead by

our group. Therefore, a sample size of n = 6/group was needed to detect a 20% increase in kidney microcirculatory Po_2 (estimated to be 31 mmHg, Δ of 5 mmHg) associated with the administration of polyethylene-glycolated carboxyhemoglobin (compared with balanced hydroxyethyl starch) and provide a power (1-β) of 80% with a type I error rate (α) of 5% using a two-group Satterthwaite two-sided *t* test.

Results

A total of 42 animals were included in the present study (n = 6–8/group). Eight animals were included in the polyethylene-glycolated carboxyhemoglobin group, and four served to established reliability in the protocol. No animal died during the experiment. The volume of blood withdrawn to induce hemorrhagic shock at T1 (6.7 ± 0.8 ml) was similar in all groups. The withdrawn volume represented $32.2 \pm 4.4\%$ of the total blood volume. The hemorrhagic shock produced similar alterations in hemodynamics (MAP and renal blood flow) and similar reductions in kidney microcirculatory Po_2 associated with a metabolic acidosis and hyperlactatemia in all groups compared with controls ($P < 0.0001$). The hemodynamic data throughout the experiment are presented in table 1. MAP was restored to ~80 mmHg in all animals that underwent resuscitation after hemorrhagic shock, according to the protocol. At the end of experiment, the renal blood flow recovered similarly to controls except

Table 1. Systemic and Renal Hemodynamics and Urine Excretion

	Baseline	Shock	Rescue Phase (30 min after Resuscitation)	Maintenance (60 min after Resuscitation)
MAP, mmHg				
Control	94 ± 10	81 ± 5	84 ± 6	90 ± 6
Nonresuscitated	92 ± 5	32 ± 2*	32 ± 1*	30 ± 1*
6% balanced hydroxyethyl starch	92 ± 10	32 ± 1*	81 ± 4	83 ± 6
PEGylated carboxyhemoglobin	91 ± 7	32 ± 2*	76 ± 11	81 ± 3
Blood transfusion	89 ± 4	32 ± 2*	86 ± 8	87 ± 10
Diluted blood transfusion	90 ± 6	33 ± 2*	81 ± 1	79 ± 4*
Renal blood flow, ml · min⁻¹				
Control	9.8 ± 2.3	7.9 ± 2.6	7.4 ± 2.4	7 ± 2.4
Nonresuscitated	10.5 ± 3.2	3.0 ± 1*	3.2 ± 1.1*	3.0 ± 1*
6% balanced hydroxyethyl starch	9.7 ± 1.3	3.9 ± 2.4*	7.4 ± 2.4	8.4 ± 2.9
PEGylated carboxyhemoglobin	11.6 ± 2	2.8 ± 0.5*	6.3 ± 1.2	8.3 ± 1.6
Blood transfusion	10.0 ± 1.7	2.3 ± 0.3*	5.0 ± 0.3	5.4 ± 0.5†
Diluted blood transfusion	11.6 ± 1.2	3.1 ± 1.5*	7.0 ± 1.7	7.3 ± 1.6
Urine output, ml · h⁻¹				
Control	2.80 ± 0.73	2.2 ± 1.42	2.46 ± 0.94	1.87 ± 0.53
Nonresuscitated	4.68 ± 1.8	0 ± 0‡	0 ± 0‡	0 ± 0‡
6% balanced hydroxyethyl starch	2.77 ± 0.91	0 ± 0‡	2.55 ± 0.75	3.93 ± 2.35
PEGylated carboxyhemoglobin	4.01 ± 2.3	0 ± 0‡	0.76 ± 0.55‡	3.97 ± 1.43
Blood transfusion	3.29 ± 1.94	0 ± 0‡	0.85 ± 0.52‡	2.2 ± 1.43
Diluted blood transfusion	4.04 ± 1.31	0 ± 0‡	1.63 ± 1.20‡	3.63 ± 1.75

The values are presented as means ± SD. Repeated measures two-way ANOVA test used with Bonferroni correction to adjust for multiple comparisons.

*Adjusted $P < 0.01$ *versus* control group at the same time point. †Adjusted $P < 0.05$ *versus* 6% balanced hydroxyethyl starch group at the same time point. ‡$P < 0.01$ *versus* baseline value within the same group.

MAP, mean arterial pressure; PEGylated, polyethylene glycolated.

Table 2. Acid–Base Balance and Arterial Lactate Levels during Experiment

	Baseline	Shock	Rescue Phase (30 min after Resuscitation)	Maintenance (60 min after Resuscitation)
pH				
Control	7.41 ± 0.04	7.38 ± 0.04	7.38 ± 0.02	7.37 ± 0.01
Nonresuscitated	7.39 ± 0.02	7.23 ± 0.02*	7.16 ± 0.05*	7.13 ± 0.05*
6% balanced hydroxyethyl starch	7.40 ± 0.04	7.22 ± 0.05*	7.36 ± 0.03*	7.40 ± 0.04
PEGylated carboxyhemoglobin	7.38 ± 0.02	7.20 ± 0.06*	7.29 ± 0.04*†	7.35 ± 0.02
Blood transfusion	7.40 ± 0.03	7.22 ± 0.03*	7.26 ± 0.04*†	7.33 ± 0.06
Diluted blood transfusion	7.39 ± 0.04	7.22 ± 0.06*	7.28 ± 0.05*†	7.32 ± 0.05†
Base excess, mmol · l^{-1})				
Control	−4.5 ± 2	−4.6 ± 1	−4.4 ± 0.9	−3.3 ± 0.6
Nonresuscitated	−2.4 ± 1.3	−12.5 ± 1.9*	−15 ± 3.0*	−16.6 ± 2.7*
6% balanced hydroxyethyl starch	−3.5 ± 2.0	−13.6 ± 0.8*	−5.6 ± 1.4*	−2.2 ± 1.4
PEGylated carboxyhemoglobin	−3.7 ± 0.8	−13.4 ± 2.3*	−9.3 ± 2.0*†	−4.5 ± 0.8
Blood transfusion	−2.5 ± 1.7	−13.2 ± 1.4*	−9.5 ± 1.3*†	−6.6 ± 1.6
Diluted blood transfusion	−1.7 ± 1.9	−12.2 ± 3.8*	−8.7 ± 3.1*	−6.9 ± 2.5*
HCO$_3^-$, mmol · l^{-1})				
Control	19.3 ± 1.1	19.5 ± 0.5	19.8 ± 1.1	20.8 ± 0.8
Nonresuscitated	21.6 ± 1.8	13.8 ± 1.9*	12.7 ± 2.4*†	11.4 ± 2.2*
6% balanced hydroxyethyl starch	20.0 ± 1.9	12.9 ± 1.1*	18.5 ± 1.4	22.1 ± 1.3
PEGylated carboxyhemoglobin	20.1 ± 1.2	13.6 ± 1.2*	16.3 ± 1.9*	20.2 ± 0.9†
Blood transfusion	21.1 ± 1.9	13.2 ± 1.8*	16.6 ± 1*	18.3 ± 0.9†
Diluted blood transfusion	22.4 ± 1.4	14.2 ± 2.8*	16.9 ± 2.6	18.0 ± 2.5†
Lactate$^-$, mmol · l^{-1}				
Control	0.9 ± 0.2	1.4 ± 0.3	1.9 ± 0.4	2.1 ± 0.5
Nonresuscitated	0.8 ± 0.2	5.1 ± 0.5*	5.8 ± 0.6*	7.2 ± 1.1*
6% balanced hydroxyethyl starch	1.1 ± 0.4	5.3 ± 1.3*	3.3 ± 0.6*	2.7 ± 0.5
PEGylated carboxyhemoglobin	0.8 ± 0.2	4.5 ± 0.7*	3.9 ± 0.8*	3.6 ± 0.8*
Blood transfusion	0.8 ± 0.1	5.1 ± 0.5*	3.0 ± 0.2	2.3 ± 0.3
Diluted blood transfusion	0.7 ± 0.1	5.0 ± 1.4*	3.0 ± 0.8	1.9 ± 0.4

The values are presented as means ± SD. Repeated measures two-way ANOVA test was used with Bonferroni's correction to adjust for multiple comparisons.
*Adjusted $P < 0.01$ *versus* control group at the same time point. †Adjusted $P < 0.01$ *versus* 6% balanced hydroxyethyl starch group at the same time point.
PEGylated, polyethylene glycolated.

for the blood transfusion group, which was lower than in the hydroxyethyl starch group ($P = 0.015$). The changes in acid–base status and lactate levels differed according to the resuscitation fluid administered (table 2). Balanced hydroxyethyl starch was the most efficient fluid at restoring the acid–base status at the end of the rescue phase compared with other therapies. Polyethylene-glycolated carboxyhemoglobin corrected the pH, but base excess, bicarbonate levels, and lactate levels remained significantly higher than controls (mean difference, −1.5; 95% CI, −2.6 to −0.4; $P = 0.0018$; table 2).

Significant differences in urine output at baseline were observed between groups. Therefore, only intragroup comparisons were performed. Notably, urine output after the rescue phase in the blood transfusion group was lower than at baseline (mean difference, 2.44; 95% CI, 0.93 to 3.95; $P = 0.0006$), and urine output was readily restored 30 min after resuscitation in the hydroxyethyl starch group (mean difference, 0.23; 95% CI, −1.28 to 1.74; $P = 0.969$; table 1).

Kidney Microcirculatory Po$_2$ and Renal Function

Figure 2 shows that hemorrhagic shock significantly reduced baseline renal cortical Po$_2$ in all groups compared with baseline and controls. The mean renal cortical Po$_2$ was 13 ± 5 mmHg 60 min after hemorrhagic shock induction. Similar MAP and renal blood flow were observed between groups, but none of the fluid therapies restored renal cortical Po$_2$ to levels similar to controls 30 or 60 min after resuscitation. Whole blood transfusion and diluted blood transfusion outperformed 6% balanced hydroxyethyl starch in increasing renal cortical Po$_2$ at the end of experiment to 51 ± 5 mmHg (mean difference, −19; 95% CI, −26 to −11; $P < 0.0001$) and 47 ± 5 mmHg (mean difference, −23; 95% CI, −31 to −15; $P < 0.0001$) *versus* 29 ± 8 mmHg, respectively. However, there were no differences between polyethylene-glycolated carboxyhemoglobin and balanced hydroxyethyl starch in renal cortical Po$_2$ in the early rescue phase (32 ± 7 mmHg *vs.* 27 ± 4 mmHg; mean difference, −6, 95% CI, −13 to 2; $P = 0.209$) or at the end of the experiment (33 ± 7 mmHg *vs.* 29 ± 8 mmHg; mean difference, −5; 95% CI, −12 to 3; $P = 0.387$).

Hemorrhagic shock induced acute kidney injury with a significant increase in plasma creatinine levels (greater than twofold increase of baseline values; fig. 3A) compared with controls ($P < 0.0001$). Creatinine levels remained higher 60 min after resuscitation compared with the control
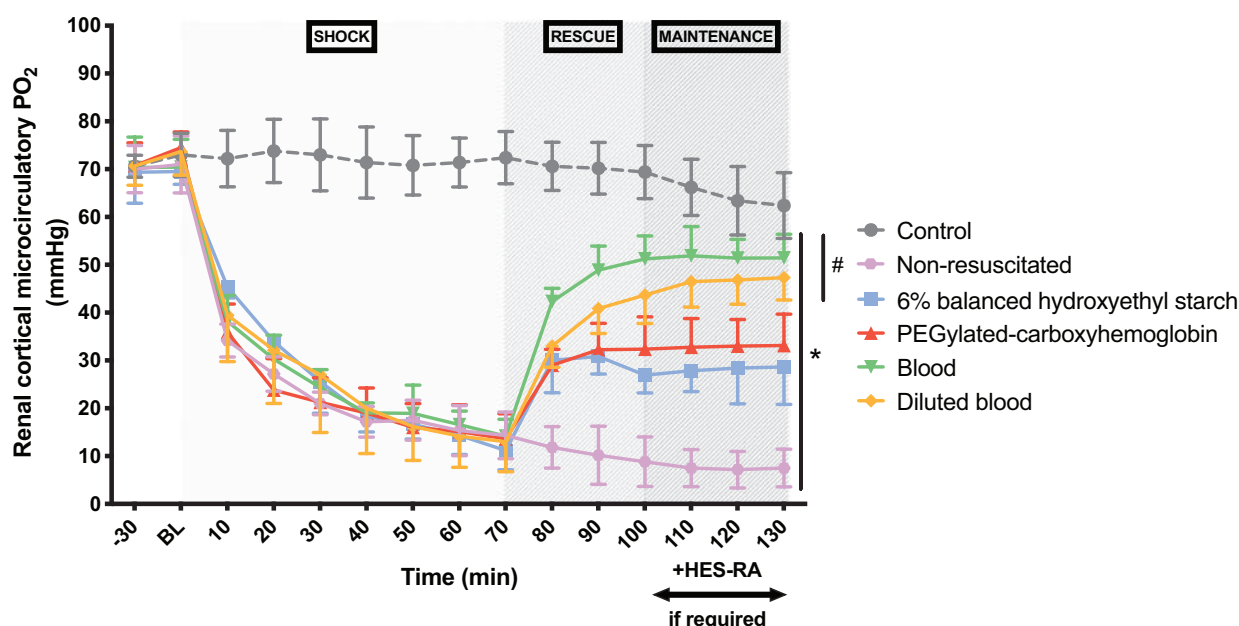
Fig. 2. Renal cortical microcirculatory Po$_2$ during the experiment. For the sake of clarity, only significant differences were shown at the end of the experiment. Repeated measures two-way ANOVA test was used with Bonferroni correction to adjust for multiple comparisons. *Adjusted $P < 0.005$ *versus* control group. #Adjusted $P < 0.05$ compared with 6% balanced hydroxyethyl starch at the same time point. The data are presented as means ± SD (n = 8 for PEG-carboxyhemoglobin group and n = 6/group for other groups). HES-RA, 6% hydroxyethyl starch balanced in ringer acetate; PEGylated, polyethylene glycolated.

rats, regardless of the therapy used ($P < 0.0001$). There were no differences regarding plasma creatinine between hydroxyethyl starch and polyethylene-glycolated carboxyhemoglobin 60 min after resuscitation (44.0 ± 6.7 µmol/l *vs.* 49.9 ± 6.4 µmol/l, respectively; mean difference, 5.9; 95% CI, −3.0 to 14.8; $P = 0.230$). Conversely, the measured creatinine clearance (fig. 3B) was not different at 60 min between groups.

The Cao$_2$ (table 3) was calculated for all time points. However, the contribution of polyethylene-glycolated carboxyhemoglobin's hemoglobin to arterial oxygen content was only considered at the last time point of the experiment (60 min after resuscitation) for technical reasons. Therefore, we did not investigate the contribution of polyethylene-glycolated carboxyhemoglobin's hemoglobin to Cao$_2$ 30 min after resuscitation. Blood and diluted blood transfusions produced Cao$_2$ values similar to controls. The Cao$_2$ was superior in the polyethylene-glycolated carboxyhemoglobin group compared with balanced hydroxyethyl starch only after the rescue period (30 min; 12.5 ± 1.3 g · dl^{-1} *vs.* 9.4 ± 1.1 g · dl^{-1}; mean difference, −3.0; 95% CI, −5.4 to −0.6; $P = 0.005$). This difference was offset at the end of the experiment. The renal oxygen delivery was significantly lower in all groups at the end of experiment compared with controls except in the diluted blood transfusion group (Supplemental Digital Content 2, http://links.lww.com/ALN/C42).

Fluid Volume

The stacked (total) fluid volume and separate volumes of each therapy plus balanced hydroxyethyl starch are presented in figure 4. The fluid volumes needed to achieve similar MAP targets varied significantly between groups, 8.5 (7.7 to 11.4), 4.5 (3.3 to 6.2), 7.3 (6.4 to 7.8), and 13.2 (11.9 to 15.35) ml, for the balanced hydroxyethyl starch, polyethylene-glycolated carboxyhemoglobin, blood transfusion, and diluted blood transfusion groups, respectively ($P < 0.0002$). The polyethylene-glycolated carboxyhemoglobin group required significantly less volume than balanced hydroxyethyl starch (mean rank difference, −11.98; $P = 0.011$) to achieve similar MAP and renal cortical Po$_2$. No additional volume of balanced hydroxyethyl starch was required in the diluted blood transfusion group to maintain MAP over the observation time of the experiment. However, this latter group required the largest amount of fluid administration.

Fate of Polyethylene-glycolated Carboxyhemoglobin

Polyethylene-glycolated carboxyhemoglobin partially released its carbon monoxide molecules after injection in the cardiovascular system (table 3). The percentage of carboxyhemoglobin decreased from ~85% measured *in vitro* (manufacturer data) to 23.6 ± 9% ($P < 0.0001$; as determined in the Supplemental Digital Content 1, http://links.lww.com/ALN/C41).
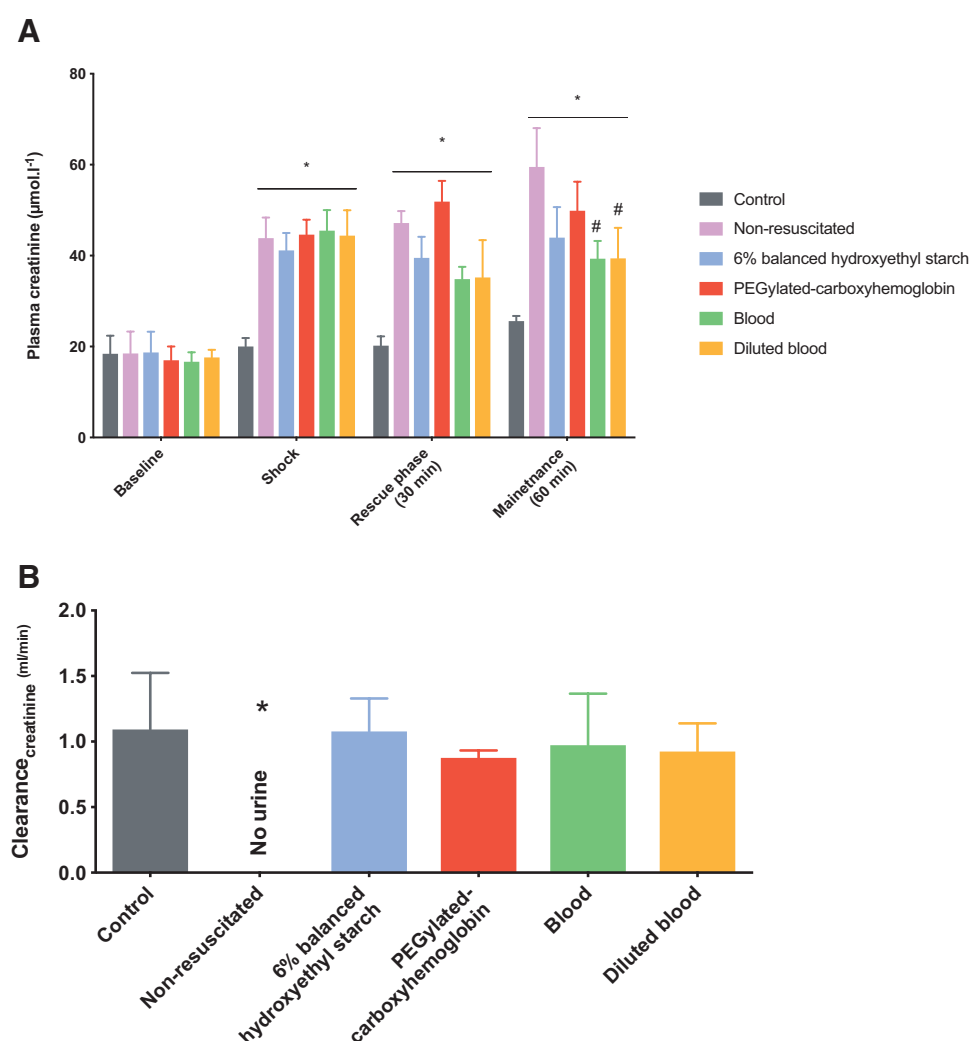
Fig. 3. Time course of changes in plasma creatinine (*A*) and creatinine clearance at the end of the experiment (*B*). (*A*) Repeated measures two-way ANOVA test was used with Bonferroni correction to adjust for multiple comparisons. *Adjusted $P < 0.001$ *versus* control group. #Adjusted $P < 0.05$ compared with 6% balanced hydroxyethyl starch at the same time point. The data are presented as means ± SD. (*B*) Kruskal–Wallis test with Dunn posttest was used. *Adjusted $P < 0.0001$ compared with control group. The data are presented as medians [interquartile range] (n = 8 for PEG-carboxyhemoglobin group and n = 6/group for other groups). PEGylated, polyethylene glycolated.

The oxygen-binding capacity of polyethylene-glycolated carboxyhemoglobin *in vitro* was determined to be $0.29 \pm 0.09\,\mathrm{ml}\ O_2 \cdot g^{-1}$ of hemoglobin (seven measurements) in its original form. This oxygen-binding capacity increased significantly after full oxygenation of the molecule to induce carbon monoxide release, reaching $0.92 \pm 0.02\,\mathrm{ml}\ O_2 \cdot g^{-1}$ of hemoglobin ($P < 0.0001$).

Nitric Oxide and Heme Oxygenase 1 in Plasma

The administration of polyethylene-glycolated carboxyhemoglobin did not result in a decrease in plasma nitric oxide concentrations, excluding a nitric oxide-scavenging effect. Plasma nitric oxide levels were higher in the blood transfusion, diluted blood transfusion, and nonresuscitated hemorrhagic shock groups compared with balanced hydroxyethyl starch ($P < 0.05$). However, there was no significant difference between the control and balanced hydroxyethyl starch or polyethylene-glycolated carboxyhemoglobin groups (Supplemental Digital Content 2A, http://links.lww.com/ALN/C42). Blood and diluted blood increased the bioavailability of NO in the plasma compared with balanced hydroxyethyl starch (mean rank difference, −20.97; $P = 0.004$; and mean rank difference, −17.13; $P = 0.029$, respectively).

CO is produced *via* the heme oxygenase 1 pathway. Therefore, we investigated the levels of heme oxygenase

Table 3. Hemoglobin Levels, Arterial Oxygen Content, and Fractions of PEGylated Carboxyhemoglobin's Derivatives

	Baseline	Shock	Rescue Phase (30 min after Resuscitation)	Maintenance (60 min after Resuscitation)
Total hemoglobin concentration, g · dl⁻¹				
Control	14.4 ± 0.9	14.5 ± 0.5	14.3 ± 0.8	13.0 ± 0.3
Nonresuscitated	15.1 ± 0.9	10.5 ± 2.0*	10.3 ± 1.2*†	9.3 ± 0.7*†
6% balanced hydroxyethyl starch	14.8 ± 1.5	9.7 ± 1.0*	6.6 ± 0.8*	5.5 ± 0.5*
PEGylated carboxyhemoglobin	15.6 ± 0.9	11.2 ± 1.0	8.6 ± 0.9	7 ± 1
Blood transfusion	15.3 ± 0.5	9.9 ± 0.8	13.5 ± 1.3	12.4 ± 0.9
Diluted blood transfusion	14.8 ± 0.2	10.1 ± 0.7	11.0 ± 0.6	10.9 ± 0.2
Hematocrit, %				
Control	44 ± 3	45 ± 1	43 ± 3	40 ± 1
Nonresuscitated	46 ± 3	32 ± 6*	32 ± 4*†	29 ± 2*†
6% balanced hydroxyethyl starch	45 ± 5	30 ± 3*	21 ± 2*	17 ± 2*
PEGylated carboxyhemoglobin	48 ± 3	35 ± 3	28 ± 3	22 ± 3
Blood transfusion	47 ± 1	31 ± 3	41 ± 4	38 ± 3
Diluted blood transfusion	45 ± 0.5	31 ± 2	34 ± 1.7	34 ± 0.7
PEGylated carboxyhemoglobin and derivatives				
Plasma PEGylated carboxyhemoglobin concentration, g · dl⁻¹			ND	0.6 ± 0.1
PEGylated carboxyhemoglobin fraction (%)			ND	23.6 ± 9
Cao₂, ml O₂ · dl⁻¹				
Control	19.8 ± 1.2	20.1 ± 0.6	19.8 ± 1	18.1 ± 0.4
Nonresuscitated	20.8 ± 1.2	14.7 ± 2.7*	14.5 ± 1.6*†	13.1 ± 0.9*†
6% balanced hydroxyethyl starch	20.4 ± 2.10	13.6 ± 1.40*	9.5 ± 1.10*	8.0 ± 0.7*
PEGylated carboxyhemoglobin	21.6 ± 1.1	15.7 ± 1.2	13 ± 1.3	10.4 ± 1.5
Blood transfusion	21.1 ± 0.6	13.9 ± 1.1	18.7 ± 1.7	17.2 ± 1.2
Diluted blood transfusion	20.4 ± 0.2	14.2 ± 1	15.3 ± 0.8	15.3 ± 0.3

The values are presented as means ± SD. Repeated measures two-way ANOVA test was used with Bonferroni correction to adjust for multiple comparisons.
*Adjusted $P < 0.01$ *versus* control group at the same time point. †Adjusted $P < 0.01$ *versus* 6% balanced hydroxyethyl starch group at the same time point.
Cao₂, arterial oxygen content; ND, not determined; PEGylated, polyethylene glycolated.

1 in the plasma. We were unable to detect any significant changes in plasma heme oxygenase 1 between groups, but there was a trend toward higher heme oxygenase 1 levels in the blood transfusion group (Supplemental Digital Content 2B, http://links.lww.com/ALN/C42).

Kidney Immunohistochemistry and Histology

Figure 5 depicts the tumor necrosis factor-α (fig. 5A) and neutrophil gelatinase–associated lipocalin (fig. 5B) histologic scores in the kidney. Hemorrhagic shock produced a significant increase in neutrophil gelatinase–associated lipocalin histologic score compared with control rats (270 [210 to 300] *vs.* 225 [197.5 to 251]; mean rank difference, −46; $P = 0.006$, respectively). Only polyethylene-glycolated carboxyhemoglobin and blood transfusion prevented the alterations in neutrophil gelatinase–associated lipocalin histologic score after hemorrhagic shock and outperformed fluid resuscitation with balanced hydroxyethyl starch ($P < 0.0005$). Consistently, the damage scores in kidney (brush border loss, vacuolization, and luminal casts) were markedly decreased in the polyethylene-glycolated carboxyhemoglobin, diluted blood transfusion, and blood transfusion groups (4 [3.3 to 4.3], 4 [3.7 to 4.7], and 3.7 [3.3 to 4], respectively) compared with balanced hydroxyethyl starch (5 [4.7 to 5]; $P < 0.001$) but remained significantly higher than the controls ($P < 0.001$; fig. 5C).

Fig. 4. Total volume of fluid administered in each group. The volumes are stacked for the sake of clarity. The height of the stacked bins represents the total volume administered. The total volumes (rescue therapy + maintenance) were compared between groups. #Adjusted $P < 0.05$ compared with 6% balanced hydroxyethyl starch. Kruskal–Wallis test with Dunn posttest was used. The data are presented as median [interquartile range] (n = 8 for PEG-carboxyhemoglobin group and n = 6/group for other groups). PEGylated, polyethylene glycolated.

Fig. 5. Kidney immunochemistry and tubular histologic damage scores. (*Left panels*) The histologic scores of tumor necrosis factor-α (*A*) and neutrophil gelatinase-associated lipocalin (*B*) are presented (see Supplemental Digital Content 1, http://links.lww.com/ALN/C41, for additional information). Tubular histologic damage graph (*C*) was drawn by calculating mean of damages of tubular brush border loss, presence of luminal cast and vacuoles. (*Right panels*) Observation of the corresponding histologic images are shown at magnification ×400. The *scale bar* is 50 µm. (*A*) Kidney sections were incubated overnight at 4°C with rabbit polyclonal tumor necrosis factor-α (1:100; Abcam 6671) or (*B*) incubated for 1 h at room temperature with Lipocalin 2 antibody (neurophil gelatinase-associated lipocalin, 1/150; Abcam 41105). (*C*) The kidney sections were stained with periodic acid–Schiff reagent and hematoxyline to depict tubular damage defined as loss of brush border, vacuolar degeneration, and cast formation. *Adjusted $P < 0.05$ compared with control group. #Adjusted $P < 0.05$ compared with 6% balanced hydroxyethyl starch. Kruskal–Wallis test with Dunn's posttest was used. A minimum of 40 tubules were examined per group, with n = 6/ kidney per animal minimum. The data are presented as the median [interquartile range]. PEGylated, polyethylene glycolated.

Discussion

The present study demonstrated that low-volume rescue therapy with polyethylene-glycolated carboxyhemoglobin provided efficient restoration of the macrohemodynamics and maintained a similar kidney microcirculatory Po_2 compared with synthetic balanced hydroxyethyl starch after resuscitation of pressure-controlled hemorrhagic shock in a rodent model. Polyethylene-glycolated carboxyhemoglobin also modulated tubular damages in the kidney, which was associated with a mitigated expression of neutrophil gelatinase–associated lipocalin, because of its embedded carbon monoxide molecules. Polyethylene-glycolated carboxyhemoglobin may blunt the risk for acute kidney injury in this setting. The small increase in oxygen-carrying capacity provided by this molecule did not affect kidney tissue oxygenation or improve lactate or creatinine clearance, but the low volume administered sustained urine output and produced very stable hemodynamic conditions for 60 min after hemorrhagic shock. We also demonstrated that diluted blood transfusion at the same level as polyethylene-glycolated carboxyhemoglobin provided significantly higher renal cortical Po_2 in the kidney but required markedly more volume to reach the targeted MAP.

Low-volume strategies were considered for decades to limit aggressive fluid resuscitation using large uncontrolled fluid boluses that increased bleeding, hemodilution, coagulopathy, hemodynamic decompensation, fluid overload, and excess in mortality.[1,2,23] Our group already published several studies on fluid resuscitation after hemorrhagic shock comparing crystalloids with colloids, balanced or unbalanced, and their consequences on renal cortical Po_2.[24–26] Balanced hydroxyethyl starch solution was found more efficient at restoring macrohemodynamics and renal cortical Po_2 than crystalloids. In the present study, we sought to evaluate an alternative colloid in the context of ongoing controversies about hydroxyethyl starch use. The European Medicines Agency recently issued several restrictions on the use of hydroxyethyl starch solutions in different clinical settings across the European Union.[5] Hydroxyethyl starch solutions are contraindicated in patients with renal impairment and should be administered to manage hemorrhage only when crystalloids alone are not considered sufficient. Therefore, new fluid therapies that outperform crystalloids and compete with the latest synthetic colloid solutions are desired to improve and sustain volume status to promptly restore blood pressure and flow in the context of hemorrhage. However, blood product transfusions remain the gold standard treatment of hemorrhage, but this treatment is not always readily available. Transfusion thresholds have been raised during the last decade with the implementation of restrictive transfusion strategies,[27,28] and fewer patients are likely to receive blood transfusions in the perioperative setting.

Previous generations of hemoglobin-based oxygen carriers failed in clinical practice primarily because of major side effects, such as hypertension and myocardial injury, that were overlooked in preclinical models.[29,30] These effects were presumably related to the extravasation of tetrameric hemoglobin and nitric oxide-scavenging effects. The formulation of hemoglobin-based oxygen carriers has changed to comply with more physiologic prerequisites. Polyethylene-glycolated carboxyhemoglobin provides a larger expansion volume than balanced hydroxyethyl starch partially because of its oncotic effect.[11] The increase in MAP was likely due to the PEGylation process of the carboxyhemoglobin rather than a nitric oxide-scavenging effect. Polyethylene-glycolated carboxyhemoglobin infusion produced similar plasma nitric oxide levels as blood transfusion in our experiment. However, the administration of hydroxyethyl starch alone as a resuscitation fluid significantly decreased the bioavailability of nitric oxide. This result suggests a decreased shear stress–mediated nitric oxide release from the endothelium.[31]

Our group previously demonstrated that a low volume of diaspirin–cross-linked hemoglobin restored epicardial and gut serosal and mucosal microvascular oxygenation.[32,33] Similarly, diaspirin–cross-linked hemoglobin restored pancreatic functional capillary density in a rodent model of hemorrhagic shock.[34] However, increasing the dose of diaspirin–cross-linked hemoglobin produced two main consequences: hyperoxia in the gut serosa and mucosae and severe hypertension. The latter effect was caused by vasoconstriction *via* the inhibition of nitric oxide. Similarly, the extensive literature on bovine hemoglobin–based oxygen carrier (HBOC-201, Hemopure, HbO_2 Therapeutics LLC, USA) reported improvements in tissue oxygenation and macrohemodynamics with induced vasoconstriction.[35] Little data on hemoglobin-based oxygen carriers and kidney oxygenation and function are available. Polyethylene-glycolated carboxyhemoglobin should not be regarded as a hemoglobin-based oxygen carrier *per se*, but more as a colloid with additional beneficial effects. Our experiment highlighted that polyethylene-glycolated carboxyhemoglobin cannot compete with blood transfusion (even diluted at the same hemoglobin concentration) in terms of quality of oxygen-carrying capacity and delivery to hypoxic tissue. In contrast, polyethylene-glycolated carboxyhemoglobin may be an interesting alternative when blood is not an option or in cases of delayed blood transfusion. First, the P_{50} is lower than the one of whole blood (8 to 16 mmHg *vs.* 26 to 30 mmHg, respectively),[11] which impedes oxygen release to moderately hypoxic tissue. Second, we demonstrated that (1) the oxygen-binding capacity of native polyethylene-glycolated carboxyhemoglobin was significantly lower than rat (or human) hemoglobin oxygen-binding capacity even after carbon monoxide release and (2) the concentration of polyethylene-glycolated carboxyhemoglobin in the plasma was too low to expect any significant increase in oxygen delivery compared with whole blood. To test whether polyethylene-glycolated carboxyhemoglobin can release more oxygen, its P_{50} could be theoretically altered by the use of RSR13 (2-[4-[[(3, 5-dimethylanilino)carbonyl]methyl]

phenoxyl]–2-methylproprionic acid),[36] a synthetic allosteric modifier of oxygen–hemoglobin affinity that increases oxygen release (rightward shift of oxygen-hemoglobin dissociation curve) to tissue by allosterically stabilizing deoxyhemoglobin. However, two limits have to be considered: (1) RSR13 will also alter the P_{50} of native hemoglobin, and (2) it could exacerbate acute renal dysfunction.[36,37]

The renal damage was mitigated with the use of polyethylene-glycolated carboxyhemoglobin compared with the administration of hydroxyethyl starch alone. These effects may be attributed to carbon monoxide release. Previous studies demonstrated possible anti-inflammatory effects of carbon monoxide mediated *via* heme oxygenase 1 activation.[38] Nassour *et al.*[16] demonstrated that carbon monoxide-releasing molecules protected against hemorrhagic shock and resuscitation-induced organ injury and inflammation in a similar rodent model of hemorrhagic shock. However, the carbon monoxide-releasing molecules used could not carry oxygen, which highlights the central role of carbon monoxide and heme oxygenase 1 in ischemia and reperfusion injury after hemorrhage.

The off-loading of carbon monoxide molecules from polyethylene-glycolated carboxyhemoglobin is not instantaneous. Approximately 23% of polyethylene-glycolated carboxyhemoglobin remained saturated with carbon monoxide 60 min after the start of resuscitation in our study. Vandegriff *et al.*[39] demonstrated that the percentage of carboxyhemoglobin of coboxyhemoglobin maleimide-activated poly(ethylene) glycol (another polyethylene-glycolated carboxyhemoglobin) decreased to 20 to 25% 30 min after mixing coboxyhemoglobin maleimide-activated poly(ethylene) glycol with erythrocyte *in vitro* which is similar to our observations. In contrast, Zhang *et al.*[40] observed different results in an *in vivo* top-load rodent model. Whole blood and plasma gas analyses revealed a low percentage (5 to 7%) of carboxyhemoglobin 1 and 3 min after polyethylene-glycolated carboxyhemoglobin injection, which suggests a rapid exchange of carbon monoxide between polyethylene-glycolated carboxyhemoglobin and native erythrocyte.[40] Overall, these results demonstrated a disassociation of carbon monoxide molecules from polyethylene-glycolated carboxyhemoglobin, which subsequently re-equilibrates with native erythrocyte. The remaining polyethylene-glycolated hemoglobin (without carbon monoxide) binds oxygen or may be oxidized in the context of hemorrhagic shock. However, whether this chemical process persists after 60 min is not known.

Parrish *et al.*[41] previously brought evidence that the cell-impermeant polyethylene glycol–20 kDa significantly affected colloid pressure and improved survival in low volume resuscitation hemorrhagic shock models. However, the effects of the administration of this polyethylene glycol–20 kDa on the kidney were not assessed. Polyethylene-glycolated carboxyhemoglobin provides additional benefits with its ability to release carbon monoxide molecules and oxygen-binding capacity.

Limitations

First, we focused only on the golden hour after resuscitation after hemorrhagic shock, and long-term results on the use of polyethylene-glycolated carboxyhemoglobin therefore cannot be anticipated. Second, lactate and creatinine levels were higher in the polyethylene-glycolated carboxyhemoglobin group after 1 h because of low-volume resuscitation even when the preset target of resuscitation, namely MAP, was reached. The changes in creatinine clearance may not be reflected at 1 h. However, there is evidence that low-volume resuscitation may improve survival, although it may delay the correction of biologic parameters.[42] The delay in correction of biologic parameters is unlikely to be related to carbon monoxide toxicity because of the very small amount released. However, we cannot exclude a direct toxicity of polyethylene-glycolated hemoglobin. Third, it may be difficult to distinguish the cytoprotective effects of carbon monoxide from the colloidal effect of polyethylene-glycolated carboxyhemoglobin that restores blood flood and pressure. Nevertheless, previous works demonstrated that the administration of hemoglobin-based oxygen carrier in the carboxy state protected against focal ischemia.[39,40] Fourth, 6% balanced hydroxyethyl starch was used in each group to maintain the targeted MAP after the rescue phase, and it may be considered a bias. However, this study design provided a more realistic approach. The use of a crystalloid or colloid to maintain blood volume in a context of hemorrhage occurs daily in clinical practice. Fifth, the observation time was set at 1 h after resuscitation, which may be short. However, the present study sought to specifically assess the effects of polyethylene-glycolated carboxyhemoglobin on renal oxygenation and function in the early phase of resuscitation after hemorrhagic shock. A survival benefit of polyethylene-glycolated carboxyhemoglobin was demonstrated previously.[43] Further research is warranted to determine the mid– to long-term effects on the kidney. An observation period of 3 to 7 days of recovery after resuscitation of hemorrhagic shock with polyethylene-glycolated carboxyhemoglobin would be relevant in this context.

Conclusions

Polyethylene-glycolated carboxyhemoglobin exhibited interesting properties for the resuscitation of hemorrhagic shock, especially because of the limited volume administered. Biomarkers of shock were not corrected as quickly as expected, but there is evidence that polyethylene-glycolated carboxyhemoglobin's dual action protects the kidney against ischemia and reperfusion damages after resuscitation through its carbon monoxide molecules and sustained urine output, which partially contribute to an increase in the oxygen delivery to tissues during the first hour of resuscitation. Further research on colloidal solutions should be intensified to provide the anesthesiologists and intensive care unit physicians an interesting option for meeting unmet clinical needs. Finally, whether polyethylene-glycolated carboxyhemoglobin exhibits long-term protective

effects on the kidney without adverse effects on the other organs should be determined in future studies.

Competing Interests

Dr. Ince owns an Internet site microcirculationacademy.org, which offers services (*e.g.*, training, courses, analysis) related to clinical microcirculation and has received honoraria and independent research grants from Fresenius-Kabi (Bad Homburg, Germany), Baxter Health Care (Deerfield, Illinois), Prolong Pharmaceuticals (South Plainfield, New Jersey), and AM-Pharma (Bunnik, The Netherlands); has developed Sidestream Dark Field imaging; is listed as an inventor on related patents commercialized by MicroVision Medical (Amsterdam, The Netherlands) under a license from the Academic Medical Center; and has been a consultant for MicroVision Medical in the past but has not been involved with this company for more than 5 yr. The company that developed the CytoCam-IDF imaging system, Braedius Medical (Huizen, The Netherlands), is owned by a relative of Dr. Ince. Dr. Ince has no financial relationship with Braedius Medical (*i.e.*, never owned shares or received consultancy or speaker fees). Dr. Jubin is an employee of Prolong Pharmaceuticals. Dr. Jan Bakker is a consultant for Prolong Pharmaceuticals. The other authors declare no competing interests.

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ANESTHESIOLOGY REFLECTIONS FROM THE WOOD LIBRARY-MUSEUM

From Fixing Photos to Fixing Cyanide Poisoning: Herschel's "Hypo" or Sodium Thiosulfate
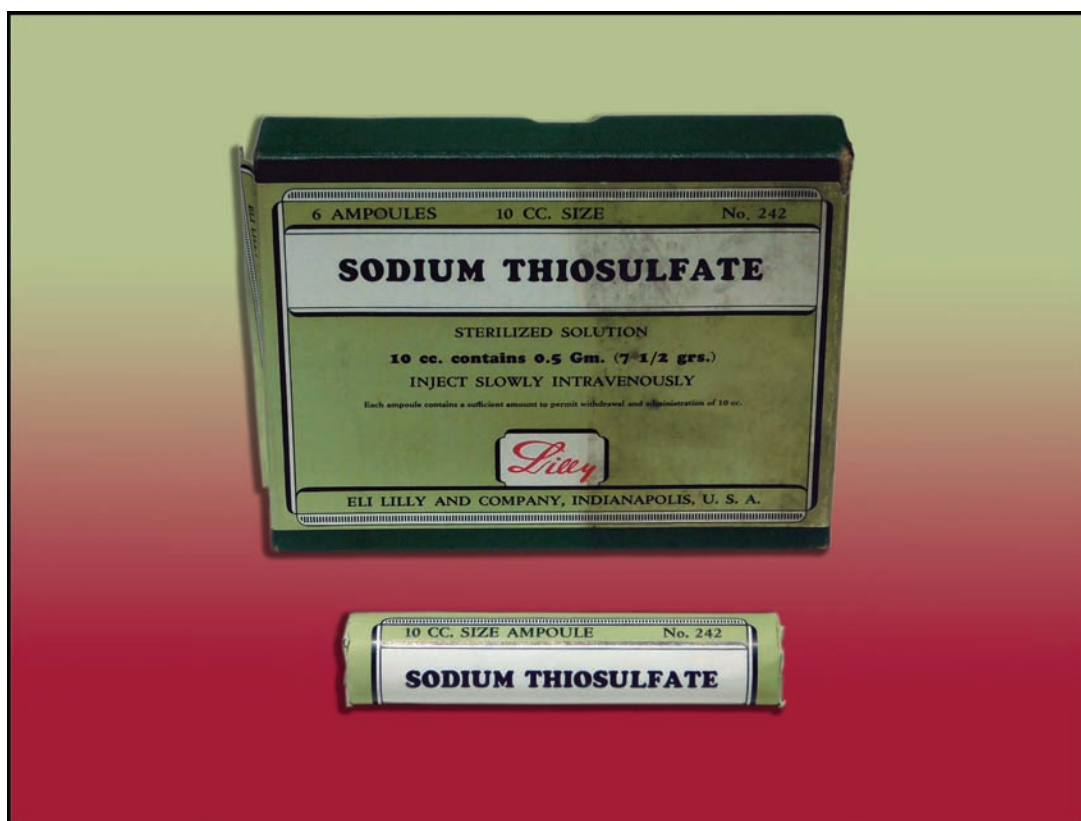
In 1819, a soon-to-be-knighted John Herschel (1792 to 1871) discovered that hyposulfite of soda could "fix" or make photographic images permanent by dissolving away unexposed and otherwise insoluble silver salts. This British genius soon coined terms for his astronomical or botanical images as "negatives" or "positives" and for the art as "photography." Even after it was renamed sodium thiosulfate, hyposulfite of soda retained photographers' nickname for it: "hypo." An antidote for cyanide poisoning, sodium thiosulfate was supplied in ampoules to physicians by Eli Lilly (*above*) and other pharmaceutical firms. Eventually, an editor-in-chief of ANESTHESIOLOGY, Dr. John Michenfelder, would help popularize the antidotal availability of sodium thiosulfate for treating cyanide toxicity resulting from overdoses of antihypertensive infusions of sodium nitroprusside. (Copyright © the American Society of Anesthesiologists' Wood Library–Museum of Anesthesiology.)

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ANESTHESIOLOGY

Vascular Endothelial Growth Factor A Signaling Promotes Spinal Central Sensitization and Pain-related Behaviors in Female Rats with Bone Cancer

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Pain resulting from cancer metastatic to bone is a major clinical problem
- Vascular endothelial growth factor receptors are involved in tumor angiogenesis and are felt to be analgesic targets

What This Article Tells Us That Is New

- In a female rat model of metastatic breast cancer, expression of vascular endothelial growth factor A and its receptor vascular endothelial growth factor receptor 2 were upregulated in spinal tissue
- Blocking vascular endothelial growth factor signaling improved several measures of nociception and function in this model suggesting a role for vascular endothelial growth factor antagonists in reducing cancer-related pain

ABSTRACT

Background: Cancer pain is a pervasive clinical symptom impairing life quality. Vascular endothelial growth factor A has been well studied in tumor angiogenesis and is recognized as a therapeutic target for anti-cancer treatment. This study tested the hypothesis that vascular endothelial growth factor A and vascular endothelial growth factor receptor 2 contribute to bone cancer pain regulation associated with spinal central sensitization.

Methods: This study was performed on female rats using a metastatic breast cancer bone pain model. Nociceptive behaviors were evaluated by mechanical allodynia, thermal hyperalgesia, spontaneous pain, and CatWalk gait analysis. Expression levels were measured by real-time quantitative polymerase chain reaction, western blot, and immunofluorescence analysis. Excitatory synaptic transmission was detected by whole-cell patch-clamp recordings. The primary outcome was the effect of pharmacologic intervention of spinal vascular endothelial growth factor A/vascular endothelial growth factor receptor 2–signaling on bone cancer pain behaviors.

Results: The mRNA and protein expression of vascular endothelial growth factor A and vascular endothelial growth factor receptor 2 were upregulated in tumor-bearing rats. Spinal blocking vascular endothelial growth factor A or vascular endothelial growth factor receptor 2 significantly attenuated tumor-induced mechanical allodynia (mean ± SD: vascular endothelial growth factor A, $7.6 ± 2.6$ g *vs.* $5.3 ± 3.3$ g; vascular endothelial growth factor receptor 2, $7.8 ± 3.0$ g *vs.* $5.2 ± 3.4$ g; n = 6; $P < 0.0001$) and thermal hyperalgesia (mean ± SD: vascular endothelial growth factor A, $9.0 ± 2.4$ s *vs.* $7.4 ± 2.7$ s; vascular endothelial growth factor receptor 2, $9.3 ± 2.5$ s *vs.* $7.5 ± 3.1$ s; n = 6; $P < 0.0001$), as well as spontaneous pain and abnormal gaits. Exogenous vascular endothelial growth factor A enhanced excitatory synaptic transmission in a vascular endothelial growth factor receptor 2–dependent manner, and spinal injection of exogenous vascular endothelial growth factor A was sufficient to cause pain hypersensitivity *via* vascular endothelial growth factor receptor 2–mediated activation of protein kinase C and Src family kinase in naïve rats. Moreover, spinal blocking vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 pathways suppressed protein kinase C-mediated *N*-methyl-D-aspartate receptor activation and Src family kinase-mediated proinflammatory cytokine production.

Conclusions: Vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 contributes to central sensitization and bone cancer pain *via* activation of neuronal protein kinase C and microglial Src family kinase pathways in the spinal cord.

(*Anesthesiology* 2019; 131:1125–47)

Bone cancer pain is the most common and intractable type of cancer pain symptoms and is associated with metastatic breast, lung, and prostate cancer in the clinic, all of which generally consist of ongoing pathologic and break-through pain.[1] Mounting oncologic evidence demonstrates that effective pain control is not only an essential aspect of comprehensive cancer management, but is also linked to prolonged survival.[2] Unfortunately, conventional pharmacologic therapies for bone cancer pain management are inadequate due to either limited analgesic effects or inevitable side effects. Thus, the exact cellular and molecular mechanisms underlying bone cancer pain should be examined within preclinical pain research for active pharmacologic

development. Our and other laboratories have established rat or mouse models of bone cancer pain *via* local injection of tumor cells into the femur, tibia, or calcaneus in order to elicit long-lasting pain hypersensitivity associated with apparent tumor growth and bone destruction.[3,4] Of interest, in these models pain behaviors were detected at the affected hind paw, rather than at the tumor-injected area, which was not directly exposed to tumor infiltration. This phenomenon suggests that although the pathogenic site is derived from the cancer microenvironment and implicated in peripheral sensitization, the bone cancer pain, as a form of secondary hypersensitivity in this context, is particularly dependent on central sensitization, which refers to enhanced nociceptive process *via* a state of neuronal hyperexcitability and glial activation in the spinal dorsal horn.[5]

Vascular endothelial growth factor A is a key mediator of angiogenesis that promotes tumor neovascularization *via* binding to vascular endothelial growth factor receptor 2, which is recognized as a potential target for anti-cancer therapeutics.[6,7] Given that vascular endothelial growth factor A, as a multifunctional cytokine, also plays an important role in the excitability and plasticity of primary sensory neurons in the dorsal root ganglion,[8–10] we hypothesized that vascular endothelial growth factor A in the spinal cord may also be involved in bone cancer pain regulation.

In this study, using a Walker 256 tumor–induced bone cancer pain model in female rats, we determined the expression and localization of vascular endothelial growth factor A and vascular endothelial growth factor receptor 2 in the spinal dorsal horn after tumor inoculation. We also investigated the effect of pharmacologic intervention of vascular endothelial growth factor A and vascular endothelial growth factor receptor 2 in neuronal protein kinase C–mediated *N*-methyl-ᴅ-aspartate (NMDA) receptor activation and microglial Src family kinase–mediated proinflammatory cytokine production, as well as bone cancer pain behaviors induced by tumor inoculation. These new findings extend our understanding of the nonangiogenic roles of vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 signaling pathways in the spinal cord concerning central sensitization and bone cancer pain hypersensitivity.

Materials and Methods
Animals

Adult (8 to 12 weeks; 180 to 220 g) and young (4 to 6 weeks, for electrophysiologic recordings only) Sprague–Dawley rats were purchased from Shanghai Experimental Animal Center of Chinese Academy of Sciences (China), and were housed under a standard 12-h light/dark cycle (light from 8:00 AM to 8:00 PM) at constant room temperature (23 ± 0.5°C) with food and water available *ad libitum*. Animals were numbered and randomly assigned to different experimental groups by use of a random number table, then tested in sequential order. All experimental protocols were approved by Medical Experimental Animal Administrative Committee of Fudan University (Shanghai, China) and were conducted in accordance with the ethical guidelines of the National Institutes of Health and the International Association for the Study of Pain for care and use of laboratory animals under pain research.[11] All efforts were made to minimize the number of animals used as well as their suffering. After the experiments rats were euthanized *via* carbon dioxide inhalation. Researchers were blinded to model condition and drug treatment during behavioral tests. All experiments were conducted between 9:00 AM and 6:00 PM. The primary outcome was the effect of pharmacologic intervention of spinal vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 signaling on bone cancer pain behaviors.

Metastatic Breast Cancer Bone Pain Model

Tumor cells were extracted from the ascitic fluid of rats having received Walker 256 mammary gland carcinoma cells (Institute for Biomedical Research of Shanghai, China); then a cell suspension of 1×10^5 cells/μl in sterile phosphate–buffered saline was prepared. The operating procedure was performed as described in our previous study with some modifications. Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal). The superficial skin of the right hindlimb was shaved and disinfected with 75% (v/v) ethanol, and 5 μl of tumor cells (1×10^5 cells/μl) were slowly injected into the tibia cavity using a 10 μl microinjection syringe. After withdrawing the needle, the injection site was sealed with bone wax to prevent leakage of the tumor cells outside of the bone injection site. For sham control, 5 μl of phosphate–buffered saline was injected into rat right tibia.

Drugs and Administration

Rat vascular endothelial growth factor A neutralizing antibody (AF564) and control antibody (normal goat immunoglobulin G, AB-108-C) were purchased from R&D Systems (USA). Vascular endothelial growth factor receptor 2 inhibitor ZM 323881 (S2896), protein kinase C inhibitor GF109203X (S7208), and Src family kinase inhibitor PP2 (S7008) were purchased from Selleck Chemicals (China). Rat recombinant vascular endothelial growth factor A was purchased from ProSpec (CYT-392; USA). Vascular endothelial growth factor A neutralizing antibody, control immunoglobulin G, and recombinant vascular endothelial growth factor A were dissolved in phosphate–buffered saline. ZM 323881, GF109203X, and PP2 were initially dissolved in dimethyl sulfoxide, then diluted in phosphate–buffered saline (with a final concentration of 1% dimethyl sulfoxide). All reagents were delivered at a volume of 10 μl into the cerebral spinal fluid *via* intrathecal injection, with doses determined by preliminary experiments. For intrathecal

injection, lumbar puncture was performed using a 25-μl Hamilton syringe with a 30-gauge needle in the L5-6 interspace. Reagent doses and treatment time points are described in the figure 2, 3, 5, and 6–9 legends.

Bone Histology

After demineralization in 10% EDTA for 2 weeks, the ipsilateral tibia was embedded in paraffin and cut into 5-mm–thick sections using a microtome with a tungsten carbide blade. Sections were then stained with hematoxylin and eosin to visualize the extent of tumor infiltration and bone destruction, or stained with tartrate-resistant acid phosphatase to visualize activated osteoclasts. Histochemistry images were captured using a digital slide scanner (NanoZoomer S210; Hamamatsu, Japan).

Mechanical Allodynia

Paw withdrawal threshold (threshold) in response to von Frey filament (Aesthesio; Danmic Global, USA) stimulation was measured to represent mechanical allodynia. In brief, after 3 consecutive days for habituation, the rat was placed in a plexiglass chamber on a wire net floor and allowed 10 to 15 min to habituate before experiment. A series of filaments (0.4, 0.6, 1.4, 2, 4, 6, 8, 10, and 15 g) were applied to the mid-plantar surface of the hind paw with sustaining pressure to bend the filament for 5 s or elicit a paw withdrawal reflex within 5 s. Each filament was applied five times, and the 50% threshold (g) was calculated using the following formula: maximum bending force value − [(maximum bending force value − minimum bending force value)/(positive rate of the maximum bending force − positive rate of the minimum bending force)] × (positive rate of the maximum bending force − 50%).

Thermal Hyperalgesia

Paw withdrawal latency (latency) in response to noxious thermal stimulation generated as by a Plantar Analgesia Meter (Model 390, Series 8; IITC Life Science, USA) was measured to represent thermal hyperalgesia. In brief, after 3 consecutive days for habituation, the rat was placed in a plexiglass chamber on a glass plate and allowed 10 to 15 min to habituate before the experiment. The duration from the onset of radiant heat stimulus to the withdrawal of the hind paw was defined as the latency (s), and a 20-s cut-off was set to avoid potential tissue damage. The heat stimulus was repeated three times to determine the latency with a 10-min interval.

Spontaneous Pain

The rat was placed in a transparent plastic cylinder and allowed to habituate for 20 min. After acclimatization, the time spent in spontaneous pain behaviors (s) was measured during a 5-min observation period.[10] Spontaneous pain

behaviors were defined as follows: (1) spontaneous flinching (*i.e.,* lifting the affected limb); (2) spontaneous guarding (*i.e.,* holding aloft the affected limb); and (3) sporadic hopping or limping (*i.e.,* intermittent jumping without using the affected limb during movement).

Limb Use Score

The rat was placed on a glass plate, observed during spontaneous ambulation, and scored on a scale of 0 to 4 as follows: 0 = complete loss of limb usage; 1 = partial loss of limb usage; 2 = clear limping and guarding; 3 = slight limping and guarding; and 4 = normal walking.[12]

CatWalk Automated Gait Analysis

Gait analysis was performed using a CatWalk XT system (Noldus, The Netherlands) based on the voluntary movement of rodents in an enclosed walkway, which has proven to be a reliable method for measuring pain-associated behaviors.[13] The rat was first placed in the open end of the enclosed glass platform under a red ceiling light-emitting diode light and allowed to walk voluntarily through the walkway. While the rat walked across the glass floor, a high-speed camera positioned underneath the apparatus captured images of the illuminated area of each paw and transferred the data to the gait analysis software (CatWalk XT, version 10.0; Noldus). A minimum of three serial step cycles, or complete passes through the tunnel, were gathered as valid data. In this study, four available parameters were identified to evaluate dynamic behaviors associated with bone cancer pain: (1) "max contact area" as the print area during maximun hind paw contact; (2) "max contact max intensity" as the maximum intensity during maximum hind paw contact; (3) "single stance" as the duration of ground contact of a single hind paw where the contralateral hind paw did not touch the glass plate; and (4) "swing" as the duration of no hind paw contact with the glass plate. Data were calculated as the percentage of ipsilateral (right)/contralateral (left) hind paw.

Real-Time Quantitative Polymerase Chain Reaction

Total RNA from L4-6 spinal dorsal horn tissues was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions, and then 1 μg of total RNA was reverse transcribed into cDNA using PrimeScript RT Master Mix (RR036A; Takara, Japan). Each process was carried out in triplicate with a 20-μl reaction mixture (containing 2 μl of cDNA and 10 μM of gene-specific primers) using a SYBR Premix Ex Taq kit (RR420A; Takara) and was run on a 7300 Plus Real-Time PCR system (Applied Biosystems, USA) with thermocycling conditions of 95°C for 30 s followed by 40 amplification cycles (5 s at 95°C and 30 s at 60°C). The mRNA expression levels were analyzed using the $2^{-\Delta\Delta Ct}$ method, in which *glyceraldehyde-3-phosphate dehydrogenase* was used as an endogenous control. The detailed primer sequences (BioTNT, China) are listed in

table S1 (Supplemental Digital Content, http://links.lww.com/ALN/C50).

Western Blot

L4–6 spinal dorsal horn tissues were collected and homogenized in ice-cold radio immunoprecipitation assay lysis buffer containing 1% phenylmethylsulfonyl fluoride and a protease/phosphatase inhibitor cocktail (5872, CST, USA). Protein concentrations were determined by bicinchoninic acid protein assay (23227, Thermo Scientific, USA), and 20 μg in 5 μl of protein per lane was loaded and separated *via* 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The separated proteins were then transferred onto a 0.22-μm polyvinylidene fluoride or polyvinylidene difluoride membrane (Millipore, USA). After blocking with 5% nonfat dry milk in 0.01M tris-buffered saline containing 0.1% Tween-20 for 1h at room temperature, membranes were incubated overnight at 4°C with primary antibodies (table S2, Supplemental Digital Content, http://links.lww.com/ALN/C50). After washing, membranes were incubated with specific horseradish peroxidase–conjugated secondary antibodies (1:10000, Proteintech, China). Proteins were detected using immobilon western chemiluminescent horseradish peroxidase substrate (WBKLS0500; Millipore) and ImageQuant LAS 4000 mini system (GE Healthcare, USA). Blots were analyzed using Quantity One analysis software (Version 4.6.5; Bio-Rad Laboratories, USA), and protein band values of intensity × area (mm²) were normalized to those of glyceraldehyde 3-phosphate dehydrogenase or β–actin. The fold change of the control group was set as 1 for quantification.

Immunofluorescence and Image Analysis

Rats were anesthetized and perfused intracardially with saline and 4% paraformaldehyde in 0.1 M phosphate buffer. L4–6 spinal tissues were removed, fixed in 4% paraformaldehyde overnight at 4°C, then transferred to 30% sucrose in 0.1 M phosphate buffer at 4°C. Subsequently, 30-μm-thick serial sections were prepared using a cryostat and blocked with 10% normal donkey serum in 0.01 M phosphate-buffered saline containing 0.3% Triton X-100 for 2h at room temperature. Spinal sections were then incubated overnight at 4°C with primary antibodies (table S3, Supplemental Digital Content, http://links.lww.com/ALN/C50). After washing, free-floating sections were incubated for 2h at room temperature with the corresponding Alexa Fluor 488-, 594-, or 647-conjugated secondary antibodies (1:1000; Invitrogen). For blocking peptide preabsorption experiment, five-fold blocking peptide of vascular endothelial growth factor A (ab46160; Abcam, USA) or vascular endothelial growth factor receptor 2 (ab39255; Abcam) was mixed with corresponding antibody by weight and incubated overnight at 4°C, then the staining protocol was performed as previously described. After mounting on slides, sections were sealed in mounting medium with 4′,6–diamidino-2-phenylindole (Fluoromount-G; SouthernBiotech, USA)

for storage and visualization. Fluorescence images were captured using a confocal scanning laser microscope (FV1000; Olympus, Japan), and images are shown as merged Z-stack projections consisting of approximately 10 optical slices (1 μm per slice). The number of c-Fos-positive neurons and mean fluorescence intensity of ionized calcium-binding adaptor molecule 1, phospho-protein kinase C, and phospho-Src family kinase within the entire spinal dorsal horn (lamina I-V), including 16 spinal sections from four rats in each group, were measured using ImageJ software (open source software from the National Institutes of Health, USA). For cell counts, images were converted into 16-bit format. The density threshold was adjusted to distinguish cells from background (lower threshold level: approximately 30; upper threshold level: 85), with particles of 10 to 100 pixel units. For mean fluorescence intensity analysis, images were quantified using the default parameters.

Short Hairpin RNA Plasmid Transfection and Immunocytochemistry

Transfection was performed using Lipofectamine 3000 reagent (Invitrogen), following the manufacturer's instruction. In brief, BL6–B6 cells were plated in a six-well plate with 2 ml of Dulbecco Modified Eagle Medium (Invitrogen) containing 10% fetal bovine serum under humidified 95% O_2 and 5% CO_2 at 37°C in for 3 days and allowed to transfect until 70 to 90% confluence. The plasmid DNA-lipid complex was prepared with the two recommended doses of lipid (3.75 μl and 7.5 μl). Transfection was initiated by testing these two concentrations of Lipofectamine 3000 reagent, with selection of the optimum volume of 7.5 μl. Both plasmid DNA and Lipofectamine 3000 reagent were diluted in 125 μl of serum-free Opti-MEM (Invitrogen) medium separately. Then, diluted DNA was added to each tube of the diluted Lipofectamine 3000 Reagent (1:1 ratio) and incubated for 5 min. After incubation, the DNA-lipid complex was mixed gently, added to each well, and incubated for 48 h. The detailed primer sequences (OBiO Technology, China) are listed in table S4 (Supplemental Digital Content, http://links.lww.com/ALN/C50). For immunocytochemistry, the transfected BL6–B6 cells were rinsed for 10 min with 0.01 M phosphate–buffered saline and fixed with 4% paraformaldehyde for 20 min at room temperature. Then, the cells were blocked with 10% normal donkey serum for 2h at room temperature, and subsequently incubated overnight at 4°C with the primary antibody: vascular endothelial growth factor A (1:500, ab46154; Abcam) or vascular endothelial growth factor receptor 2 (1:500, sc-505; Santa Cruz, USA). After washing, the cells were incubated with Alexa Fluor 594–conjugated secondary antibodies (1:1000, Invitrogen) for 2h, sealed in mounting medium with 4′,6-diamidino-2-phenylindole.

Spinal Cord Slice Preparation

Female Sprague–Dawley rats (4 to 6 weeks) were anesthetized and transcardially perfused with preoxygenated

ice-cold sucrose–based artificial cerebrospinal fluid containing the following (in mM): 80 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, 3.5 $MgCl_2$, 25 $NaHCO_3$, 75 sucrose, 1.3 sodium ascorbate, and 3.0 sodium pyruvate. The lumbar spinal segment (L4–L6) was placed in an agarose block and then cut on a vibratome (VT 1200S; Leica, Germany). Spinal slices (300 µm) were then incubated for approximately 1 h at 32°C in a recording solution saturated by a mixture of 95% O_2 and 5% CO_2 containing the following (in mM): 126 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 25 D-glucose, 1.3 sodium ascorbate, and 3.0 sodium pyruvate. Slices were subsequently maintained at room temperature for at least 30 min and then transferred into a recording chamber and perfused with oxygenated recording solution at 3 ml/min during electrophysiologic recordings.

Whole-cell Patch-clamp Recordings

The spinal lamina IIo neurons were recorded under whole-cell patch–clamp mode. The resistance of the pipette electrode was 5 to 8 MΩ when filled with an internal solution containing the following (in mM): 120 potassium gluconate, 20 KCl, 2.0 $MgCl_2$, 2.0 Na_2-ATP, 0.5 Na_2-GTP, 20 HEPES, and 0.5 EGTA (pH 7.3; 300 to 310 mOsm). The membrane current was processed at 30°C using a MultiClamp 700B amplifier (Axon Instruments, USA). Signals were filtered at 4 kHz (lowpass filter frequency) and digitized at 10 kHz (sampling rate) with a Digidata 1440A digitizer (Axon Instruments). A seal resistance (greater than 2 GΩ) within the patch stage and an access resistance (less than 35 MΩ) within the cell stage were considered to be acceptable. Spontaneous excitatory postsynaptic currents were recorded at a holding potential of −70 mV in the presence of 10.0 µM gabazine (SR95531, abcam) and 1.0 µM strychnine (45661; Sigma-Aldrich, USA) to block γ-aminobutyric acid type A and glycine receptors, respectively. Data were collected for approximately 5 min to obtain at least 200 events as the baseline or treatment values in each neuron with pClamp10.3 software (Axon Instruments) and were then processed with Igor Pro 6.02 software (WaveMetrics, USA).

Statistical Analysis

All data are expressed as the mean ± SD. No statistical power calculation was conducted before the study. The sample sizes were based on our previous knowledge and research. There were no missing data, with the exception of five rats that did not survive before the end of the behavior experiments, and were thus excluded from the analysis. All data from different groups were verified for normality and homogeneity of variance using Kolmogorov–Smirnov and Brown–Forsythe tests before analysis. For pain behavioral experiments, differences between groups for threshold and latency results were determined using two-way repeated-measures ANOVA followed by *post hoc* Bonferroni multiple comparison test, and Pearson correlation test was used for the linear correlation analysis; differences between groups for spontaneous pain, limb use score, and CatWalk gait results were determined using one-way ANOVA followed by *post hoc* Dunnett multiple comparison test. For real-time quantitative polymerase chain reaction, western blot, and immunofluorescence experiments, group differences were determined using one-way ANOVA followed by *post hoc* Dunnett multiple comparison test. For electrophysiologic experiments, group differences were determined using a paired or independent unpaired two-tailed Student's *t* test. Differences were considered statistically significant if $P < 0.05$. Outliers, if any, were not evaluated, and no data were excluded from statistical analyses. All statistical analyses were performed using GraphPad Prism 6.0 software.

Results

Intratibial Tumor Inoculation Induces Bone Destruction, Pain Hypersensitivity, and Spinal Central Sensitization

To verify the establishment of the bone cancer pain model in female rats, we evaluated the microscopic characteristics of tumor growth, osteoclast activation, and bone destruction in the bone microenvironment after tumor inoculation. Hematoxylin and eosin staining showed progressive tumor cell growth in the bone marrow cavity and destruction of the cortical and trabecular bone in a time-dependent manner on days 3, 7, and 14 post–tumor inoculation. In addition, tartrate-resistant acid phosphatase staining also showed gradually progressive bone destruction on days 3, 7, and 14 post–tumor inoculation, with obvious reactive osteoclasts, the principal bone-resorbing cells, on day 14 post–tumor inoculation. However, these microscopic characteristics were not observed in sham rats (fig. 1A). Consistent with our previous studies and others, tumor-bearing rats exhibited progressive nociceptive hypersensitivity after tumor inoculation, which was characterized by mechanical allodynia and thermal hyperalgesia in the affected limb.[4,14] As a sign of mechanical allodynia, the threshold caused by von Frey filament stimulation was decreased significantly on day 5 and maintained until day 21 post–tumor inoculation (fig. 1B). As a sign of thermal hyperalgesia, the latency caused by radiant heat stimulation was also decreased significantly on day 7 and maintained until day 21 post–tumor inoculation (fig. 1C). In addition, male rats suffering from tumor inoculation showed similar behavioral changes (fig. S1, Supplemental Digital Content, http://links.lww.com/ALN/C50).

It is well established that persistent noxious stimulus from the bone cancer microenvironment can enhance spinal central sensitization through primary afferent fibers projecting to the L4–L6 spinal dorsal horn, which is a critical pathogenic mechanism underlying bone cancer pain.[15,16]
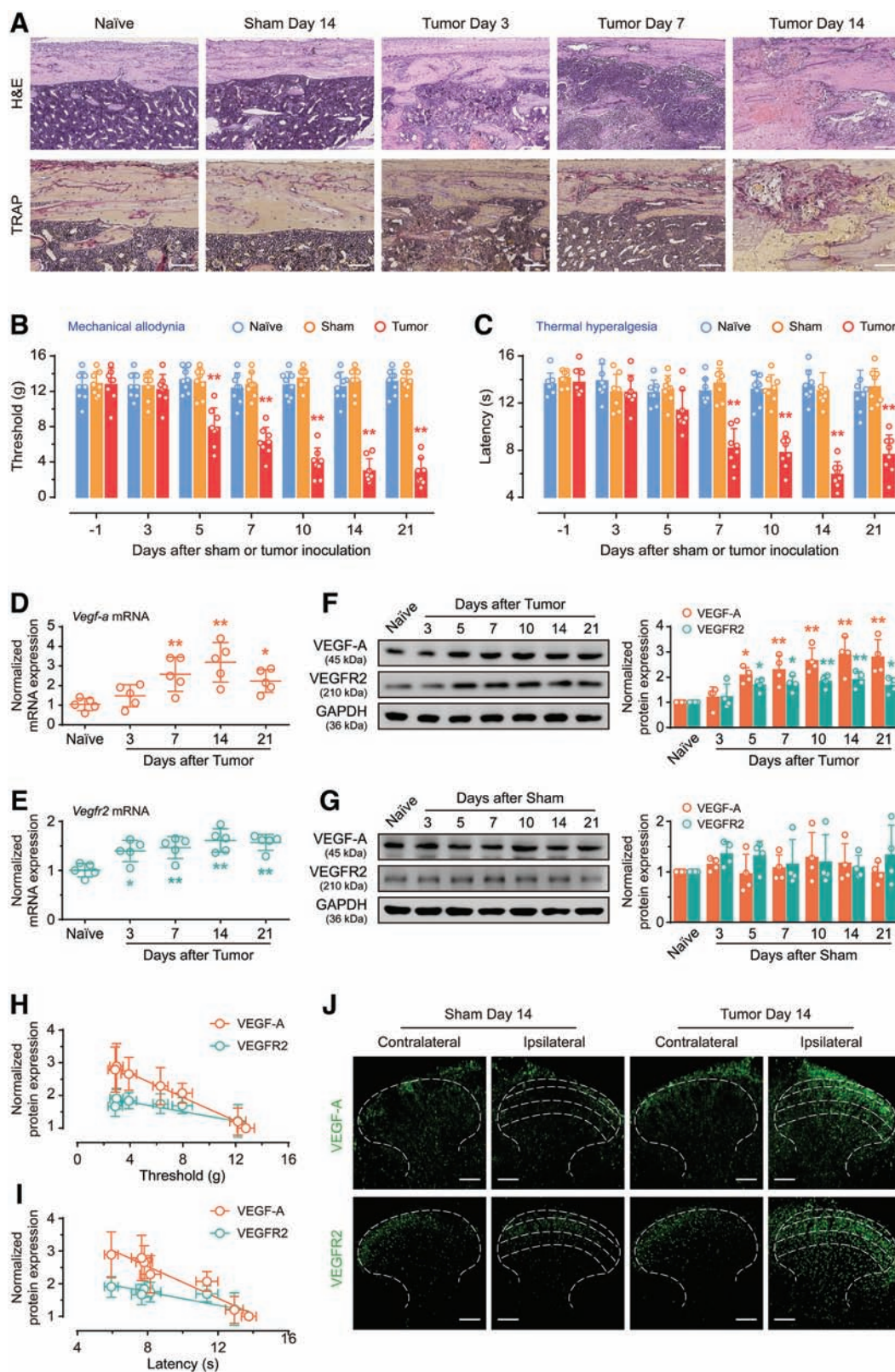
Fig. 1. *(Continued)*

Fig. 1. Expression and distribution of vascular endothelial growth factor A (VEGF-A) and vascular endothelial growth factor receptor 2 (VEGFR2) in the spinal cord after tumor inoculation. (*A*) Hematoxylin and eosin (H&E) staining shows that bone substance and bone marrow were destroyed and infiltrated by cancer cells after tumor inoculation. Tartrate-resistant acid phosphatase (TRAP) staining shows that bone destruction and osteoclasts were increased and activated by cancer cells after tumor inoculation. Scale bars: 200 µm. (*B, C*) Behavioral analysis shows that tumor inoculation induced reduction of threshold (*B*) and latency (*C*) in the affected hind paw. Data are expressed as mean ± SD. **$P < 0.01$ *versus* naïve group, N = 8 for each group, two-way repeated-measures ANOVA with *post hoc* Bonferroni test. (*D, E*) Real-time quantitative polymerase chain reaction analysis shows the time-course expression of *Vegf-a* (*D*) and *Vegfr2* (*E*) mRNA in rats before (naïve) and after tumor inoculation. Data were normalized to the housekeeping gene *Gapdh* (N = 5 for each time point). (*F, G*) Western blot analysis shows the time-course expression of VEGF-A and VEGFR2 protein in rats before (naïve) and after tumor inoculation (*F*) or sham operation (*G*). Data were normalized to the housekeeping protein GAPDH (N = 4 for each time point). (*D* to *G*) Data are expressed as mean ± SD. *$P < 0.05$, **$P < 0.01$ *versus* naïve group, one-way ANOVA with *post hoc* Dunnett test. (*H, I*) Pearson correlation test shows that the relative levels of VEGF-A and VEGFR2 protein expression were negatively correlated with the threshold (*H*, VEGF-A: Y = −0.1813 × X + 3.397; R^2 = 0.9912; $P < 0.0001$; VEGFR2: Y = −0.07389 × X + 2.101; R^2 = 0.8277; $P = 0.0045$) or latency (*I*, VEGF-A: Y = −0.2431 × X + 4.476; R^2 = 0.9229; $P = 0.0006$; VEGFR2: Y = −0.1016 × X + 2.565; R^2 = 0.8111; $P = 0.0057$), respectively. (*J*) Immunofluorescence shows that VEGF-A and VEGFR2 were low in both sides of the spinal dorsal horn in sham rats; but increased in the ipsilateral spinal dorsal horn compared with the contralateral spinal dorsal horn in tumor-bearing rats. Tissues were collected on day 14 after sham or tumor inoculation. Scale bars: 100 µm.

In the present study, we further confirmed that the immunofluorescence expression of c-Fos (marker for neuronal sensitization), glial fibrillary acidic protein (marker for astrocytic activation), and ionized calcium-binding adaptor molecule 1 (marker for microglial activation) in the ipsilateral spinal dorsal horn were all distinctly increased on day 14 post–tumor inoculation in both female and male rats, which demonstrated spinal central sensitization in the bone cancer pain condition (fig. S2, Supplemental Digital Content, http://links.lww.com/ALN/C50).

Vascular Endothelial Growth Factor A/Vascular Endothelial Growth Factor Receptor 2 Is Upregulated in the Spinal Cord after Tumor Inoculation

To investigate the expression and distribution of vascular endothelial growth factor A and vascular endothelial growth factor receptor 2 in the spinal dorsal horn after tumor inoculation, we first analyzed changes in the mRNA expression of *vascular endothelial growth factor A* and *vascular endothelial growth factor receptor 2* in the spinal dorsal horn during the precancerous (day 3), early (day 7), advanced (day 14), and late (day 21) phases of bone cancer pain. Compared with naïve rats, *vascular endothelial growth factor A* mRNA in the spinal dorsal horn was significantly upregulated on day 7 and remained at high levels until day 21 post–tumor inoculation (fig. 1D). Similarly, *vascular endothelial growth factor receptor 2* mRNA in the spinal dorsal horn was persistently upregulated from day 3 to day 21 post–tumor inoculation (fig. 1E), suggesting upregulation of *vascular endothelial growth factor A* and *vascular endothelial growth factor receptor 2* genes *in situ* throughout the course of bone cancer pain. Consistent with real-time quantitative polymerase chain reaction analysis, western blot analysis also showed that tumor inoculation induced rapid-onset and long-lasting expression of vascular endothelial growth factor A and vascular endothelial growth factor receptor 2 proteins in the spinal dorsal horn, which was significantly increased on

day 5 and maintained through day 21 (fig. 1F). However, protein expression levels were not changed in sham rats at any detected time point (fig. 1G). Meanwhile, for all time points observed, the correlation analysis showed that the time course of tumor-induced vascular endothelial growth factor A and vascular endothelial growth factor receptor 2 protein upregulation shown in figure 1F was closely related to that of tumor-induced reduction in the threshold shown in figure 1B (fig. 1H) and the latency shown in figure 1C (fig. 1I), respectively. Furthermore, immunofluorescence revealed that the increased expression of vascular endothelial growth factor A and vascular endothelial growth factor receptor 2 was distributed predominately in the ipsilateral, but not contralateral spinal dorsal horn, on day 14 post–tumor inoculation and specifically distributed in the superficial spinal dorsal horn, a region critical for processing nociceptive signal transmission (fig. 1J). According to published proposals,[9,14,17] the vascular endothelial growth factor A and vascular endothelial growth factor receptor 2 antibody specificity was verified by short hairpin RNA plasmid transfection experiment in B16–BL6 cells and by blocking peptide preabsorption experiment in rat spinal cord (fig. S3, Supplemental Digital Content, http://links.lww.com/ALN/C50). Taken together, these results suggest that spinal vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 may be functionally upregulated and is relevant for bone cancer pain behaviors.

Vascular Endothelial Growth Factor A/Vascular Endothelial Growth Factor Receptor 2 Mediates Bone Cancer Pain Behaviors

Next, we employed pharmacologic approaches to examine the regulatory role of spinal vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 in the maintenance of bone cancer pain. A single intrathecal injection of vascular endothelial growth factor A neutralizing antibody or vascular endothelial growth
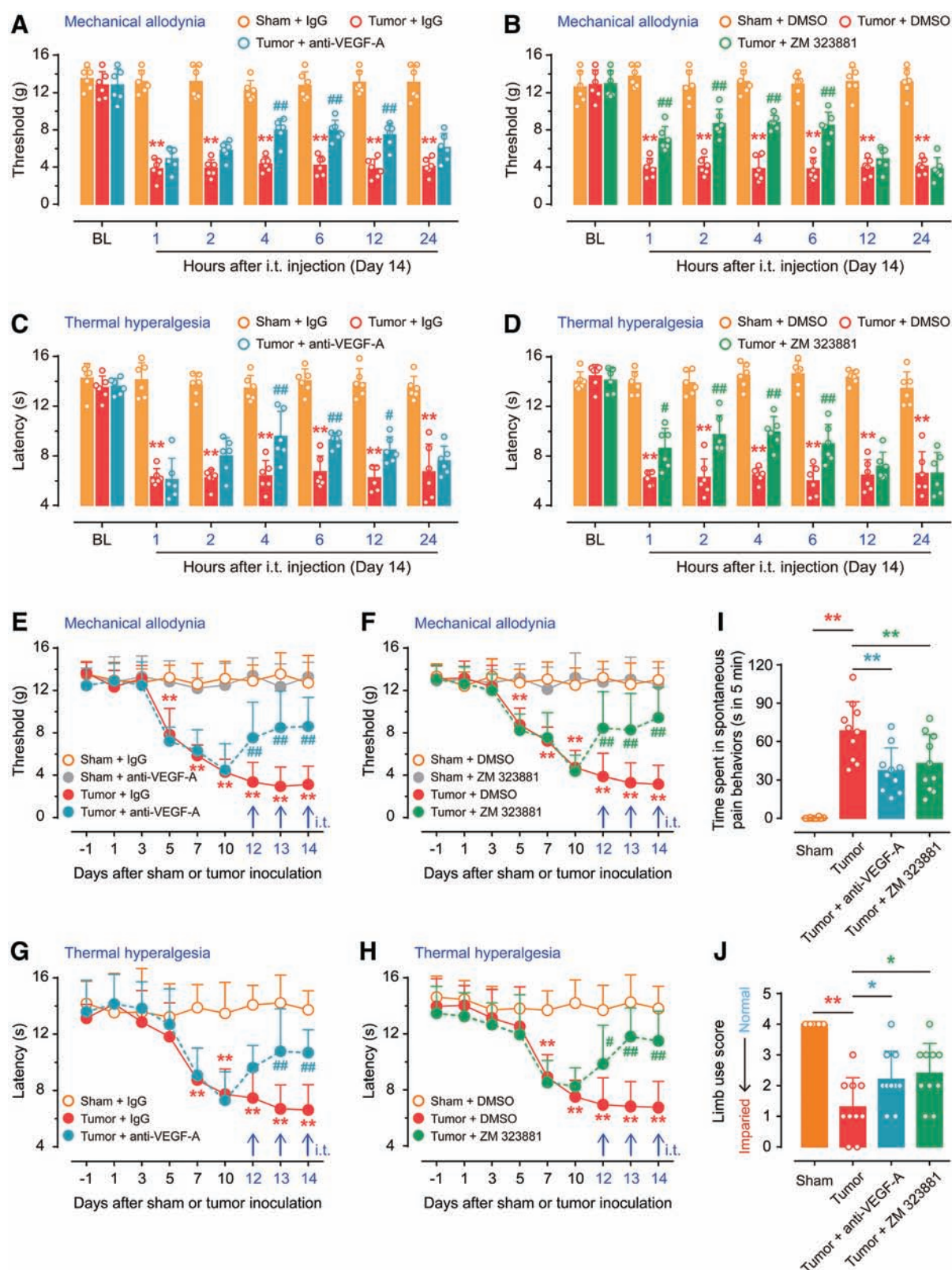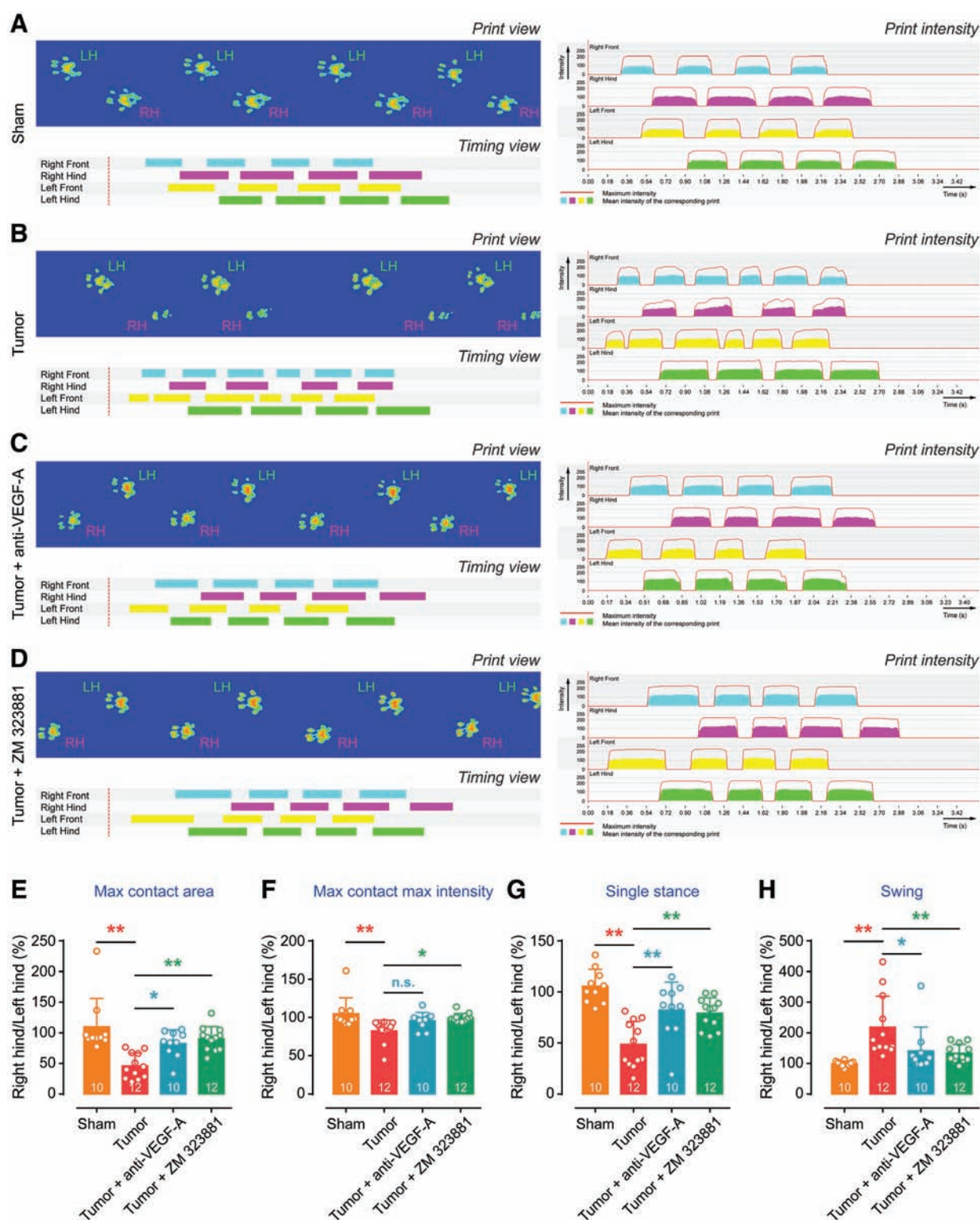
Fig. 2. *(Continued)*

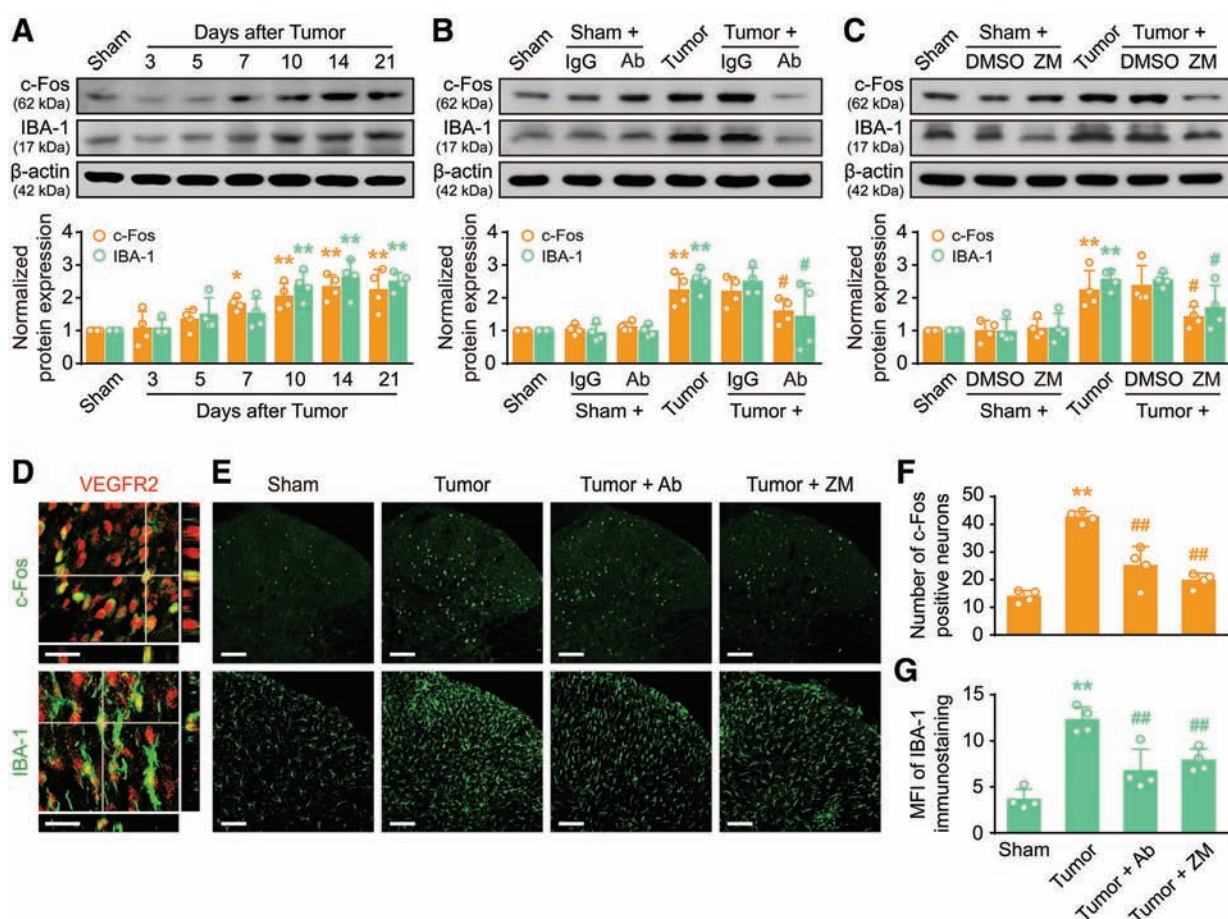**Fig. 2.** Spinal blockade of vascular endothelial growth factor A (VEGF-A)/vascular endothelial growth factor receptor 2 (VEGFR2) attenuates mechanical allodynia, thermal hyperalgesia and spontaneous pain in tumor-bearing rats. (*A* to *D*) Single administration of VEGF-A neutralizing antibody or VEGFR2 inhibitor ZM 323881 time-dependently reversed tumor-induced reduction in the threshold (*A, B*) and latency (*C, D*) in tumor-bearing rats. VEGF-A neutralizing antibody (anti-VEGF-A, 2 µg, intrathecal), immunoglobulin G (IgG; vehicle control for anti-VEGF-A, 2 µg, intrathecal [i.t.]), ZM 323881 (100 nM, i.t.) or dimethyl sulfoxide (DMSO)(vehicle control for ZM 323881, 1%, i.t.) was injected on day 14 post–tumor inoculation. Data are expressed as mean ± SD. **$P < 0.01$ *versus* sham + IgG (or DMSO) group; #$P < 0.05$, ##$P < 0.01$ *versus* tumor + IgG (or DMSO) group, N = 6 for each group, two-way repeated-measures ANOVA with *post hoc* Bonferroni test. (*E* to *H*) Repetitive administration of VEGF-A neutralizing antibody or ZM 323881 significantly attenuated tumor-induced reduction in the threshold (*E, F*) and latency (*G, H*) in tumor-bearing rats. VEGF-A neutralizing antibody (anti-VEGF-A, 2 µg, i.t.), IgG (2 µg, i.t) ZM 323881 (100 nM, i.t.) or DMSO (1%, i.t.) was injected once daily on days 12, 13, and 14 post–tumor inoculation. Behavioral tests were performed 4 h after each injection. Data are expressed as mean ± SD. **$P < 0.01$ *versus* sham + IgG (or DMSO) group; #$P < 0.05$, ##$P < 0.01$ *versus* tumor + IgG (or DMSO) group, N = 8 for each group, two-way repeated-measures ANOVA with *post hoc* Bonferroni test. (*I, J*) Repetitive administration of VEGF-A neutralizing antibody or ZM 323881 significantly attenuated tumor-induced spontaneous pain behaviors (*I*) and limb uselessness (*J*) in tumor-bearing rats. VEGF-A neutralizing antibody (anti-VEGF-A, 2 µg, i.t.) or ZM 323881 (100 nM, i.t.) was injected once daily on days 12, 13, and 14 post–tumor inoculation. Behavioral tests were performed 4 h after the last injection on day 14. Data are expressed as mean ± SD. *$P < 0.05$, **$P < 0.01$, N = 10 for each group, one-way ANOVA with *post hoc* Dunnett test.

factor receptor 2–specific inhibitor ZM 323881 on day 14 post–tumor inoculation time-dependently alleviated tumor-induced established mechanical allodynia (fig. 2A: 7.6 ± 2.6 g *vs.* 5.3 ± 3.3 g; fig. 2B: 7.8 ± 3.0 g *vs.* 5.2 ± 3.4 g; n = 6; $P < 0.0001$) and thermal hyperalgesia (fig. 2C: 9.0 ± 2.4 s *vs.* 7.4 ± 2.7 s; fig. 2D: 9.3 ± 2.5 s *vs.* 7.5 ± 3.1 s; n = 6; $P < 0.0001$). Similar results were also observed in male rats on day 14 post–tumor inoculation (fig. S1, Supplemental Digital Content, http://links.lww.com/ALN/C50). Furthermore, repetitive intrathecal injection of vascular endothelial growth factor A neutralizing antibody or ZM 323881 once daily for 3 consecutive days on days 12, 13, and 14 post–tumor inoculation effectively and persistently attenuated tumor-induced mechanical allodynia (fig. 2, E and F) and thermal hyperalgesia (fig. 2, G and H). In addition, tumor-related spontaneous pain (fig. 2I) and limb uselessness (fig. 2J) were also relieved by repeated intrathecal administration of vascular endothelial growth factor A neutralizing antibody or ZM 323881 on day 14 post–tumor inoculation.

CatWalk gait analysis is suggested to be a valuable method for the objective assessment of chronic pain behavior in several neuropathic and inflammatory pain models.[18,19] Here, we employed CatWalk analysis in the context of bone cancer pain research, with consideration of its comprehensive and detailed analysis of the affected hind paw during voluntary movement. We selected four specific parameters that were significantly altered in tumor-bearing rats: (1) "max contact area," considered as the hind paw print area during max contact; (2) "max contact max intensity," considered as the maximum intensity of the maximum hind paw contact; (3) "single stance," considered as the duration of ground contact for a single hind paw during which the contralateral hind paw did not touch the glass plate; and (4) "swing," considered as the duration of no hind paw contact with the glass plate. Percentages of ipsilateral (right)/contralateral (left) hind paw ratio of max contact area, max contact max intensity, single stance, and swing were approximately 100%

in sham rats on day 14 (fig. 3A). After tumor inoculation, percentages of max contact area, max contact max intensity, and single stance were decreased significantly, whereas percentage of swing was increased significantly on day 14, which could be representative of bone cancer pain behaviors from different aspects (fig. 3B). However, repeated intrathecal injection of vascular endothelial growth factor A neutralizing antibody (fig. 3C) or ZM 323881 (fig. 3D) partially, but significantly, reversed tumor-induced gait alterations (fig. 3, E to H).

Vascular Endothelial Growth Factor A/Vascular Endothelial Growth Factor Receptor 2 Is Responsible for Neuronal Sensitization and Microglial Activation in Tumor-bearing Rats

The findings above (figs. 2 and 3) indicated a modulatory role of vascular endothelial growth factor A and vascular endothelial growth factor receptor 2 in the spinal processing of bone cancer pain. However, the specific cellular mechanism underlying vascular endothelial growth factor A/vascular endothelial growth factor receptor 2–mediated bone cancer pain remains unclear. Thus, we further defined the cellular localization of vascular endothelial growth factor A and vascular endothelial growth factor receptor 2 *via* immunostaining with various cell markers in the spinal dorsal horn on day 14 post–tumor inoculation. Immunofluorescence demonstrated that vascular endothelial growth factor A predominantly colocalized with the central terminals of primary sensory neurons (calcitonin gene–related peptide and isolectin B4) and spinal neurons (neuronal nuclei), and occasionally colocalized with oligodendrocytes (oligodendrocyte lineage transcription factor 2) (fig. 4A). Meanwhile, vascular endothelial growth factor receptor 2 predominantly colocalized with spinal neurons and microglia (ionized calcium–binding adaptor molecule 1) (fig. 4B). Similar results were also observed in male rats on day 14 post–tumor inoculation (fig. S4, Supplemental Digital Content, http://links.lww.com/ALN/C50).

Fig. 3. *(Continued)*

Fig. 3. Spinal blockade of vascular endothelial growth factor A (VEGF-A)/vascular endothelial growth factor receptor 2 (VEGFR2) attenuates abnormal gait in tumor-bearing rats. (*A* to *D*) Representative CatWalk gait, including print view, timing view, and print intensity, in sham (*A*), tumor (*B*), tumor + anti-VEGF-A (*C*), and tumor + ZM 323881 (*D*) group. (*E* to *H*) Spinal administration of VEGF-A neutralizing antibody or VEGFR2 inhibitor ZM 323881 significantly attenuated tumor-induced decrease in max contact area (*E*), max contact max intensity (*F*) and single stance (*G*), as well as increase in swing (*H*) in tumor-bearing rats. VEGF-A neutralizing antibody (anti-VEGF-A, 2 µg, intrathecal [i.t.]) or ZM 323881 (100 nM, i.t.) was injected once daily on days 12, 13, and 14 post–tumor inoculation. Behavioral tests were performed 4 h after the last injection. Data were calculated as percentage of ipsilateral (right)/contralateral (left) hind paw. Data are expressed as mean ± SD. *P < 0.05, **P < 0.01, one-way ANOVA with *post hoc* Dunnett test. The number of detected animals is indicated inside of each column. LH, left hind; n.s., no statistical significance; RH, right hind.
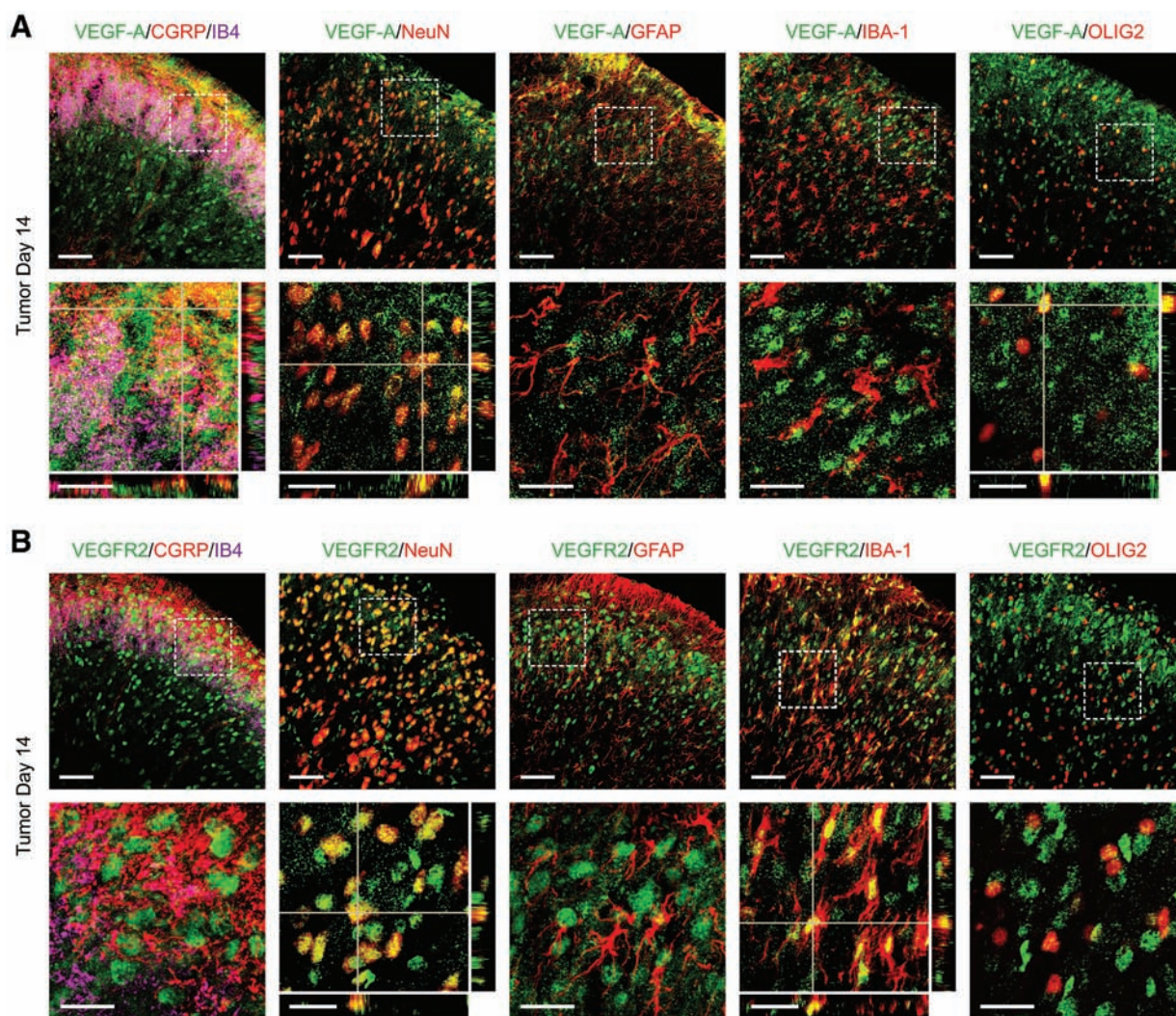
Fig. 4. Cellular localization of vascular endothelial growth factor A (VEGF-A) and vascular endothelial growth factor receptor 2 (VEGFR2) in the spinal cord after tumor inoculation. (*A, B*) Immunofluorescence staining of VEGF-A (*A*) and VEGFR2 (*B*) combined with cell markers in the spinal dorsal horn: calcitonin gene-related peptide (CGRP; peptidergic primary afferent terminals), isolectin B4 (IB4; nonpeptidergic primary afferent terminals), neuronal nuclei (NeuN; neurons), glial fibrillary acidic protein (GFAP; astrocytes), ionized calcium-binding adaptor molecule 1 (IBA-1; microglia), and oligodendrocyte lineage transcription factor 2 (OLIG2; oligodendrocytes). (*A*) VEGF-A immunoreactivity predominantly colocalized with CGRP, IB4 and NeuN, occasionally colocalized with OLIG2; (*B*) VEGFR2 immunoreactivity predominantly colocalized with NeuN and IBA-1. Tissues were collected on day 14 post–tumor inoculation. Scale bars: 50 µm and 20 µm (zoom).

Given that vascular endothelial growth factor receptor 2 was expressed and increased in both spinal neurons and microglia, we next investigated whether vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 mediates neuronal sensitization and microglial activation in bone cancer pain conditions. Western blot analysis showed that tumor inoculation caused a persistent and evident upregulation of c-Fos and ionized calcium–binding adaptor molecule 1, indicating activation of neurons and microglia in the spinal dorsal horn, respectively (fig. 5A). However, this tumor-induced c-Fos and ionized calcium–binding adaptor molecule 1 upregulation was remarkably suppressed by repeated intrathecal injection of vascular endothelial growth factor A neutralizing antibody (fig. 5B) or ZM 323881 (fig. 5C). In addition, we observed that c-Fos and ionized calcium–binding adaptor molecule 1 were clearly coexpressed with vascular endothelial growth factor receptor 2 (fig. 5D), and immunofluorescence analysis showed that tumor-induced increase in number of c-Fos-positive neurons and mean fluorescence intensity of ionized calcium–binding adaptor molecule 1-labeled microglia were both suppressed by vascular endothelial growth factor A neutralizing antibody or ZM 323881 in the spinal dorsal horn (fig. 5, E to G). Taken together, these

Fig. 5. Spinal blockade of vascular endothelial growth factor A (VEGF-A)/vascular endothelial growth factor receptor 2 (VEGFR2) suppresses tumor-induced neuronal sensitization and microglial activation. (*A*) Tumor inoculation induced upregulation of c-Fos and ionized calcium-binding adaptor molecule 1 (IBA-1) in a time-dependent manner (N = 4 for each time point). Data are expressed as mean ± SD. *$P <$ 0.05, **$P <$ 0.01 *versus* sham group, one-way ANOVA with *post hoc* Dunnett test. (*B, C*) Spinal injection of VEGF-A neutralizing antibody (*B*) or VEGFR2 inhibitor ZM 323881 (*C*) significantly suppressed tumor-induced upregulation of c-Fos and IBA-1 expression in the spinal dorsal horn. (*D* to *G*) VEGFR2 immunoreactivity was primarily coexpressed with c-Fos or IBA-1 in the spinal dorsal horn (*D*), and spinal injection of VEGF-A neutralizing antibody or ZM 323881 significantly suppressed tumor-increased number of c-Fos (*E, F*) and mean fluorescence intensity (MFI) of IBA-1 (*E, G*). Scale bars: 20 μm (*D*) and 100 μm (*E*). (*B, C, F, G*) VEGF-A neutralizing antibody (Ab, 2 μg, intrathecal), IgG (2 μg, intrathecal), ZM 323881 (ZM, 100 nM, intrathecal) or DMSO (1%, intrathecal) was injected once daily on days 12, 13, and 14 post–tumor inoculation. Tissues were collected 4 h after the last injection on day 14. Data are expressed as mean ± SD. **$P <$ 0.01 *versus* sham group; #$P <$ 0.05, ##$P <$ 0.01 *versus* tumor group, N = 4 for each group, one-way ANOVA with *post hoc* Dunnett test.

results suggest that spinal vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 may directly impact neuronal sensitization and microglial activation underlying the bone cancer pain state.

Vascular Endothelial Growth Factor A/Vascular Endothelial Growth Factor Receptor 2 Enhances Excitatory Synaptic Transmission in Spinal Neurons

Since vascular endothelial growth factor receptor 2 could regulate neuronal sensitization and microglial activation, we hypothesized that vascular endothelial growth factor receptor 2 could enhance excitatory synaptic transmission *via* activating neurons and microglial cells. Thus, we further examined the effects of vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 on spontaneous excitatory postsynaptic currents in lamina IIo neurons in naïve rats. After whole cell configuration was established, recombinant vascular endothelial growth factor A perfusion induced an evident increase in both the frequency and amplitude of spontaneous excitatory postsynaptic currents (fig. 6, A to C). However, vascular endothelial growth factor A–increased frequency and amplitude of spontaneous excitatory postsynaptic currents

were remarkably suppressed by ZM 323881 perfusion (fig. 6, D to H). These results indicate that vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 enhances excitatory synaptic transmission *via* promoting presynaptic glutamate vesicle release (*i.e.,* presynaptic effect) and postsynaptic glutamate receptor expression and function (*i.e.,* postsynaptic effect).[20]

Protein Kinase C and Src Family Kinase Signals Are Functionally Activated in Vascular Endothelial Growth Factor A/Vascular Endothelial Growth Factor Receptor 2–Mediated Pain Hypersensitivity in Naïve Rats

Since exogenous vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 could enhance the excitatory synaptic transmission of superficial spinal neurons, we investigated whether vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 at the spinal level was sufficient to evoke pain hypersensitivity in naïve rats. After recombinant vascular endothelial growth factor A intrathecal administration, rats manifested an obvious mechanical allodynia and thermal hyperalgesia in a time-dependent manner within 1 h, with persistence for at least 12 h. However, intrathecal delivery of ZM
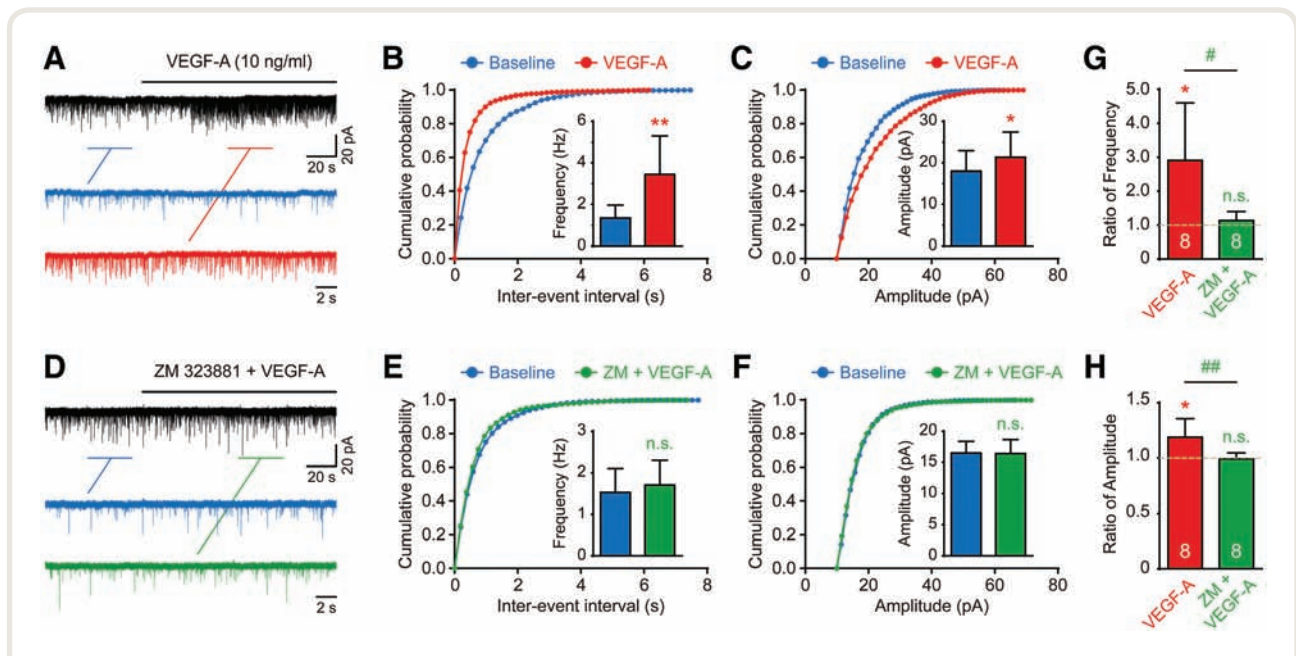
Fig. 6. Vascular endothelial growth factor A (VEGF-A) increases spontaneous excitatory postsynaptic currents in lamina IIo spinal neurons *via* vascular endothelial growth factor receptor 2 (VEGFR2). (*A*) Patch-clamp recording showing the frequency and amplitude of spontaneous excitatory postsynaptic currents after perfusion with VEGF-A (10 ng/ml). (*B, C*) Corresponding cumulative distribution and quantification of spontaneous excitatory postsynaptic current frequency (*B*) and amplitude (*C*). (*D*) Patch-clamp recording showing the frequency and amplitude of spontaneous excitatory postsynaptic currents after perfusion with ZM 323881 (ZM, 10 nM) + VEGF-A (10 ng/ml). (*E, F*) Corresponding cumulative distribution and quantification of spontaneous excitatory postsynaptic current frequency (*E*) and amplitude (*F*). (*G, H*) Ratio of the frequency (*G*) and amplitude (*H*) of spontaneous excitatory postsynaptic currents following treatment with VEGF-A (10 ng/ml) or ZM 323881 (ZM, 10 nM) + VEGF-A (10 ng/ml). *Dashed line* indicates baseline (set as 1 for normalization). Data are expressed as mean ± SD. *$P < 0.05$, **$P < 0.01$ *versus* baseline; #$P < 0.05$, ##$P < 0.01$ *versus* VEGF-A group, N = 8 neurons from 4 rats, Student's paired (*B, C, E, F*) or independent unpaired (*G, H*) two-tailed *t* test.
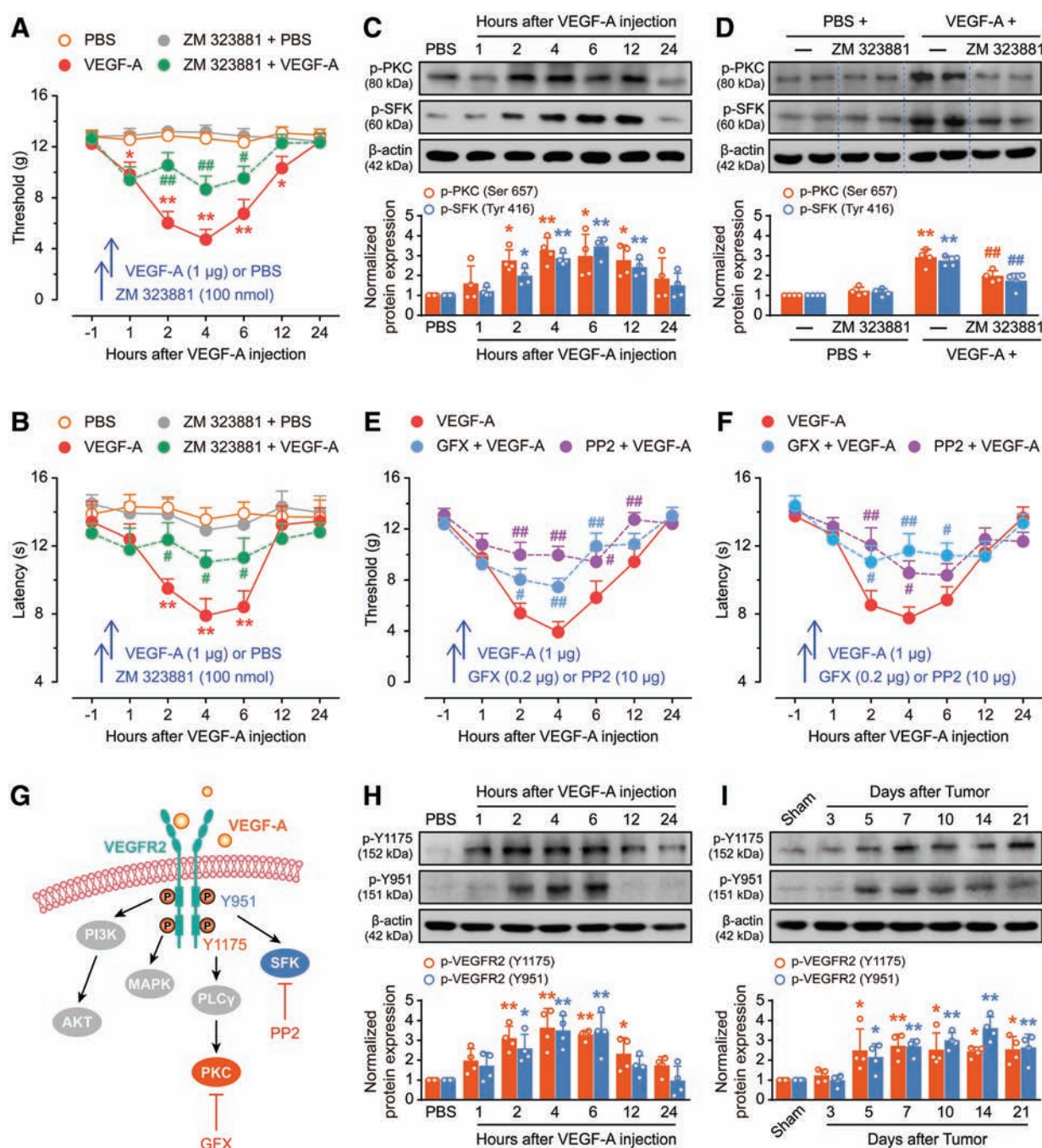
Fig. 7. Vascular endothelial growth factor A (VEGF-A) induces vascular endothelial growth factor receptor 2 (VEGFR2)–dependent pain hypersensitivity in naïve rats *via* activating protein-kinase C (PKC) and Src family kinase (SFK). (*A, B*) Spinal administration of exogenous VEGF-A–induced time-dependent mechanical allodynia (*A*) and thermal hyperalgesia (*B*) in naïve rats, while preadministration of VEGFR2 inhibitor ZM 323881 30 min before VEGF-A injection partially attenuated VEGF-A–induced pain hypersensitivity. Data are expressed as mean ± SD. *$P < 0.05$, **$P < 0.01$ *versus* phosphate-buffered saline group; #$P < 0.05$, ##$P < 0.01$ *versus* VEGF-A group, N = 8 for each group, two-way repeated-measures ANOVA with *post hoc* Bonferroni test. (*C, D*) Exogenous VEGF-A induced the upregulation of phospho- (p-)-PKC and p-SFK in the spinal dorsal horn (*C*), while preadministration of ZM 323881 30 min before VEGF-A injection significantly suppressed VEGF-A–increased p-PKC and p-SFK expression (*D*). Data are expressed as mean ± SD. *$P < 0.05$, **$P < 0.01$ *versus* phosphate-buffered saline group; #$P < 0.05$, ##$P < 0.01$ *versus* VEGF-A group, N = 4 for each group, one-way ANOVA with *post hoc* Dunnett test. (*E, F*) Preadministration of PKC inhibitor GF109203X or SFK inhibitor PP2 30-min before VEGF-A injection partially attenuated VEGF-A–induced mechanical allodynia (*E*)

(Continued)

(**Fig. 7.** *Continued*) and thermal hyperalgesia (*F*) in naïve rats. Data are expressed as mean ± SD. #*P* < 0.05, ##*P* < 0.01 *versus* VEGF-A group, N = 8 for each group, two-way repeated-measures ANOVA with *post hoc* Bonferroni test. (*G*) A schematic overview of VEGF-A–mediated VEGFR2 activation and intracellular signaling pathways. (*H*) Exogenous VEGF-A induced the upregulation of p-Y951 and p-Y1175 in the spinal dorsal horn (N = 4 for each time point). Data are expressed as mean ± SD. *P* < 0.05, **P* < 0.01 *versus* phosphate-buffered saline group, one-way ANOVA with *post hoc* Dunnett test. (*I*) Tumor inoculation induced the upregulation of p-Y951 and p-Y1175 in a time-dependent manner (N = 4 for each time point). *P* < 0.05, **P* < 0.01 *versus* sham group, one-way ANOVA with *post hoc* Dunnett test.

323881 30 min before vascular endothelial growth factor A injection partially prevented vascular endothelial growth factor A–induced mechanical allodynia (fig. 7A) and thermal hyperalgesia (fig. 7B). These results indicate that spinal vascular endothelial growth factor A is sufficient to trigger nociceptive behaviors *via* vascular endothelial growth factor receptor 2 in naïve rats.

We then examined the specific molecular signaling mechanisms underlying vascular endothelial growth factor A/vascular endothelial growth factor receptor 2–mediated pain hypersensitivity. As described in previous studies, vascular endothelial growth factor receptor 2 has the ability to activate four major intracellular signaling pathways: phospholipase Cγ-protein kinase C, Src family kinase, phosphatidylinositol 3-kinase–Akt, and mitogen-activated protein kinases. In this study, we primarily focused on protein kinase C and Src family kinase based on the following considerations: (1) protein kinase C is primarily expressed in neurons and involved in membrane translocation and phosphorylation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, which is the main molecular mechanism for increased spontaneous excitatory postsynaptic currents; and (2) Src family kinase is primarily expressed in microglial cells and involved in activation of extracellular signal-regulated kinase and p38 signaling pathways, which are the major molecular mechanisms for microglia-mediated inflammatory responses. Here, we first examined the activation of protein kinase C and Src family kinase in the spinal dorsal horn through detection of phospho-protein kinase C (Ser657) and phospho-Src family kinase (Tyr416) expression after intrathecal injection of recombinant vascular endothelial growth factor A in naïve rats. As shown by western blot analysis, phospho-protein kinase C and phospho-Src family kinase were increased within 2 h and 4 h, respectively. In addition, this upregulation of phospho-protein kinase C and phospho-Src family kinase persisted for at least 12 h and returned to normal levels at 24 h post–vascular endothelial growth factor A injection (fig. 7C). Furthermore, intrathecal administration of ZM 323881 30 min before vascular endothelial growth factor A injection significantly suppressed vascular endothelial growth factor A–induced upregulated expression of phospho-protein kinase C and phospho-Src family kinase (fig. 7D), suggesting a conceivable link between vascular endothelial growth factor receptor 2–mediated protein kinase C and Src family kinase activation in the spinal dorsal horn and vascular endothelial growth factor A–evoked nociceptive hypersensitivity in naïve rats. To

further address these upstream and downstream relationships, we administered protein kinase C inhibitor GF109203X or Src family kinase inhibitor PP2 30 min before vascular endothelial growth factor A injection and observed changes in pain behaviors. Similarly, both GF109203X and PP2 could partially, but significantly, attenuate the vascular endothelial growth factor A–induced mechanical allodynia (fig. 7E) and thermal hyperalgesia (fig. 7F). In addition, previous studies demonstrated that the tyrosine phosphorylation sites of vascular endothelial growth factor receptor 2 at positions Y1175 and Y951 regulate protein kinase C and Src family kinase activity, respectively (fig. 7G), and thereby influences vascular endothelial growth factor A–mediated biologic function.[21–23] Herein, we further confirmed that the phosphorylation levels of phospho-Y1175 and phospho-Y951 in the spinal cord were time-dependently increased after both vascular endothelial growth factor A injection in naïve rats (fig. 7H) and tumor inoculation in tumor-bearing rats (fig. 7I). The above findings demonstrate that spinal protein kinase C and Src family kinase are critical pathways underlying vascular endothelial growth factor A/vascular endothelial growth factor receptor 2–evoked pain hypersensitivity in naïve rats.

Vascular Endothelial Growth Factor A/Vascular Endothelial Growth Factor Receptor 2 Activates Neuronal Protein Kinase C and Microglial Src Family Kinase Pathways in Tumor-bearing Rats

We continued to verify whether spinal protein kinase C and Src family kinase signaling pathways were downstream of vascular endothelial growth factor receptor 2 in tumor-bearing rats. Compared with sham rats, tumor inoculation produced persistent phospho-protein kinase C and phospho-Src family kinase upregulation in a time-dependent manner in the spinal dorsal horn, which commenced on days 7 to 10 and were maintained at a high level until day 21 (fig. 8A). However, the tumor-induced upregulation of phospho-protein kinase C and phospho-Src family kinase was apparently reduced by repeated intrathecal administration of vascular endothelial growth factor A neutralizing antibody (fig. 8B) or ZM 323881 (fig. 8C). In addition, immunofluorescence staining confirmed that phospho-protein kinase C was predominantly expressed in neurons (fig. 8D), but not in astrocytes or microglia (fig. 8E); whereas phospho-Src family kinase was predominantly expressed in microglia (fig. 8F), but not in neurons or astrocytes (fig. 8G), in the spinal dorsal horn on day 14 post–tumor inoculation.
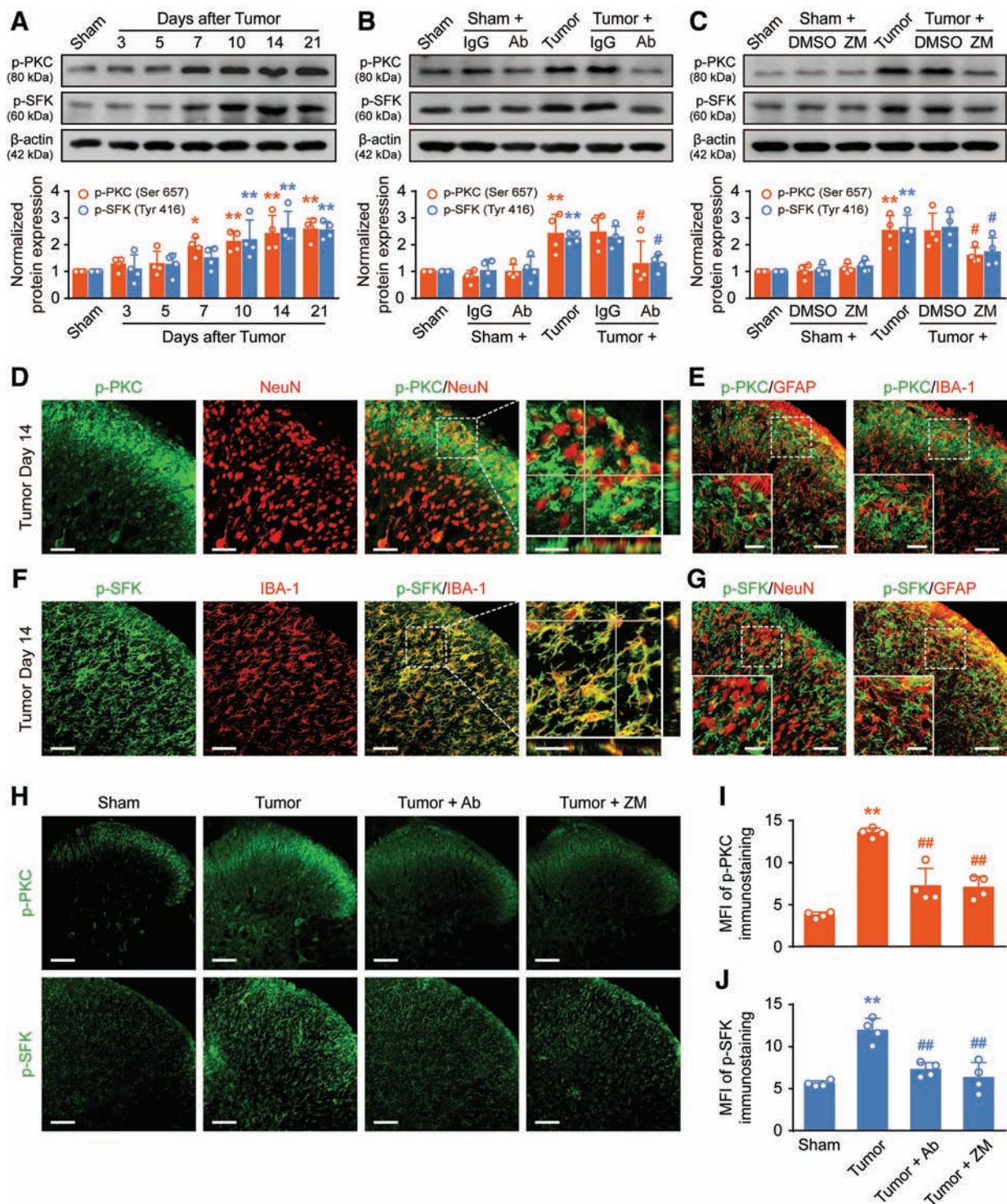
Fig. 8. Protein kinase C (PKC) and Src family kinase (SFK) signals are activated by vascular endothelial growth factor A (VEGF-A)/vascular endothelial growth factor receptor 2 (VEGFR2) in tumor-bearing rats. (*A*) Tumor inoculation induced the upregulation of phosphor- (p-)PKC and p-SFK in a time-dependent manner (N = 4 for each time point). Data are expressed as mean ± SD. *$P < 0.05$, **$P < 0.01$ *versus* sham group, one-way ANOVA with *post hoc* Dunnett test. (*B, C*) Spinal injection of VEGF-A neutralizing antibody (*B*) or VEGFR2 inhibitor ZM 323881 (*C*) significantly suppressed tumor-induced upregulation of p-PKC and p-SFK expression in the spinal dorsal horn. (*D* to *G*) p-PKC immunoreactivity colocalized with NeuN (*D*), but not with glial fibrillary acidic protein (GFAP) or ionized calcium-binding adapter molecule 1 (IBA-1)
(Continued)

(**Fig. 8.** *Continued*) (*E*) in the spinal dorsal horn; meanwhile, p-SFK immunoreactivity colocalized with IBA-1 (*F*), but not with NeuN or GFAP (*G*) in the spinal dorsal horn. Scale bars: 50 µm and 20 µm (zoom). (*H* to *J*) Spinal injection of VEGF-A neutralizing antibody or ZM 323881 significantly suppressed tumor-increased mean fluorescence intensity (MFI) of p-PKC (*H, I*) and p-SFK (*H, J*). Scale bars: 100 µm. (*B, C, H* to *J*) VEGF-A neutralizing antibody (Ab; 2 µg, intrathecal), IgG (2 µg, intrathecal), ZM 323881 (ZM, 100 nM, intrathecal) or dimethyl sulfoxide (1%, intrathecal) was injected once daily on days 12, 13, and 14 post–tumor inoculation. Tissues were collected 4 h after the last injection on day 14. Data are expressed as mean ± SD. **P < 0.01 *versus* sham group; #*P* < 0.05, ##*P* < 0.01 *versus* tumor group, N = 4 for each group, one-way ANOVA with *post hoc* Dunnett test.

Similarly, the effect of vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 on phospho–protein kinase C and phospho–Src family kinase upregulation was further confirmed by immunofluorescence analysis (fig. 8, H to J).

Vascular Endothelial Growth Factor A/Vascular Endothelial Growth Factor Receptor 2/Protein Kinase C Signaling Enhances NMDA Receptor Activation in Spinal Neurons

Previous reports have shown that in the process of chronic pain formation, NMDA receptors in the postsynaptic neuronal membranes are widely expressed at the spinal cord level. After activation, NMDA receptors can increase the concentrations of intracellular secondary messenger Ca^{2+} and then activate downstream Ca^{2+}-dependent signaling molecules (*e.g.,* extracellular signal-regulated kinase, calcium/calmodulin-dependent protein kinase II, and cyclic-AMP response element-binding protein), which plays a key role in inducing and maintaining central sensitization. Among them, extracellular signal-regulated kinase and calcium/calmodulin-dependent protein kinase II enhance neuronal excitability *via* phosphorylation of AMPA and NMDA receptors, as well as ion channels;[24,25] meanwhile, cyclic-AMP response element-binding protein as a transcription factor promotes the synthesis of NMDA receptor substrates.[26] Previous reports have demonstrated that protein kinase C pathway can not only indirectly activate NMDA receptors by promoting the synergistic effect of AMPA receptors, but also directly activate NMDA receptors *via* phosphorylation of the NR1 subunit.[27–29] Therefore, we speculated that vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 signaling facilitates the activation of NMDA receptors and Ca^{2+}-dependent signals *via* protein kinase C pathway in spinal neurons. To explore this neuronal mechanism of vascular endothelial growth factor A/vascular endothelial growth factor receptor 2/protein kinase C in bone cancer pain, we further examined changes of protein expression of phospho–NMDA receptor subunit 2B (Tyr1472), phospho–extracellular signal-regulated kinase (Thr202/Tyr204), phospho–calcium/calmodulin-dependent protein kinase II (Thr286), and phospho–cyclic-AMP response element-binding protein (Ser133) in the spinal dorsal horn *via* spinal blockade of vascular endothelial growth factor A, vascular endothelial growth factor receptor 2, or protein kinase C. As shown by western blot analysis, the protein expression levels of phospho–NMDA receptor subunit 2B, phospho–extracellular signal-regulated kinase, phospho–calcium/calmodulin-dependent protein kinase II, and phospho–cyclic-AMP response element-binding protein were all pronouncedly increased on day 14 post–tumor inoculation. However, intrathecal administration of vascular endothelial growth factor A neutralizing antibody, vascular endothelial growth factor receptor 2 inhibitor ZM 323881, or protein kinase C inhibitor GF109203X effectively reduced tumor-induced protein expression of phospho–NMDA receptor subunit 2B (fig. 9A), phospho–extracellular signal-regulated kinase (fig. 9B), phospho–calcium/calmodulin-dependent protein kinase II (fig. 9C), and phospho–cyclic-AMP response element-binding protein (fig. 9D). These findings suggest that the neuronal mechanism of vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 in relation to bone cancer pain regulation is dependent on protein kinase C-mediated NMDA receptor activation.

Vascular Endothelial Growth Factor A/Vascular Endothelial Growth Factor Receptor 2/Src Family Kinase Signaling Promotes Proinflammatory Cytokine Production in Spinal Microglia

Reactive microglial cells in the spinal cord promote persistent production of proinflammatory cytokines *via* activation of extracellular signal-regulated kinase and p38 pathways under different pain situations.[30,31] On the one hand, these proinflammatory cytokines promote glia-mediated "inflammation soup" formation *via* acting on the corresponding receptors located in their own, as well as surrounding, microglia and astrocytes in the spinal microenvironment.[32] On the other hand, they directly mediate synaptic plasticity and neuronal excitability *via* acting on the corresponding receptors located in presynaptic and/or postsynaptic membranes.[33] Previous reports have demonstrated that activity of Src family kinase pathway can increase the phosphorylation of p38 or extracellular signal-regulated kinase pathways in spinal microglia in various chronic pain models.[34,35] Thus, we speculated that vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 signaling facilitates proinflammatory cytokine production *via* Src family kinase pathway in spinal microglia. To explore this microglial mechanism of vascular endothelial growth factor A/vascular endothelial growth factor receptor 2/
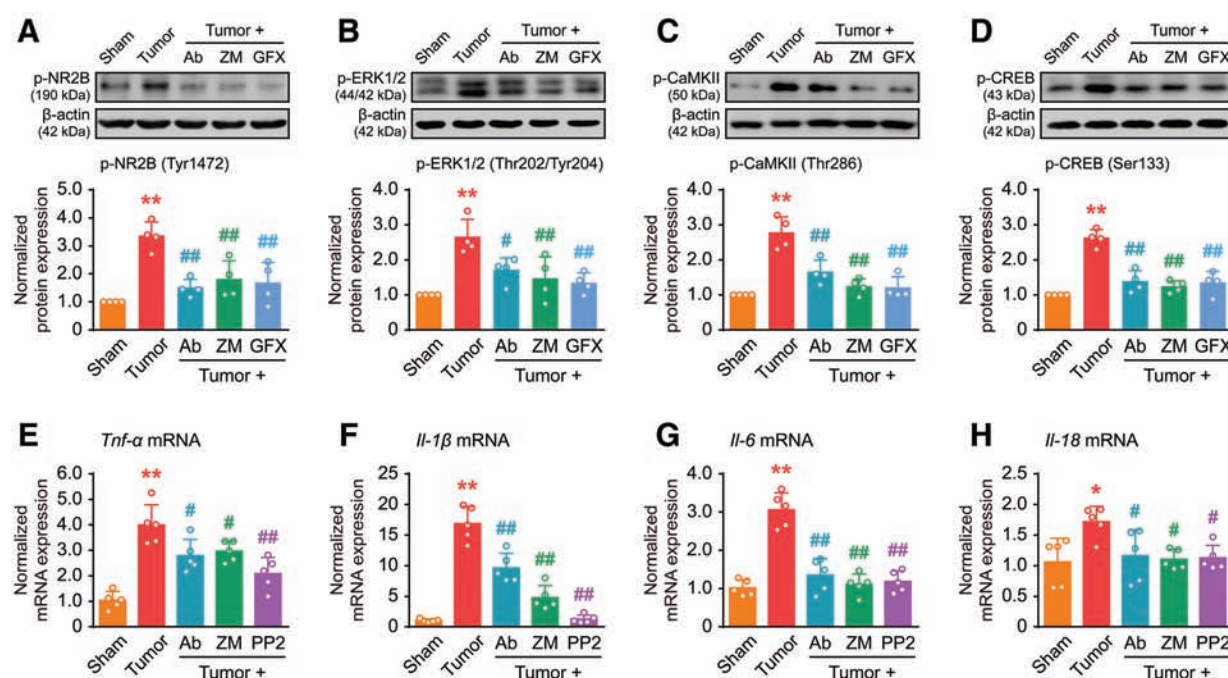
Fig. 9. Spinal blockade of vascular endothelial growth factor A (VEGF-A)/vascular endothelial growth factor receptor 2 (VEGFR2) suppresses tumor-induced NMDA receptor activation and proinflammatory cytokine production *via* protein kinase C (PKC) and Src family kinase (SFK), respectively. (*A* to *D*) Western blot analysis shows that VEGF-A neutralizing antibody, ZM 323881, or GF109203X significantly attenuated tumor-induced upregulation of phospho- (p-)NMDA receptor subunit 2B (NR2B) (*A*), p-extracellular signal-regulated kinase (ERK)1/2 (*B*), p-calcium/calmodulin-dependent protein kinase II (CaMKII) (*C*), and p-cyclic-AMP response element-binding protein (CREB) (*D*) protein expression in the spinal dorsal horn. (*E* to *H*) Real-time quantitative polymerase chain reaction analysis shows that VEGF-A neutralizing antibody, ZM 323881, or PP2 significantly attenuated tumor-induced upregulation of *Tnf-α* (*E*), *Il-1β* (*F*), *Il-6* (*G*), and *Il-18* (*H*) mRNA expression in the spinal dorsal horn. (*A* to *H*) VEGF-A neutralizing antibody (Ab; 2 μg, intrathecal), ZM 323881 (ZM, 100 nM, intrathecal), GF109203X (GFX, 0.2 μg, intrathecal), or PP2 (10 μg, intrathecal) was injected once daily on days 12, 13, and 14 post–tumor inoculation. Tissues were collected 4 h after the last injection on day 14. Data are expressed as mean ± SD. *$P < 0.05$, **$P < 0.01$ *versus* sham group; #$P < 0.05$, ##$P < 0.01$ *versus* tumor group, N = 4 (*A-D*) or 5 (*E* to *H*) for each group, one-way ANOVA with *post hoc* Dunnett test.

Src family kinase in bone cancer pain, we further examined changes of mRNA expression of proinflammatory cytokines (including TNF-α, IL-1β, IL-6, and IL-18) in the spinal dorsal horn *via* spinal blockade of vascular endothelial growth factor A, vascular endothelial growth factor receptor 2, or Src family kinase. As shown by real-time quantitative polymerase chain reaction analysis, the mRNA expression levels of *Tnf-α*, *Il-1β*, *Il-6*, and *Il-18* were all pronouncedly increased on day 14 post–tumor inoculation. However, intrathecal administration of vascular endothelial growth factor A neutralizing antibody, vascular endothelial growth factor receptor 2 inhibitor ZM 323881, or Src family kinase inhibitor PP2 effectively reduced tumor-induced mRNA expression of *Tnf-α* (fig. 9E), *Il-1β* (fig. 9F), *Il-6* (fig. 9G), and *Il-18* (fig. 9H). These findings suggest that the microglial mechanism of vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 in bone cancer pain regulation is dependent on Src family kinase–mediated proinflammatory cytokine production.

Discussion

Several recent studies have shown that vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 signaling plays an important role in peripheral sensitization in primary sensory neurons *via* activation of P2X$_{2/3}$ receptor or calcineurin in the dorsal root ganglion.[8,9] However, there are no relevant reports concerning whether vascular endothelial growth factor A also modulates bone cancer pain and central sensitization at the spinal cord level. Our findings here revealed that intrathecal injection of vascular endothelial growth factor A neutralizing antibody or vascular endothelial growth factor receptor 2 inhibitor could significantly relieve mechanical allodynia, thermal hyperalgesia, and spontaneous pain behaviors in tumor-bearing rats, as well as neuronal sensitization and microglial activation in the spinal dorsal horn. These results suggest that vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 signaling plays a critical role in the regulation of bone cancer pain at the spinal cord level.

Notably, our current study cannot exclude the possibility that intrathecal injection of neutralizing antibody or inhibitor may also block vascular endothelial growth factor A and vascular endothelial growth factor receptor 2 activity in dorsal root ganglion neurons, thereby partially contributing to the alleviation of bone cancer pain.

In addition, we applied a CatWalk gait analysis system to study abnormal gait features in tumor-bearing rats *via* observation of weight-bearing conditions (*i.e.,* max contact area and max contact max intensity) and usage conditions (*i.e.,* single stance and swing) of the affected limb during voluntary walking. CatWalk gait analysis has been reported in studies of inflammatory and neuropathic pain.[36–38] However, since intratibial tumor inoculation leads to neither hind paw swelling (*e.g.,* inflammatory pain model) nor injury to motor nerve fibers of sciatic nerve (*e.g.,* neuropathic pain model), we believe that CatWalk gait analysis is more reflective of the bone cancer pain state. In the current study, tumor-bearing rats exhibited reductions in max contact area, max contact max intensity and single stance duration, as well as increase in swing duration in the affected limbs, all of which demonstrate a loss of weight-bearing capacity and reduced limb usability. These results are consistent with clinical findings that patients suffering from bone cancer pain exhibit reduced functional abilities in daily life. Our behavioral data further revealed that intrathecal injection of vascular endothelial growth factor A neutralizing antibody or vascular endothelial growth factor receptor 2 inhibitor could significantly reverse these tumor-induced abnormal gait features.

In addition to previous observation that vascular endothelial growth factor A and vascular endothelial growth factor receptor 2 are increased in dorsal root ganglion neurons of cancer rats,[9] we also found that the mRNA and protein expression levels of vascular endothelial growth factor A and vascular endothelial growth factor receptor 2 were increased in the ipsilateral spinal dorsal horn after tumor inoculation in a time-dependent manner. Moreover, we identified that vascular endothelial growth factor A was primarily expressed in the central terminals of primary sensory neurons and spinal neurons, while vascular endothelial growth factor receptor 2 was primarily expressed in spinal neurons and microglia, and spinal blockade of vascular endothelial growth factor A or vascular endothelial growth factor receptor 2 could significantly inhibit tumor-induced upregulation of c-Fos and ionized calcium-binding adapter molecule 1 in the spinal dorsal horn. These results confirm our hypothesis that vascular endothelial growth factor A was released from central terminals of primary sensory neurons and spinal neurons, acts on vascular endothelial growth factor receptor 2 at the surface of spinal neurons and microglia *via* the "ligand–receptor" formation, and mediates neuronal sensitization and microglial activation *via* "neuronal–neuronal" and "neuronal–microglial" crosstalks during the maintenance of bone cancer pain.

Recent evidence demonstrated sex dimorphism in the activation and roles of microglia in inflammatory and neuropathic pain models. For example, spinal TLR4, P2X4R, and p38 mediate inflammatory and/or neuropathic pain in male, but not female mice and/or rats, suggesting the possibility of male-dominant microglial signaling in the spinal dorsal horn.[39–41] However, it is well documented that the sex dimorphism in microglia-mediated pain regulation may be dependent on certain sex-specific signaling activation and pain models.[31] In order to closely mimic the human pathophysiology of breast cancer–induced pain, female rats are the most commonly used animal species for this model with Walker 256 tumor inoculation.[4] Our data provided several lines of evidence to suggest equivalent pain hypersensitivity and spinal central sensitization within male and female rats in response to tumor inoculation, accompanied by similar reactions to vascular endothelial growth factor A/vascular endothelial growth factor receptor 2–mediated bone cancer pain behaviors. First, at the behavioral level, tumor inoculation induced apparent and time-dependent mechanical allodynia and thermal hyperalgesia occurred to a similar degree in both sexes. Second, at the cellular level, tumor inoculation increased expression of c-Fos, glial fibrillary acidic protein, and ionized calcium-binding adapter molecule 1 in the spinal dorsal horn occurred to a similar degree in both sexes. Third, at the molecular level, tumor inoculation elicited upregulation and localization of vascular endothelial growth factor A and vascular endothelial growth factor receptor 2 in the spinal dorsal horn occurred to a similar degree in both sexes, and spinal blockade of vascular endothelial growth factor A or vascular endothelial growth factor receptor 2 could significantly reverse bone cancer pain behaviors in both sexes.

Spinal excitatory synaptic transmission is the material basis of central sensitization.[42,43] In this study, exogenous vascular endothelial growth factor A increased both the frequency and amplitude of spontaneous excitatory postsynaptic currents in spinal lamina IIo neurons in naïve rats, and vascular endothelial growth factor receptor 2 inhibitor largely prevented these changes, suggesting that vascular endothelial growth factor A enhances excitatory synaptic transmission in superficial spinal dorsal horn neurons *via* vascular endothelial growth factor receptor 2. Considering these *in vitro* results, we continued to detect the algogenic effect of vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 on naïve rats *in vivo*. Behavioral data further indicated that intrathecal injection of exogenous vascular endothelial growth factor A simultaneously reduced threshold and latency in naïve rats, but pretreatment with vascular endothelial growth factor receptor 2 inhibitor could inhibit this phenomenon. These findings suggest that vascular endothelial growth factor A is sufficient to induce allodynia and hyperalgesia *via* vascular endothelial growth factor receptor 2, and the underlying causative mechanism may be related to the enhancement of spinal excitatory synaptic transmission.

For patch–clamp recordings, vascular endothelial growth factor A–induced spontaneous excitatory postsynaptic currents increases were primarily mediated by AMPA receptors. In the central nervous system, protein kinase C mediates AMPA receptor membrane translocation and increases AMPA receptor activation *via* binding to serine 831 phosphorylation site on its GluA1 subunit.[44] Therefore, we speculated that protein kinase C might be involved in the regulation of vascular endothelial growth factor A/vascular endothelial growth factor receptor 2–mediated neuronal sensitization and pain behaviors. Our results showed that exogenous vascular endothelial growth factor A upregulated phospho–protein kinase C expression in the spinal dorsal horn *via* vascular endothelial growth factor receptor 2, and that intrathecal injection of protein kinase C inhibitor GF109203X inhibited vascular endothelial growth factor A/vascular endothelial growth factor receptor 2–induced pain sensitization in naïve rats. Moreover, we found that phospho–protein kinase C was upregulated after tumor inoculation and could be significantly inhibited by vascular endothelial growth factor A neutralizing antibody or vascular endothelial growth factor receptor 2 inhibitor. In addition, the central sensitization maintenance is mainly dependent on the synergistic effect of AMPA and NMDA receptors in combination,[45] and protein kinase C also plays a key regulatory role in activation of NMDA receptors *via* binding to two phosphorylation sites of serine 890 and 896

on NMDA receptors, or *via* relief of Mg^{2+} blocking effect on NMDA receptors.[29,46,47] Therefore, we further focused on the effect of vascular endothelial growth factor A/vascular endothelial growth factor receptor 2/protein kinase C signaling pathway on the activation of NMDA receptors, as well as downstream Ca^{2+}-dependent signals in the spinal cord after tumor inoculation. Western blot analysis showed that spinal blockade of vascular endothelial growth factor A, vascular endothelial growth factor receptor 2, or protein kinase C remarkably suppressed tumor-induced protein expression of phospho–NMDA receptor subunit 2B, phospho–extracellular signal-regulated kinase, phospho–calcium/calmodulin-dependent protein kinase II, and phospho–cyclic-AMP response element-binding protein in tumor-bearing rats. These findings suggest that neuronal protein kinase C mediates the role of vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 in bone cancer pain regulation *via* promotion of the activation of NMDA receptor subunit 2B and Ca^{2+}-dependent signals in the spinal cord (fig. 10).

Among the four vascular endothelial growth factor receptor 2–mediated signaling pathways, the role of Src family kinase in microglial activation is clear. Previous studies have shown that Src family kinase is selectively activated in spinal microglia after formalin paw injection or spinal nerve ligation, and intrathecal injection of Src family kinase inhibitor PP2 can significantly attenuate both inflammatory and neuropathic
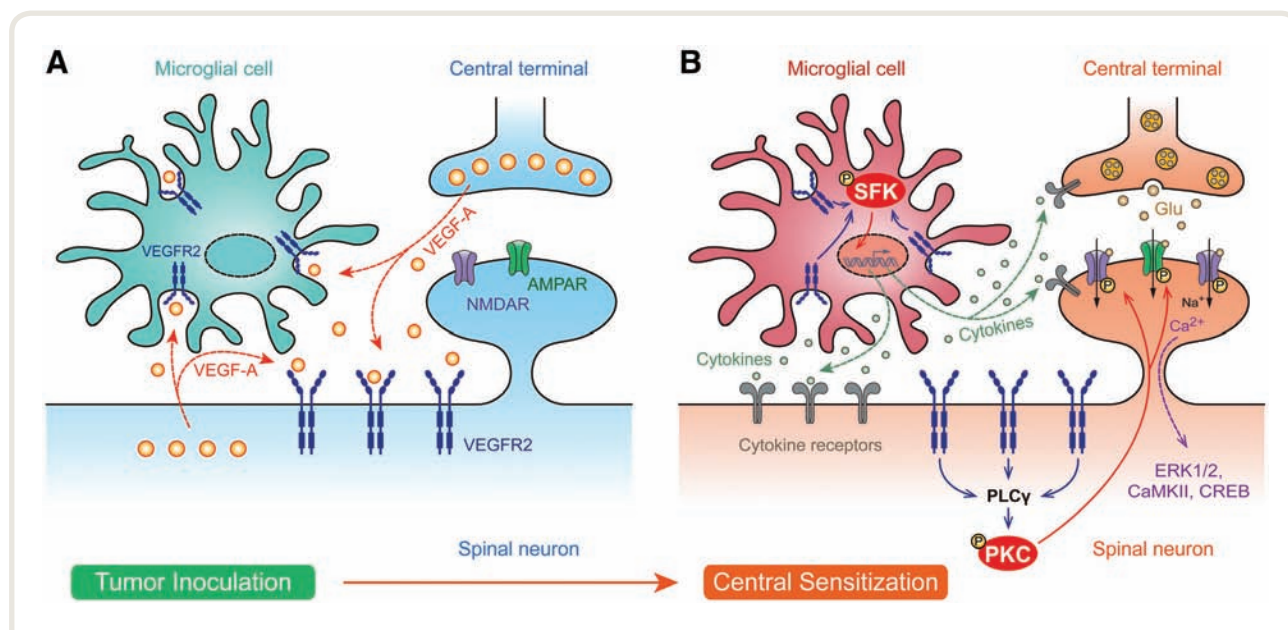
Fig. 10. Schematic illustration of the mechanisms underlying contributions of vascular endothelial growth factor A (VEGF-A)/vascular endothelial growth factor receptor 2 (VEGFR2) to spinal central sensitization in bone cancer pain. (*A*) After tumor inoculation, VEGF-A is primarily produced and released from presynaptic and postsynaptic neurons, and its receptor VEGFR2 is upregulated in spinal neurons and microglial cells. (*B*) After ligand-receptor binding, VEGF-A/VEGFR2 enhances neuronal sensitization *via* protein kinase C (PKC)-mediated NMDA receptor activation, and promotes microglial activation *via* Src family kinase (SFK)-mediated proinflammatory cytokine production. As a result, VEGF-A/VEGFR2 signaling regulates neuron-neuron and neuron–glia interactions in the development of spinal central sensitization, and contributes to bone cancer pain hypersensitivity. *Solid lines* indicate the pathways in this study. *Dashed lines* indicate possible pathways in other studies.

pain behaviors in rat models.[34,35] Therefore, we speculated that Src family kinase may be involved in vascular endothelial growth factor A/vascular endothelial growth factor receptor 2–regulated pain behaviors. Our results showed that exogenous vascular endothelial growth factor A could upregulate phospho-Src family kinase expression in the spinal dorsal horn *via* vascular endothelial growth factor receptor 2, and that intrathecal injection of PP2 suppressed vascular endothelial growth factor A/vascular endothelial growth factor receptor 2–induced pain hypersensitivity in naïve rats. Moreover, we found that phospho-Src family kinase was upregulated after tumor inoculation and could be significantly inhibited by application of vascular endothelial growth factor A neutralizing antibody or vascular endothelial growth factor receptor 2 inhibitor. In addition, the regulatory role of microglia in spinal neuronal sensitization is primarily dependent on release of proinflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IL-18.[48] Among these, TNF-α, IL-1β, and IL-18 can increase excitatory synaptic transmission, whereas IL-1β and IL-6 can attenuate inhibitory synaptic transmission. Spinal injection of TNF-α, IL-1β, IL-6 or IL-18 can significantly induce mechanical allodynia and/or thermal hyperalgesia in naïve rats.[14,49] Therefore, we further investigated the effect of vascular endothelial growth factor A/vascular endothelial growth factor receptor 2/Src family kinase signaling pathway on the production of proinflammatory cytokines in the spinal cord after tumor inoculation. Real-time quantitative polymerase chain reaction data showed that spinal blockade of vascular endothelial growth factor A, vascular endothelial growth factor receptor 2, or Src family kinase could remarkably suppress tumor-induced mRNA expression of *Tnf-α*, *Il-1β*, *Il-6*, and *Il-18* in tumor-bearing rats. These findings suggest that microglial Src family kinase mediates the role of vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 in bone cancer pain regulation *via* promotion of the inflammatory response in the spinal cord (fig. 10).

In summary, remarkable achievements have been made in both basic and clinical research in the field of oncology on vascular endothelial growth factor A and vascular endothelial growth factor receptor 2. Antiangiogenic drugs against vascular endothelial growth factor A and/or vascular endothelial growth factor receptor 2 have been employed clinically for antitumor therapy.[50] This study reports a novel role for vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 signaling in bone cancer pain regulation and the implicated mechanisms in the spinal cord. Thus, blockade of vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 signaling could be a potential pharmaceutical therapy used not only to treat cancers, but also to relieve cancer pain symptoms within comprehensive cancer treatment.

Competing Interests

The authors declare no competing interests.

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Address correspondence to Dr. Wang: Department of Integrative Medicine and Neurobiology, Fudan University, 138 Yi-Xue-Yuan Road, Shanghai 200032, China. wang-yanqing@shmu.edu.cn. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

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The Lung Point

Early Identification of Pneumothorax on Point of Care Ultrasound

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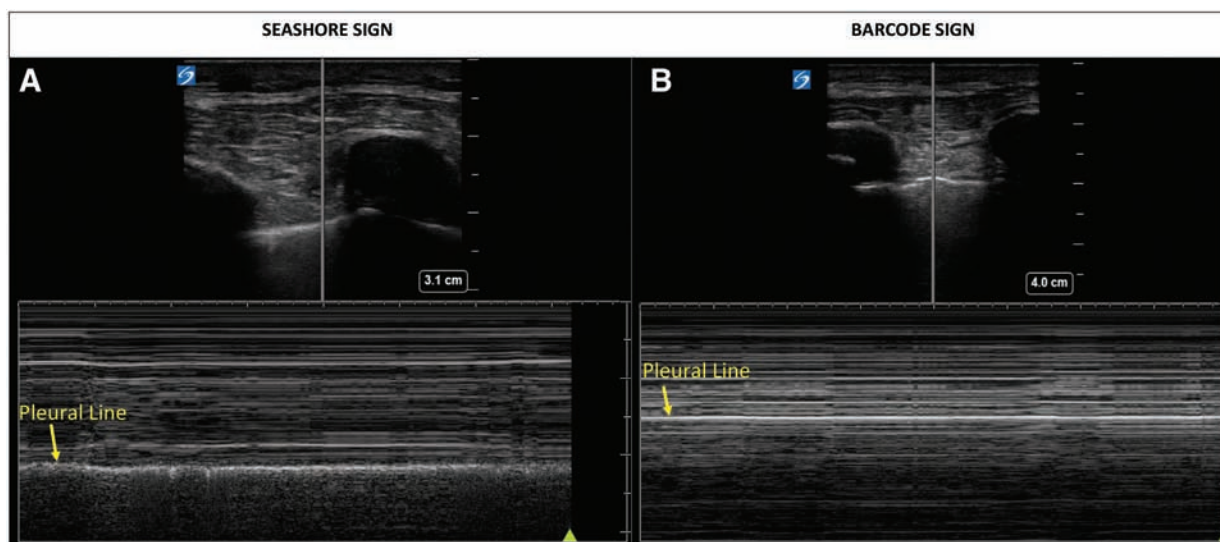
Pneumothorax is a potentially life-threatening emergency that can be difficult to diagnose intraoperatively. Unrecognized pneumothorax can result in hypoxia, and positive pressure ventilation can lead to the development of tension pneumothorax. Perioperative lung ultrasonography can aid in the rapid diagnosis of pneumothorax.[1]

These lung ultrasound images demonstrate the presence of the lung point sign, the interface between normal lung and pneumothorax, after an attempted central venous line placement.[2] The images were obtained with a high-frequency linear probe placed in the longitudinal direction on the chest with the probe marker pointing cephalad. The pleura is seen as a hyperechoic line extending between rib shadows. Lung sliding—the shimmering of the hyperechoic pleura due to the sliding of visceral pleura relative to the parietal pleura—is seen in healthy lungs. In lung point, normal pleural sliding is seen on one side of the interface and absence of sliding on the other, which is diagnostic for pneumothorax (Supplemental Digital Content, http://links.lww.com/ALN/B987). On motion mode, with the beam placed at the point of normal lung sliding, the seashore sign is seen with lines above the hyperechoic pleura and a speckled pattern beyond it (*panel A*). When the ultrasound beam is moved to the site with absent lung sliding, a linear pattern both above and below the pleura, the bar code sign, is noted (*panel B*).

Although the identification of lung point sign is 100% specific for the diagnosis of pneumothorax, the sensitivity is only around 66%.[3] Lung point is not seen in cases of total lung collapse.

Competing Interests

The authors declare no competing interests.

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Resection of an Adrenocortical Carcinoma Invading the Inferior Vena Cava Extending into the Right Ventricle

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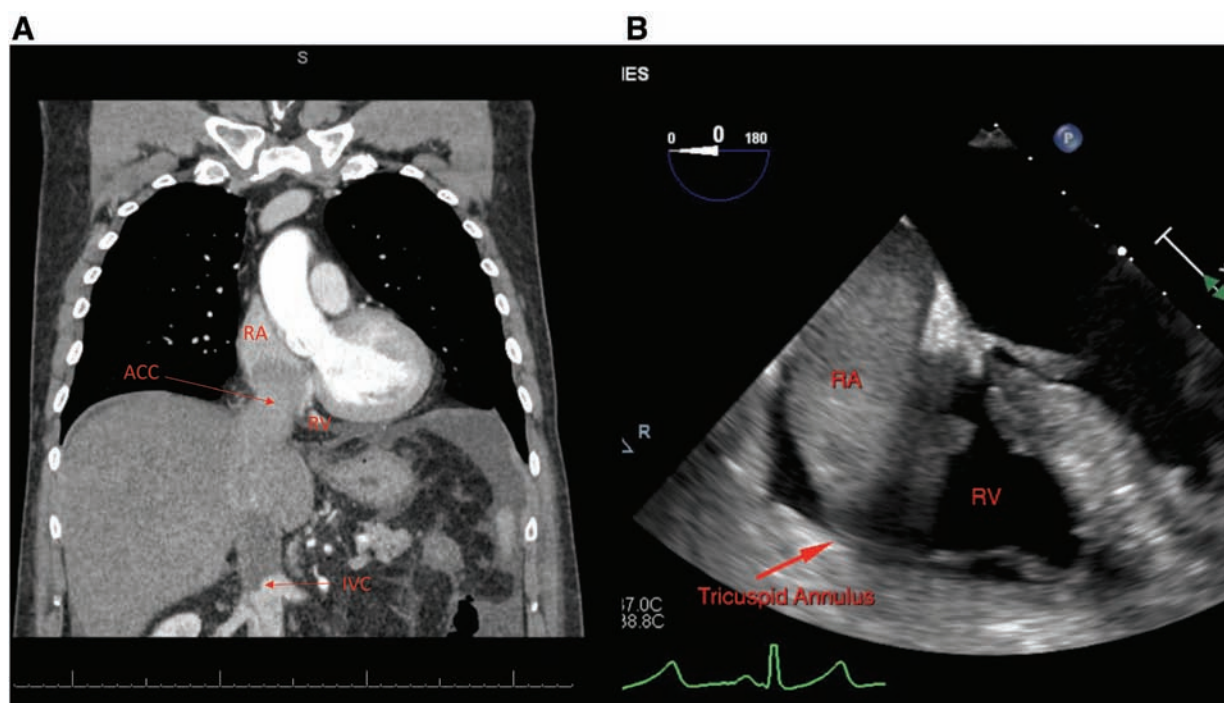
Adrenocortical carcinoma (ACC) is an uncommon and aggressive tumor with the potential to invade the inferior vena cava (IVC) and spread to the right atrium (RA) and right ventricle (RV) (image A).

Perioperative evaluation of a patient with a tumor invading the inferior vena cava includes a comprehensive analysis of the preoperative imaging studies (echocardiogram, computer tomography, or magnetic resonance) to assess for tumor extension and structures affected. Ultimately, tumor extension will determine the anesthetic management. Tumors occluding the inferior vena cava require large-bore central venous access above the diaphragm. Pulmonary artery catheter placement may not be recommended if the tumor is spreading into the right atrium because of the risk of tumor embolism.[1] Hepatic vein invasion may affect drug metabolism and coagulation. Increased collateral circulation will also potentiate the risk of intraoperative bleeding. Significant ascites places the patient at a higher risk for aspiration.

Mechanical obstruction may compromise preload significantly. The induction agents should be chosen to minimize cardiovascular depression, and the anesthesiologist may consider maintaining spontaneous ventilation to preserve preload to the heart. Cardiac surgery and perfusion teams should be on standby for emergency cardiopulmonary bypass.

Tumor pulmonary embolism is a feared intraoperative complication that can result in right ventricular failure, shock, and death.[2] Intraoperative transesophageal echocardiogram (image B; transesophageal echocardiogram exposing adrenocortical carcinoma protruding through the tricuspid annulus) provides instantaneous information about heart function and monitoring for embolic phenomenon during tumor manipulation.[3] Early detection of tumor embolism using transesophageal echocardiogram may lead to inferior vena cava clamping and the addition of cardiopulmonary bypass with or without deep hypothermic circulatory arrest.[2]

Competing Interests

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Pitfalls of a Shared Neuraxial Space

Wandering Epidural Catheter

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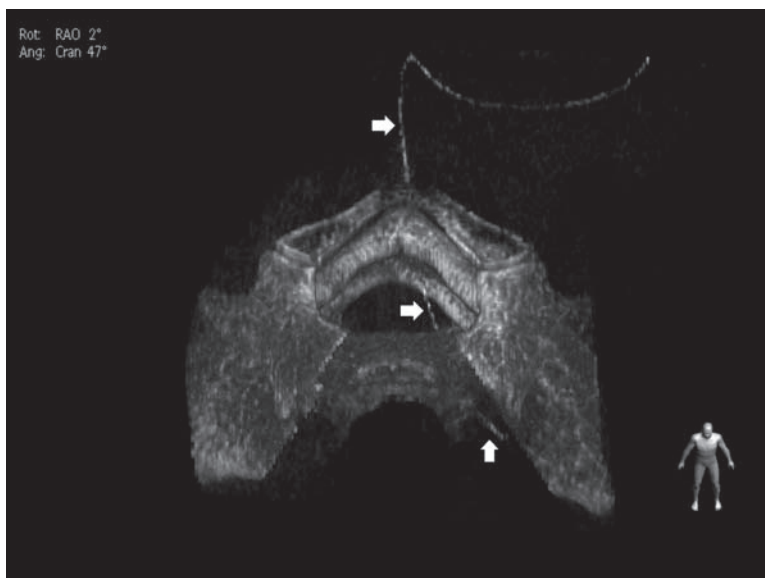
Children with severe spasticity with indwelling baclofen pumps often present for extensive orthopedic procedures to improve mobility and comfort. Procedural pain can be effectively managed by epidural infusions. Epidurals are sited at surgically congruent levels either above or below the level of insertion of the intrathecal catheter.[1,2]

The three-dimensional reconstructed view demonstrates the course of epidural catheter traveling caudally in the epidural space (image). The catheter travels from the L4 level coursing antero-laterally at the L5 level, "wandering" toward the left L5–S1 foramen to lie anterior to the sacrum.

The epidural catheter is placed in the L4–L5 interspace with the bevel of the Touhy needle directed cephalad in a patient with a longstanding intrathecal catheter at the L3 level.

Risks of placing an epidural with an existing intrathecal catheter include catheter damage with interruption of baclofen delivery and subsequent baclofen withdrawal syndrome, intrathecal migration of the epidural catheter, and misdirected epidural catheter due to a scarred epidural space. Any one of these scenarios would lead to suboptimal pain control in a patient population that is often nonverbal or cognitively delayed.

This image underscores the potential for epidural catheter malposition in a scarred epidural space and make a case for routine postepidural placement epidurogram in patients with intrathecal catheters. A computed tomography scan conspicuously displays small–caliber epidural catheters and may be further augmented by contrast.[3]

Competing Interests

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Split Larynx

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A 30-YEAR-OLD male, intubated due to respiratory distress after cervical spine injury, required endotracheal tube exchange due to high airway pressures. In this unique laryngoscopic video (C-MAC; Karl Storz-Endoscope, Germany) view, the glottis seems to be split into an anterior triangular and a posterior circular portion secondary to the presence of bilateral vocal cord granulomas. The posterior portion may be mistaken for the esophageal inlet, and attempts to insert the endotracheal tube into the smaller anterior portion can result in trauma and complicate airway management.

Granulomas are soft, lobulated, benign masses usually found on the arytenoids due to perichondral inflammation after traumatic or prolonged intubation, oversized endotracheal tube, excessive cuff pressure, and/or laryngopharyngeal reflux.[1] Airway management is challenging, since proper identification of the laryngeal inlet is made difficult by the altered anatomy. On laryngoscopy, visualizing tracheal rings in the subglottic area gives a clue that the view is tracheal rather than esophageal. Repeated attempts to place the endotracheal tube either anteriorly or posteriorly should be avoided since the laryngeal cavity is not actually split. Liberal airway anesthesia, flexible bronchoscopy, and a smaller sized endotracheal tube may help to navigate beyond the lesion in fewer attempts without causing undue trauma, bleeding, or airway obstruction.[2,3] Extubation should be attempted cautiously, with large granulomas causing stridor and respiratory distress. Smaller granulomas may present innocuously with hoarseness and voice changes postoperatively. Management is essentially conservative and involves removal of the irritant or causative factor, antireflux therapy, and steroids. Most granulomas spontaneously decrease in size and resolve in 4 to 6 weeks. Surgical treatment can be offered with otorhinolaryngology consultation in unresolving lesions.

Competing Interests

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Four-factor Prothrombin Complex Concentrate for the Management of Patients Receiving Direct Oral Activated Factor X Inhibitors

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Direct oral anticoagulants (DOACs) have been approved for the prevention of stroke and systemic embolism in atrial fibrillation, treatment and secondary prevention of venous thromboembolism (VTE), and thromboprophylaxis after major orthopedic surgery. DOACs achieve anticoagulation by inhibiting specific coagulation factors; apixaban, betrixaban, edoxaban, and rivaroxaban inhibit activated factor X, whereas dabigatran inhibits thrombin (factor IIa). In contrast to vitamin K antagonists such as warfarin, DOACs have more predictable pharmacokinetics and pharmacodynamics and fewer interactions with other medications and food, and they are not associated with the problems of a narrow therapeutic window like warfarin.[1–3] This allows for fixed oral dosing once or twice per day, without the need for anticoagulation monitoring or dose adjustments.

Randomized clinical trial data demonstrate that activated factor X inhibitor therapies are noninferior to other anticoagulants such as vitamin K antagonists.[4] DOACs also have favorable safety and efficacy profiles, as demonstrated by evidence from "real-world" cohorts.[5–7] The risk of intracranial hemorrhages was lower with all the DOACs compared with warfarin,[8] although the rate of gastrointestinal bleeding might be increased with rivaroxaban.[9] Other studies have shown that outcomes after bleeding events were similar or less severe for patients receiving DOACs than those receiving vitamin K antagonists.[10,11] The number of patients using DOACs will likely increase due to their advantages over existing therapies, and thus the number of bleeding events in patients on DOAC therapy is also expected to increase. Therefore, for patients presenting with severe or life-threatening bleeding or for patients undergoing urgent surgery, a clear strategy to reverse anticoagulation and to achieve hemostasis is essential.

Management of Patients on DOAC Therapies

While recommendations for the management of vitamin K antagonist–related bleeding are well established,[12–14] guidelines for managing patients on DOACs,[15,16] which were developed more recently, are based on limited evidence. The decision to stop treatment depends on a patient's risk of bleeding *versus* their risk of thromboembolic complications. When deciding to reverse activated factor X inhibitors, their short elimination half-life should also be taken into account[16–19] (table 1); hemostasis is restored approximately 12 to 24 h after cessation of DOACs, assuming normal renal function.[16,20] As DOACs are partially eliminated via the kidney, they can accumulate in patients with impaired renal function.[16,21]

Reversal Strategies in Emergency Cases

Coagulopathy, which is common in trauma and perioperative bleeding, is associated with increased morbidity and mortality, and anticoagulation can further increase a patient's risk of developing coagulopathic bleeding.[14,22] Thus, in cases of life-threatening bleeding, restoration of hemostasis requires prompt reversal of anticoagulation in addition to a multimodal approach using hemostatic agents.[16] In the context of this review, we define severe bleeding according to the International Society on Thrombosis and Haemostasis criteria for major bleeding in nonsurgical patients having a symptomatic presentation.[23]

For reversal of dabigatran, a specific agent is available: idarucizumab, a humanized antibody fragment that specifically binds dabigatran with high affinity and reverses its effects within minutes.[24,25] For reversal of activated factor X inhibitor therapies, andexanet alfa, a recombinant modified human activated factor X protein that specifically binds activated factor X inhibitors, has been developed.[26,27] Although recently approved by the U.S. Food and Drug Administration (Silver Spring, Maryland), its labeled use is currently restricted as clinical evidence for its effectiveness was based on studies in healthy volunteers, whereas improvement in hemostasis in bleeding patients has not been established.[28] Indeed, such improvement has only been demonstrated for four-factor prothrombin complex concentrate (four-factor PCC) *versus* fresh frozen plasma in a randomized clinical trial in surgical patients.[29] In this

Table 1. Pharmacokinetic Properties of Direct Oral Anticoagulants

	Dabigatran[81]	Apixaban[19]	Rivaroxaban[17]	Edoxaban[18]
Mechanism of action	Direct thrombin inhibitor	Xa inhibitor	Xa inhibitor	Xa inhibitor
Time to peak	2 h	3–4 h	2–4 h	1–2 h
Terminal half-life	11–14 h*	12 h	5–13 h	10–14 h
Renal excretion	85%	27%	~33%	50%

*Longer in patients with renal insufficiency.

trial with 181 patients on vitamin K antagonist, "effective hemostasis" was observed in 90% of patients with PCC *versus* 75% with fresh frozen plasma (difference 14.3%; 95% CI 2.8 to 25.8) and rapid reduction of international normalized ratio was achieved in 55% and 10%, respectively (difference 45.3%; 95% CI, 31.9 to 56.4). In addition, andexanet alfa is not widely available and is expected to be costly (see Economic Considerations for the Use of Prothrombin Complex Concentrate *versus* Andexanet Alfa section).

Several nonspecific reversal strategies have been studied as potential DOAC reversal agents, including fresh frozen plasma, three-factor PCC or four-factor PCC, activated PCC, and recombinant activated factor VII.[30] Due to its well-documented efficacy and safety in the urgent reversal of vitamin K antagonist, four-factor PCC has emerged as a promising agent to antagonize the effects of activated factor X inhibitors, although the mechanism of action is different, as discussed at the end of the next paragraph.[12–14]

PCCs contain either three or four coagulation factors (factors II, IX, and X, with or without factor VII) and, depending on the formulation, similar proportions of coagulation inhibitors such as protein C, protein S, and low doses of heparin.[31–33] The mechanism of action of PCCs is important for understanding their therapeutic applications. Vitamin K antagonists such as warfarin function by reducing levels of factors II, VII, IX, and X. For acute substitution, for example in severe bleeding, where coagulopathy is caused by consumption and dilution due to massive transfusion, PCCs serve as a source of coagulation factors. In contrast, levels of coagulation factors in bleeding patients on DOAC therapies are not primarily affected by the DOAC but the function of a single factor, either thrombin or activated factor X, is inhibited. The exact mechanism whereby PCC may improve hemostasis under activated factor X inhibition has not been clarified yet. Several animal studies and a phase I trial have indicated that there is a dose-dependent effect,[34–37] but the mechanism of action can probably not be explained by a simple stoichiometric reaction. On a molar basis, the concentration of FX in PCC is not sufficient to overcome the antagonizing effect of the activated factor X inhibitor. Instead, PCCs may restore hemostasis in patients on DOACs as they increase both prothrombin and FX levels at the site of injury and may exert a compensatory prohemostatic effect with increased thrombin generation potential.

There are considerable variations among countries in the availability and licensing status of PCCs. For example, four-factor PCCs have been used for many years in Europe, where their license is not restricted to vitamin K antagonist reversal; thus, PCCs have a broad approval for the "treatment and prophylaxis of bleeding in acquired deficiency of prothrombin complex coagulation factors."[38,39] In the United States, however, four-factor PCC was only approved in 2013 and is currently restricted in use to the urgent reversal of vitamin K antagonist therapy.

PCC in the Reversal of Activated Factor X Inhibitors

The benefit of PCC for management of activated factor X inhibitor–associated bleeds was first explored in animal models of acute hemorrhage using different injury approaches (*e.g.,* kidney incision, hepatosplenic bleeding, mesenteric bleeding, and intracranial hemorrhage)[40–42] and in spiking experiments in blood from human volunteers[42–47] (table 2). These studies provide evidence for improvement of surrogate markers such as partial or full normalization of prothrombin time (useful for rivaroxaban but not for apixaban or edoxaban), endogenous thrombin potential, or thrombin generation.

Phase I studies in human volunteers involved a supratherapeutic dose of rivaroxaban, apixaban, or edoxaban followed by administration of PCC at different doses (10 to 50 IU/kg body weight) and showed similar results in improvement of surrogate markers as was seen for the animal and *ex vivo* studies.[33,37,48–50] There was a dose-dependent response whereby 10 IU PCC/kg body weight was inadequate for activated factor X inhibitor reversal, 25 IU/kg body weight had partial effect, and 37.5 IU/kg body weight gave better reversal but still not complete normalization of coagulation parameters. A randomized placebo-controlled study using a punch biopsy bleeding model in healthy subjects on edoxaban reported reversal to baseline for bleeding duration and a trend toward reduced bleeding volume after administration of four-factor PCC, although CIs for the effect on bleeding duration overlapped with those for placebo.[37] Conversely, a similar study reported that four-factor PCC partially reversed the effects of rivaroxaban on thrombin generation but had no effect on bleeding duration or volume.[51]

Table 2. Overview of *Ex Vivo* Studies and Studies in Healthy Volunteers of the Efficacy of Four-factor PCC

	Study Design	No.	Xa inhibitor	PCC Dose(s)	Outcomes
Ex vivo studies					
Fukuda *et al.,* 2012, *Thromb Haemost*[42]	Spiked human plasma	(Pooled)	Edoxaban (up to 300 ng/ml)	0.15, 0.5, 1.5 U/ml	• PCC (0.15, 0.5 and 1.5 U/mL) reduced PT
Marlu *et al.,* 2012, *Thromb Haemost*[46]	Randomized crossover study; spiked human plasma	10	Rivaroxaban (20 mg)	0.25, 0.5, 1 U/ml	• PCC dose-dependently increased ETP AUC and thrombin peak • Slight reduction in lag time • No effect on time to peak
Dinkelaar *et al.,* 2013; *J Thromb Haemost*[43]	Spiked human plasma and whole blood samples	9	Rivaroxaban (up to 800 µg/l)	Up to 4 IU/ml	• PCC normalized thrombin potential • PCC did not normalize PT
Perzborn *et al.,* 2014, *Thromb Res*[41]	Spiked human plasma and whole blood samples	6	Rivaroxaban (200–1,000 ng/ml)	0.2–1.0 U/ml	• PCC significantly reversed PT prolongation • PCC did not reverse CT prolongation • PCC concentration-dependently increased ETP
Escolar *et al.,* 2015, *Circ J*[45]	Spiked steady and circulating human blood	8	Rivaroxaban (230 ng/ml)	50 IU/kg	• PCC shortened prolongation of CT and CFT • PCC restored thrombin generation
Körber *et al.,* 2016, *Blood Transf*[47]	Spiked human blood samples	10	Rivaroxaban (median 603 ng/ml)	25, 50 IU/kg	• 25 and 50 IU/kg PCC did not significantly improve PT • 25 and 50 IU/kg PCC significantly corrected aPTT • 25 IU/kg PCC significantly corrected CT but results did not reach significance with 50 IU/kg
Studies in healthy volunteers					
Eerenberg *et al.,* 2011, *Circulation*[82]	Randomized, double-blind, placebo-controlled crossover study	12	Rivaroxaban (20 mg twice daily for 2.5 d)	50 IU/kg	• PCC normalized PT • PCC normalized ETP
Levi *et al.,* 2014, *J Thromb Haemost*[83]	Open-label, single-center, parallel-group study of 3F-PCC vs four-factor-PCC	35 (3F-PCC=12; four-factor-PCC=11)	Rivaroxaban (20 mg twice daily for 4 d)	50 IU/kg	• PCC reduced prolonged PT (four-factor-PCC>3F-PCC) • PCC increased thrombin generation (3F-PCC>four-factor-PCC) • PCC did not reverse prolonged aPTT
Cheung *et al.,* 2015, *J Thromb Haemost*[48]	Randomized, double-blind, placebo-controlled, crossover study	6	Apixaban (10 mg twice daily for 3.5 d)	25, 37.5 IU/kg	• 25 and 37.5 IU/kg PCC restored PT prolongation • 25 and 37.5 IU/kg PCC increased ETP

Zahir *et al.,* 2015, *Circulation*[37]	Phase I double-blind, randomized, placebo-controlled, two-way crossover study	110	Edoxaban (60 mg)	10, 25, 50 IU/kg	• PCC dose-dependently reversed the effects of edoxaban
					   o 50 IU/kg PCC completely reversed extended bleeding duration and ETP
					   o 50 IU/kg PCC partially reversed PT prolongation

Barco *et al.,* 2016, *Br J Haematol*[83]	Randomized, double-blind, crossover, placebo-controlled study	6	Rivaroxaban (15 mg twice daily for 2.5 d)	25, 37.5 IU/kg	• 25 and 37.5 IU/kg PCC restored PT prolongation • 37.5 IU/kg of PCC led to partial restoration of ETP but 25 IU/kg did not
Nagalla *et al.,* 2016, *Clin Transl Sci*[49]	Phase I randomized, assessor-blinded, two-period crossover study	12	Apixaban (5 mg twice daily for 2.5 d)	25 IU/kg	• PCC increased peak thrombin generation • PCC partially reversed ETP • PCC shortened PT
Song *et al.,* 2017, *J Thromb Haemost*[50]	Open-label, randomized, placebo-controlled, three-period crossover study	15	Apixaban (10 mg twice daily for 3 d)	50 IU/kg	• PCC restored ETP • PCC increased TGA peak height but had little effect on TGA lag time or time to peak • PCC reversed prolonged PT • PCC did not reverse prolonged aPTT
Levy *et al.,* 2018, *J Thromb Haemost*[61]	Randomized, double-blind, parallel-group study of four-factor PCC vs TXA	147 (four-factor-PCC=48)	Rivaroxaban (20 mg twice daily for 3 d)	50 IU/kg	• PCC partially reversed PT prolongation • PCC completely restored ETP • PCC had no effect on bleeding duration or volume

3F, three-factor; 4F, four-factor; aPTT, activated partial thromboplastin time; AUC, area under the curve; CFT, clot formation time; CT, clotting time; ETP, endogenous thrombin potential; PCC, prothrombin complex concentrate; PT, prothrombin time; TGA, thrombin generation assay; TXA, tranexamic acid.

Four-factor PCCs contain a higher content of factor VII than three-factor PCCs, and evidence suggests that four-factor PCCs are a better option for reversing anticoagulation therapies. In a study in healthy volunteers taking rivaroxaban, four-factor PCC reduced mean prothrombin time within 30 min by 2.5 to 3.5 s, *versus* 0.6 to 1.0 s with three-factor PCC.[33]

Based on the available data, PCCs are recommended by international guidelines and used clinically in patients with major or life-threatening bleeding receiving DOAC therapy.[14,16,52] However, it was not known whether reversal in animal models, healthy subjects, and *ex vivo* studies accurately reflects reversal in bleeding patients on DOAC therapy. Therefore, the "An observational multicenter cohort study on efficacy and safety of Unactivated Prothrombin complex concentrate for reversal of oral, direct factor Xa inhibitors Rivaroxaban, Apixaban or edoxaban in Treatment of major bleeding Events" (hereafter referred to as UPRATE) study was initiated to systematically investigate the effectiveness of four-factor PCC to overcome severe bleeding in patients receiving rivaroxaban or apixaban.

Studies with PCC in Patients with Severe Bleeding

A number of cohort studies with PCC for management of activated factor X inhibitor–related bleeds have been published. In addition, there are registries of DOAC-related bleeds, including different aspects and where some patients were reported to receive PCC. An overview of these studies is provided in table 3.[53–61] Published data from the Dresden Registry on patients with DOACs only included 6 cases with PCC for major bleeding.[6] A prospective cohort from the Stroke Acute Management with Urgent Risk-factor Assessment and Improvement—Non-Valvular Atrial Fibrillation (SAMURAI-NVAF) study included 10 cases, but only 9 of those had been on an activated factor X inhibitor.[62] These and other case series with fewer than 10 cases have not been included in the overview. The assessment of effectiveness was done with different methods, with the two UPRATE studies being the only ones that provided assessment according to the International Society on Thrombosis and Haemostasis recommendation.[63] Overall, the effect was considered poor in 6 to 35%. The duration of follow-up varied from "length of stay in hospital" to 180 days, and the thromboembolism event rate was 0 to 8%, whenever specified (crude pooled ratio, 10 of 254 = 4%). Similarly, for mortality, the crude pooled ratio was 56 of 274 = 20%. The two UPRATE studies, which were prospective cohorts with similar design and outcome assessment, will hereafter be described in more detail.

UPRATE Study

The UPRATE study included two cohorts, one in Sweden[55] and one in Canada.[56] PCC was administered according to hospital recommendations (Sweden, 1,500 IU for patients weighing less than 65 kg and 2,000 IU for those weighing

65 kg or more; Canada, 2,000 IU for all patients), with the intention of giving approximately 25 IU/kg. Patients could be treated with any approved four-factor PCC (Sweden: Ocplex; Canada: Octaplex [Octapharma, Switzerland] or Sweden: Confidex; Canada: Beriplex [CSL Behring, USA]). There were differences in data collection between the two cohorts, with respect to time of arrival in hospital, hemoglobin levels after treatment, and follow-up on progression of hematoma volume. Treatment effectiveness was assessed as "effective" or "ineffective," in accordance with the International Society on Thrombosis and Haemostasis recommendation.[63] (In Canada, this was a *post hoc* analysis; effectiveness was initially classified as "good," "moderate," or "poor," according to the Assessment Guide by Sarode *et al.*[64]).

Eighty-four patients were recruited for the Swedish UPRATE cohort, and 66 were recruited for the Canadian cohort (table 4).[55,56] Patient age was similar in the two countries (Sweden, median 75 yr; Canada, mean 77 yr) but, in Sweden *versus* Canada, the percentage of males was lower (57% *versus* 67%) and the median body weight was lower (75 kg *versus* 81 kg). Rivaroxaban was used by more than half of patients in both studies (Sweden, 54%; Canada, 56%). The most common type of hemorrhage necessitating anticoagulation reversal was intracranial bleeding (Sweden, 70% of patients; Canada, 55%), while gastrointestinal bleeding had occurred in 16% of the Swedish patients and 24% of the Canadian patients (table 5).[55,56] Tranexamic acid was added more frequently in Sweden than in Canada (67% *versus* 26% of patients).

Four-factor PCC therapy was found to be "effective" in similar percentages of patients in the two cohorts: 69% in Sweden and 68% in Canada (table 6).[55,56] The percentages of patients receiving additional erythrocyte transfusions were 24% in Sweden and 20% in Canada. Across the two cohorts, there were eight thromboembolic events (5%) within 30 days after PCC administration. No deaths were considered directly related to treatment with PCC; one death (in a patient who had an ischemic stroke 5 days after PCC administration) was assessed as possibly related to treatment with PCC (table 3).[55,56] Overall, the results from the UPRATE study support the use of four-factor-PCCs for treating severe DOAC-associated bleeding, but they should be interpreted with caution due to the absence of a control group.

Andexanet Alfa, a Novel Antidote to the Anticoagulation Effects of FXA Inhibitors (ANNEXA)-4 Study

The phase III study with andexanet alfa included 352 patients with major bleeding.[27] Patients had to be within an 18-h window from the last dose of the factor Xa inhibitor and for evaluation of effectiveness, the baseline anti–factor Xa activity had to be at least 75 ng/ml. Thus, 249 patients were evaluable for effectiveness, and 171 (69%) were evaluated as excellent, 33 (13%) as good, and 45 (18%) as poor/

Table 3. Overview of Studies with PCC for Management of Major Factor Xa Inhibitor–related Bleeding*

First Author, Yr, Ref	Design	Cases Treated, No.	Xa inhibitor, No.	Bleeds Included	Follow-up, d	Effectiveness Assessment	Thromboembolic Events, No. (%)	Deaths, No. (%)
Grandhi, 2015[54]	Retrospective	18	riva (16), apix (2)	ICH	90	Effective 94%, not effective 6%	1 (6)	6 (33)
Albaladejo, 2017[53]	Prospective DOAC registry	148	riva (129), apix (19)	Mainly ICH for PCC	30	Totally 44%, partially 27%, not at all 19%	Not specified for PCC	Not specified for PCC
Majeed, 2017[55]	Prospective cohort	84	riva (45), apix (39)	International Society on Thrombosis and Haemostasis major bleeds[23]	30	International Society on Thrombosis and Haemostasis–effective† 69%, not effective 31%	2 (2)	27 (32)
Schulman, 2018[56]	Prospective cohort	66	riva (37), apix (29)	International Society on Thrombosis and Haemostasis major bleeds[23]	30	International Society on Thrombosis and Haemostasis–effective† 68%, not effective 32%	5 (8)	9 (14)
Gerner, 2018[57]	Retrospective of ICH on DOAC, N=146	94	riva (81), apix (13)	ICH	90	Effective 65%, not effective 35%	Not provided	Not specified for PCC
Testa, 2018[59]	Subset of prospective registry	20	riva (15), apix (20)	ICH (90%) and GI bleed (10%)	180	Not specified for PCC	Not specified for PCC	4 (20)
Tellor, 2018[58]	Retrospective	29	riva (18), apix (11)	90% had International Society on Thrombosis and Haemostasis major bleed[23]; 6 ICH	Variable	Not done	1 (3)	6 (21)
Harrison, 2018[60]	Subset of prospective registry	14	Not specified	ICH	Length of hospital stay	Hematoma expansion 7%	0	2 (14)
Tao, 2018[61]	Retrospective	43	riva (49), apix (51)	ICH (37%), GI bleed (40%)	14	Active bleeding despite PCC 7%	1 (2)	In hospital 2 (5)

*Only studies with at least 10 cases treated with PCC have been included. †Effectiveness assessment performed according to the ISTH recommendation.[62]

apix, Apixaban; DOAC, direct oral anticoagulant; GI, gastrointestinal; ICH, intracranial hemorrhage; ISTH, International Society on Thrombosis and Haemostasis; PCC, prothrombin complex concentrate; Ref, reference; riva, rivaroxaban.

Table 4. Baseline Characteristics in the Two UPRATE Cohorts[55,56] and in ANNEXA-4[27]

Characteristic*	Sweden	Canada	ANNEXA-4
N	84	66	352
Age, yr	75.0 ± 10.9	76.9 ± 10.4	77.4 ± 10.8
Male sex, No. (%)	48 (57)	42 (67)	187 (53)
Weight, kg, median (IQR)	75 (66–80)	81 (68–90)	Not available
Creatinine clearance, ml/min, median (IQR)	69.9 (49.6–93.0)	65.3 (51.8–87.2)	
< 30 ml/min, No. (%)	3 (4)	4 (6)	33 (9)
30 to < 60 ml/min, No. (%)	27 (32)	18 (27)	137 (39)
Indication for anticoagulation, No. (%)			
Atrial fibrillation	63 (75)	54 (82)	280 (80)
Venous thromboembolism	18 (21)	8 (13)	61 (17)
Both indications	3 (4)	2 (3)	Not available
Anticoagulant treatment			
Patients on rivaroxaban, No. (%)	45 (57)	37 (56)	128 (36)
Daily dose, mg	19.6 ± 2.3	18.6 ± 3.4	19.3 ± 4.3
Patients on apixaban, No. (%)	39	29 (44)	194 (55)
Daily dose, mg	8.8 ± 2.1	8.1 ± 2.4	7.7 ± 2.7
Concomitant antiplatelet agent, No. (%)*	10 (12)	11 (17)	Not available

Results are provided as mean ± SD unless otherwise stated. *One patient in each study was on concomitant aspirin plus clopidogrel.
IQR, interquartile range.

Table 5. Characteristics of Bleeding Events and Treatment With PCC in UPRATE[55,56] and in ANNEXA-4[27]

Characteristic*	Sweden N = 84	Canada N = 66	ANNEXA-4 N = 352
Type of bleeding, No. (%)			
Intracranial	59 (70)	36 (55)	227 (64.5)
Intraspinal	0	2 (3)	NA
Gastrointestinal	13 (16)	16 (24)	90 (25.6)
Retroperitoneal	5 (6)	3 (5)	16 (11)
Intramuscular	3 (4)	2 (3)	NA
Other	4 (5)	7 (11)	NA
Trauma-related bleed, No. (%)	26 (31)	25 (38)	99 (28)
Criteria for major bleeding, No. (%)†			NA
Critical organ	60 (71)	43 (65)	
Overt bleed, transfused ≥ 2 U	11 (13)	12 (18)	
Overt bleed, hemoglobin drop ≥ 20 g/l	17 (20)	28 (42)	
Time from onset of bleed to PCC, h	6 (2–10)	8.6 (5–18)	NA
Time from last dose DOAC to PCC, h	12.5 (9–16)	16.9 (11.9–21.2)	9.5 to 13.1‡
First dose of PCC, IU	2,000 (1,500–2,000)	2,000 (2,000–2,000)	Not applicable
First dose of PCC, IU/kg	26.7 (21.4–29.9)	25.0 (22.2–31.0)	
Second dose of PCC, No. (%)	3 (4)	2 (3)	Not applicable

*Results are provided as median (interquartile range) unless otherwise stated. †Some patients fulfilled more than one criterion. ‡Mean time from last dose to andexanet alfa for the 4 groups of Xa-inhibitors studied (edoxaban 9.5 h, apixaban 12.1 h, rivaroxaban 12.3 h, enoxaparin 13.1 h).
DOAC, direct oral anticoagulant; PCC, prothrombin complex concentrate; NA, not available.

none. The composite outcome of excellent or good was slightly better for gastrointestinal bleeding (85%) than for intracranial hemorrhage (80%). Thromboembolic complications within 30 days were observed in 10%, and 14% died within that time period. Major strengths of the study are that clinically important anti–Xa activity was demonstrated in all cases adjudicated for effectiveness and the larger number of patients than in the UPRATE studies. There were, however, more exclusion criteria in ANNEXA-4—for example, intracranial hemorrhage with a Glasgow Coma Scale score of less than 7, hematoma volume of greater than 60 ml, or estimated survival of less than 1 month.

Benefit–Risk Assessment for the Use of PCC in Anticoagulated Patients

Risk of Thromboembolic Events

Most interventions in acutely ill patients incur a degree of risk as well as expenditures, and in patients receiving anticoagulants, the essential risks are bleeding and

Table 6. Effectiveness Outcomes of DOAC Reversal Management in UPRATE[55,56]

Outcome*	Sweden (n = 84)	Canada (n = 66)
Effectiveness according to ISTH recommendation[63]		
Effective	58 (69)	45 (68)
Ineffective	26 (31)	21 (32)
Hemostatic effectiveness rating†		
Good	46 (55)	43 (65)
Moderate	13 (15)	13 (20)
Poor/None	25 (30)	10 (15)
Transfusions after PCC, patients		
Red cells (1–14 units)	18 (21)	16 (24)
Platelets (1–3 apheresis or pooled units)	10 (12)	8 (12)
Fresh frozen plasma (1–11 units)	8 (10)	4 (6)
Recombinant factor VIIa	1 (1)	0
Length of hospital stay, days, median (IQR)	7 (3–15)	16 (6–30)
Length of stay in ICU, days, median (IQR)	0.5 (0–2)	0 (0–6)

*Results are No. (%) unless otherwise stated. †Evaluated using an Assessment Guide[64] in the Swedish study by two steering committee members and in the Canadian study by the treating physician.

DOAC, direct oral anticoagulant; ICU, intensive care unit; IQR, interquartile range; ISTH, International Society on Thrombosis and Haemostasis; PCC, prothrombin complex concentrate.

thromboembolism. In patients with nonvalvular atrial fibrillation, clots in the left atrial appendage were seen in 10% of patients, and twofold to fourfold that proportion were seen in patients who suffered a recent thromboembolic event.[65] The risk of finding these thrombi in the appendage varies from 5 to 17% as inversely correlated to the left atrial appendage flow velocity, and as seen on transesophageal echocardiography.[66] In patients with venous thrombosis, recurrence is prevalent, especially during the first 3 months, before it is resolved or becomes reorganized to a fibrotic scar. Thus, in patients only treated for 6 weeks, the 3-month recurrence rate is 7%,[67] and in patients with venous thromboembolism and cancer, the 3-month recurrence rate while on warfarin is 15%.[68]

When anticoagulant therapy is automatically held after a major bleeding event, the underlying thrombotic risk is exposed. The bleeding event may require surgical intervention and bed rest for at least a few days with ensuing risk of VTE. Furthermore, bleeding initiates hemostatic responses, including stress hormone-induced release of factor VIII and von Willebrand factor from the endothelium and elevated fibrinogen levels.[69] Platelets are also released in premature forms from bone marrow and in mature form from the spleen and lungs.[70] In addition, hemodilution by infusion of crystalloids attenuates procoagulant factors, as well as natural anticoagulants such as antithrombin, resulting in prolonged half-life of activated factor X and thrombin.[70] This can lead to disseminated intravascular coagulation,[70] whereby small clots form in the bloodstream.

To place the risk of thromboembolic events with PCC in context, a meta-analysis found that thromboembolic events occurred in 4.2% of patients who had warfarin reversed with PCC *versus* 4.8% in patients treated with fresh frozen plasma.[71] Fresh frozen plasma has not been considered a thrombogenic substance. In eight uncontrolled studies using PCC for activated factor X inhibitor–associated major bleeds, the crude pooled incidence of thromboembolic events was 5%.[72] It therefore seems likely that an intrinsic thromboembolic event rate of 4 to 5% in these specific patients has to be accepted when oral anticoagulants are reversed for major bleeding. This risk has been reported as 10% for andexanet alfa.[27]

Evaluation of Available Evidence

The benefit of PCC for management of activated factor X inhibitor–associated bleeds has been explored in animal models and healthy human volunteers, as discussed earlier in the PCC in the Reversal of Activated Factor X Inhibitors section. However, the ultimate evidence for hemostatic efficacy comes from studies in the target population—patients with major bleeding. Fully conclusive data must come from randomized controlled trials, which have not been performed so far, neither with PCC for reversal of activated factor X inhibitors, nor with the specific antidotes against any of the DOACs. When the effectiveness of reversal is evaluated in uncontrolled studies, several possible scenarios can occur. First, the efficacy of the DOAC reversal agent could be adjudicated as poor because this is truly the case or because the case was not informative. For example, this could occur with a large intracranial hemorrhage where the patient is already moribund due to brain herniation, or in active spurting gastric bleeding (rated 1A or 1B according to the Forrest classification[73]), when reversal of the anticoagulant should not be expected to have effect. Second, the efficacy could be adjudicated as good/excellent because there was no further increase in hematoma volume or drop in hemoglobin levels; however, without serial measurements before reversal, it is not known if bleeding had already stopped by the time the reversal agent was provided. It would obviously be unethical to delay reversal in patients with acute intracranial hemorrhage to obtain serial brain scans or in patients with hemodynamically unstable gastrointestinal bleeding to obtain serial hemoglobin levels. Third, the effectiveness of reversal could be rated as good/excellent because the bleeding stopped but concomitant procedures were performed (external ventricular drain, endoscopic cauterization, therapeutic embolization). These cases should be classified as nonevaluable unless it can be shown that the bleeding stopped or decreased significantly before the procedure. Fourth, the efficacy is rated as good/excellent and it was indeed observed by the treating physician that an overt bleeding ceased with PCC. Cases of this last kind have been described, but it would difficult to perform a study solely on patients with overt and observable major bleeding.

Comparison of the effectiveness of DOAC reversal agents between different studies and therapies is problematic due to variations in the inclusion and exclusion

criteria, as well as in the definition of effectiveness. The criteria for effectiveness recommended by the International Society on Thrombosis and Haemostasis was used in the two UPRATE cohorts, which showed remarkably similar results (PCC was rated as 68% and 69% "effective"), despite independent assessments.[55,56] Another way to address the question of effectiveness is to understand the mechanism of action. Specific antidotes neutralize (idarucizumab) or compete with (andexanet alfa) the anticoagulant effect of the thrombin or activated factor X inhibitor, which can be directly appreciated as a reduction or normalization of anti-Xa levels. Conversely, PCC has a more general prohemostatic effect with elevation of the vitamin K–dependent factor levels, which compensate for the impaired activation of the prothrombinase complex. Furthermore, some PCCs contain clinically significant concentrations of the anticoagulants protein C and protein S,[31] which may contribute to maintaining hemostatic equilibrium under bleeding conditions and thereby reduce the risk of thrombotic events.

Taken together, there is reason to believe that the use of 25 IU/kg PCC for management of activated factor X inhibitor–associated bleeding has a positive effect without significantly increasing the risk of thromboembolic events. To understand how PCC performs in comparison with a specific antidote, the effectiveness of PCC in the Canadian UPRATE cohort and the effectiveness of andexanet alfa in the interim report from ANNEXA-4[26] were compared in a *post hoc* analysis.[56] The assessment rules from the ANNEXA-4 study for intracranial hemorrhage were applied to a similar subset from UPRATE, and were based on change in hematoma volume and/or thickness. After treatment with andexanet alfa or PCC, the outcome was classified as excellent or good in a similar percentage of patients (80% and 76% of patients, respectively). In the full report on andexanet alfa, 171 patients with intracranial hemorrhage were evaluable for efficacy, and the excellent/good classification remained at 80%.[27]

Restarting Anticoagulant Therapy

Irrespective of the strategy used for management of bleeding, it is important to restart prophylaxis against VTE as early as possible. Mechanical prophylaxis can be provided while the patient is still bleeding except in case of lower limb injuries. Pharmacologic prophylaxis can be started 2 to 4 days after an intracranial hemorrhage.[74] Pharmacologic stroke prophylaxis for patients with atrial fibrillation unfortunately requires a longer interval due to the higher dose. For patients with additional risk factors for recurrent intracranial or other life-threatening bleeds, one should consider left atrial appendage closure.[75]

Economic Considerations for the Use of PCC *versus* Andexanet Alfa

Finally, economic considerations influencing the use of anticoagulation reversal agents should be taken into account. For the dose of PCC used in most participants of the UPRATE study (2,000 IU, *i.e.*, 26 IU/kg body weight), the price is €800 (France), US$2,540 (United States),[76] or approximately Can$1,500 (Canada). In comparison, initial pricing of andexanet alfa (Andexxa, Portola, USA) is US$3,300 for a vial of 100 mg.[77] The recommended dose based on the ANNEXA-4 study protocol is a 400-mg bolus, followed by 480 mg as a 2-h infusion (nine vials=$29,700), but for reversal of rivaroxaban, edoxaban, or enoxaparin within 7 h from last dose or unknown interval, the bolus should be 800 mg, followed by a 960-mg infusion (18 vials=$59,400).[77] Thus for reversal of activated factor X inhibitors, PCC compares favorably to andexanet alfa, at least in terms of direct costs, as well as demonstrating comparable effectiveness and a low level of thromboembolic complications when used for reversal of activated factor X inhibitors in clinical trials so far. Nevertheless, it should be kept in mind that there were differences between the ANNEXA-4 and the UPRATE studies regarding design, patient characteristics, and effectiveness assessments. Only a head-to-head comparison between andexanet alfa and PCC, as requested by U.S. Food and Drug Administration and now being initiated, can demonstrate whether there are significant differences in effectiveness and safety between the products.

Areas for Further Research

Results from the UPRATE studies provided estimates of the effectiveness of PCC in activated factor X inhibitor reversal, but further studies are needed to optimize treatment. The selection of PCC dose for DOAC reversal requires finding the optimal balance between effectiveness and thrombotic risk. It cannot be excluded that higher doses of PCC may increase the risk of thromboembolic events. A careful increase of the dose of PCC might improve the effectiveness without increased risk and is worth exploring, preferably in a randomized controlled study with two doses.

Another open question is whether PCC should be given at a fixed dose based on body weight. The fixed dose is easy to remember and was used in the Canadian UPRATE cohort with almost identical effectiveness results as in the Swedish cohort, which used a quasi–weight-based regimen (1,500 or 2,000 IU for body weight less than or more than 65.0 kg).[55] However, it should be noted that as patient body mass increases, plasma volume gets larger. Whereas the area under the curve for oral activated factor X inhibitors is minimally influenced by body mass, a larger dose of PCC would be required to achieve the same plasma level as in patients with a lower body mass. Thus, a weight-based regimen for PCC would be more in accordance with the pharmacokinetic data.[78–80]

Outcomes for bleeding patients might be improved further by use of adjunctive therapies. Use of tranexamic acid for patients with mucosal bleeding, although contraindicated for urinary tract bleeding, and desmopressin

or platelet transfusion in case of concomitant antiplatelet therapy, should be explored. This could initially be done by hypothesis-generating subgroup analyses of previous studies.

Finally, the management of nonbleeding patients on activated factor X inhibitors before emergency surgery is of particular interest. If it can be shown that PCC provides normal or almost normal hemostasis during surgery for patients with a clinically important plasma level of anti-Xa, we would obtain crucial evidence for effectiveness. The assessment of effectiveness should include the evaluation by the surgeon, blood loss volume, and postoperative complications.

Future studies of PCC and of DOAC reversal agents must include instructions regarding aggressive thromboprophylaxis. The effectiveness of these agents should be assessed according to the recommendation by International Society on Thrombosis and Haemostasis, with or without other assessment rules, to facilitate comparisons between studies.

Conclusions

Current data including recent results from the UPRATE study support the use of PCC for the reversal of activated factor X inhibitors in bleeding patients and suggest that PCC could become a useful and relatively affordable option for management of DOAC-associated bleeding. Further studies are needed to investigate the optimal dosing of PCC to maintain the balance between procoagulant effectiveness and low thrombotic risk.

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Competing Interests

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ANESTHESIOLOGY

Transversus Abdominis Plane Block

A Narrative Review

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Since Rafi's[1] 2001 description, transversus abdominis plane blocks have become one of the most commonly performed truncal blocks.[2] They can be used to provide postoperative analgesia for open and laparoscopic abdominal surgery as well as inpatient and outpatient surgical procedures.[3] Transversus abdominis plane blocks remain a deceptively complex topic. For instance, not only can the transversus abdominis plane compartment be targeted using various approaches and techniques, but its size also requires a judicious dose of local anesthetic to ensure adequate postoperative pain control. More importantly, most approaches for transversus abdominis plane block only provide somatic (*i.e.*, abdominal wall) and not visceral analgesia. Thus, they may confer minimal benefits when compared with standard multimodal or thoracic epidural analgesia.

In this narrative review article, we discuss the anatomy, nomenclature, history, approaches/techniques, pharmacology, and complications of transversus abdominis plane blocks. We also review the evidence supporting their clinical use for common open and laparoscopic surgical procedures. Finally, we explore possible alternative truncal blocks as well as areas requiring further investigation.

Anatomy

The anterolateral abdominal wall encompasses four muscles: the rectus abdominis, external oblique, internal oblique, and transversus abdominis muscles. The transversus abdominis plane compartment is an anatomical plane that contains the T6–L1 thoracolumbar nerves and that can be found between the internal oblique and transversus abdominis muscles.[4] Anteriorly, the compartment is located between the transversus and rectus abdominis muscles.[5] Posterolaterally, as the

ABSTRACT

In this narrative review article, the authors discuss the anatomy, nomenclature, history, approaches (posterior *vs.* lateral vs. subcostal), techniques, pharmacology, indications, and complications of transversus abdominis plane blocks, as well as possible alternative truncal blocks.

Despite the scarcity of evidence and contradictory findings, certain clinical suggestions can nonetheless be made. Overall transversus abdominis plane blocks appear most beneficial in the setting of open appendectomy (posterior or lateral approach). Lateral transversus abdominis plane blocks are not suggested for laparoscopic hysterectomy, laparoscopic appendectomy, or open prostatectomy. However, transversus abdominis plane blocks could serve as an analgesic option for Cesarean delivery (posterior or lateral approach) and open colorectal section (subcostal or lateral approach) if there exist contraindications to intrathecal morphine and thoracic epidural analgesia, respectively.

Future investigation is required to compare posterior and subcostal transversus abdominis plane blocks in clinical settings. Furthermore, posterior transversus abdominis plane blocks should be investigated for surgical interventions in which their lateral counterparts have proven not to be beneficial (*e.g.*, laparoscopic hysterectomy/appendectomy, open prostatectomy). More importantly, because posterior transversus abdominis plane blocks can purportedly provide sympathetic blockade and visceral analgesia, they should be compared with thoracic epidural analgesia for open colorectal surgery. Finally, transversus abdominis plane blocks should be compared with newer truncal blocks (*e.g.*, erector spinae plane and quadratus lumborum blocks) with well-designed and adequately powered trials.

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rectus abdominis tapers to an end, the transversus abdominis plane compartment can be found between the internal oblique and transversus abdominis muscles.

Immediately after exiting from their respective intervertebral foramina, spinal nerves divide into anterior and posterior rami (fig. 1).[6] In turn, the anterior ramus gives off two main branches: the anterior and lateral cutaneous nerves. The anterior cutaneous branch (from the T6–T11 segments) gives rise to intercostal nerves, which supply the skin and muscles of the anterior abdominal wall.[4] The T6–T8 intercostal nerves initially travel between the innermost and internal intercostal muscles before entering the transversus abdominis plane compartment at the level of the costal margin.[4] In the transversus abdominis plane compartment, intercostal nerves display extensive interconnections and anastomosis to form the upper (cephalad) portion of the transversus abdominis plane plexus. The T9–T11 intercostal and T12 subcostal nerves penetrate the transversus abdominis plane compartment posterior to the midaxillary line.[7] They also interconnect

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Fig. 1. Transverse section of the lower abdominal wall (at the T12 level) demonstrating the course of a thoracolumbar nerve.

with each other and form the lower (caudad) portion of the transversus abdominis plane plexus. The latter runs along the deep circumflex iliac artery[4] and enters the rectus sheath at the lateral edge of the rectus abdominis muscle. Within the rectus sheath, the neural plexus runs along the deep inferior epigastric artery. The lower thoracic intercostal and subcostal nerves innervate the skin of the infra-umbilical area between the midline and midclavicular lines.

The lateral cutaneous branches of the T6–T11 spinal nerves depart from their respective anterior rami near the angle of the rib, or around the midaxillary line.[3,5,6] Thus, the lateral cutaneous branches arise before the main nerves penetrate the lateral transversus abdominis plane compartment (fig. 1). They supply the skin over the lateral abdominal wall between the costal margin and iliac crest.[5,6,8]

The L1 spinal nerve divides into iliohypogastric and ilioinguinal nerves. Both leave the transversus abdominis plane compartment anterior to the middle third of the iliac crest and lie ventral to the internal oblique muscle and medial to the anterosuperior iliac spine. These nerves supply the anterior abdomen at the level of the inguinal area and the medial thigh.[5,9]

Nomenclature of Transversus Abdominis Plane Blocks

The transversus abdominis plane compartment can be accessed using various approaches and techniques. For the purposes of this review article, the term *approach* refers to

the anatomical site *where* the transversus abdominis plane compartment is targeted. The term *technique* refers to *how* (*i.e.*, loss-of-resistance, ultrasound guidance, direct surgical vision) the compartment is identified for a given approach.

The nomenclature pertaining to approaches remains controversial.[10] For the sake of simplicity and clarity, the current review employs a modified version of the 2015 classification proposed by Hebbard.[11] The *subcostal* approach targets the transversus abdominis plane compartment in the anterior abdominal wall (beneath the costal margin as its name implies) anywhere between the xyphoid process[12] and the anterosuperior iliac spine (fig. 2).[13] The *lateral* approach targets the transversus abdominis plane compartment in the lateral abdominal wall between the midaxillary[14] and anterior axillary[15] lines (fig. 3). Finally, the *posterior* approach targets the transversus abdominis plane compartment at the level of the lumbar triangle of Petit[1] or the anterolateral aspect of the quadratus lumborum muscle (fig. 4).[16]

History of Transversus Abdominis Plane Blocks

Beyond simple academic interest, a discussion of the circuitous history of transversus abdominis plane blocks allows clinicians to understand the practical problems that led to the development of the different approaches. Furthermore, the chronology eloquently illustrates the possible continuum that exists between (posterior) transversus abdominis plane blocks and its more modern counterparts (*e.g.*, quadratus lumborum blocks).
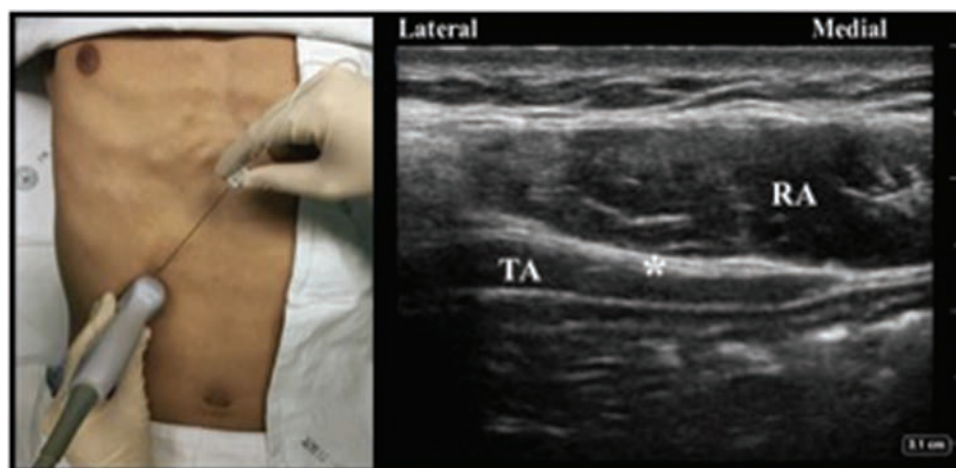
Fig. 2. Ultrasound probe position, needle puncture site, and sonographic image of the subcostal transversus abdominis plane block. Asterisk indicates needle target; RA, rectus abdominis muscle; TA, transversus abdominis muscle.
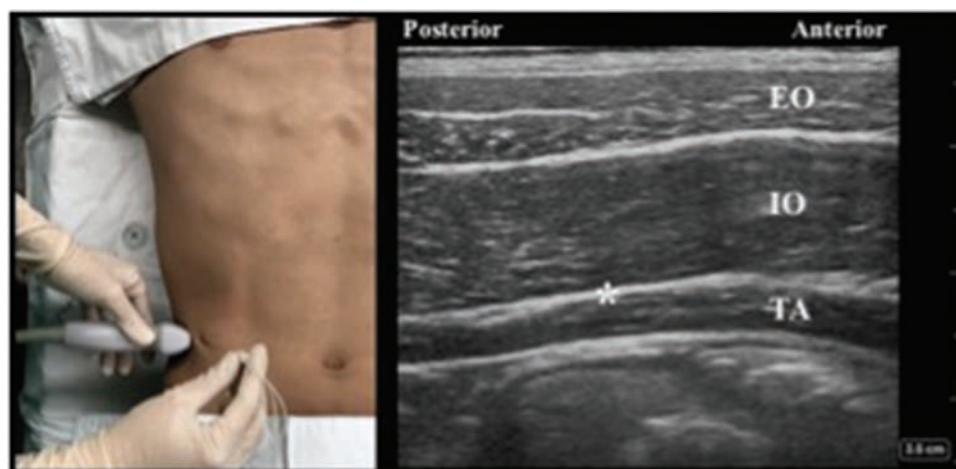
Fig. 3. Ultrasound probe position, needle puncture site, and sonographic image of the lateral transversus abdominis plane block. Asterisk indicates needle target; EO, external oblique muscle; IO, internal oblique muscle; TA, transversus abdominis muscle.

The first description of transversus abdominis plane block is generally credited to Rafi, who, in 2001, advocated the performance of abdominal field block at the level of the lumbar triangle of Petit. Rafi suggested using a blunt needle and a single pop sensation to identify the intermuscular plane between the internal oblique and transversus abdominis muscles, a compartment purported to contain the 7th to 11th intercostal nerves, the subcostal nerve, as well as the ilioinguinal and iliohypogastric nerves.[1] In 2006, O'Donnell[17] introduced the term *transversus abdominis plane block* into the literature. He also modified Rafi's original description by advocating a double pop technique to identify the planes between fascial extensions of the external oblique muscle and the internal oblique muscle (first pop),[18] and between the internal oblique and transversus abdominis muscles (second pop).[17]

The next technical development occurred in 2007: because the triangle of Petit can be difficult to identify in obese patients (because of its increased depth) and elderly subjects (because of a loss in muscle mass), Hebbard *et al.*[14] advocated the use of ultrasound guidance to identify the different intermuscular planes. Hebbard *et al.*[14] also favored a puncture site on the midaxillary line (instead of the triangle of Petit) to facilitate visualization of the abdominal wall. The technique proposed by Hebbard *et al.*[14] was indeed easy to master, as experience with only 16 blocks was required
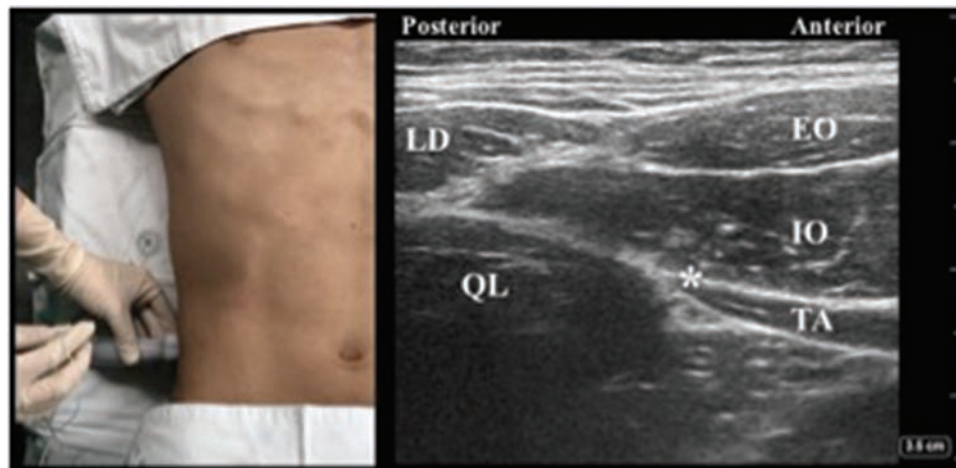
Fig. 4. Ultrasound probe position, needle puncture site, and sonographic image of the posterior transversus abdominis plane block. Asterisk indicates needle target; EO, external oblique muscle; IO, internal oblique muscle; LD, latissimus dorsi muscle; QL, quadratus lumborum muscle; TA, transversus abdominis muscle.

to achieve 90% proficiency.[19] Unfortunately, clinical experience[12,20] and cadaveric investigation[21] soon revealed that lateral transversus abdominis plane blocks cover mainly the T10 to L1 dermatomes, thereby confining their usefulness to lower abdominal surgery.

Dissatisfaction with the lateral approach spearheaded the search for better alternatives. Two (opposite) schools of thought arose. In one camp, propelled by Hebbard's subsequent 2008 report,[12] operators started experimenting with an ultrasound-guided subcostal approach for transversus abdominis plane blocks. With this method, the initial needle insertion begins near the xyphoid process and the local anesthetic is deposited between the rectus abdominis and transversus abdominis muscles. Subsequently, the needle is directed inferolaterally along the costal margin toward the anterosuperior iliac spine with incremental local anesthetic injection to distend the transversus abdominis plane compartment.[12] A 2010 confirmatory study by Lee *et al.*[22] demonstrated that, compared with its lateral counterpart, the new subcostal approach anesthetized an increased number of dermatomes (4 *vs.* 3) and yielded a higher peak of sensory blockade (T8 *vs.* T10).

In contrast, proponents of the ultrasound-guided posterior approach advocated displacing the puncture site posterior to the midaxillary line to target the anterolateral border of the quadratus lumborum muscle. The earliest description of posterior transversus abdominis plane blocks can be traced back to a 2011 study by Carney *et al.*[16] In it, the authors credited a personal communication with Dr. Rafael Blanco for the concept. In Carney *et al.*'s study, volunteers underwent ultrasound-guided posterior transversus abdominis plane blocks: local anesthetic and contrast solution were deposited at the intersection of the oblique/transversus abdominis muscles and the quadratus lumborum muscle, superficial to the transversalis

fascia. On subsequent magnetic resonance imaging, contrast spread reached the T6–T10 paravertebral spaces.[16] These findings seem to suggest that the mechanism of action of the posterior approach could be dual: blockade of the thoracolumbar nerves in the transversus abdominis plane compartment and local anesthetic spread around the quadratus lumborum muscle to the paravertebral space. Interestingly, the injection site posterior to the midaxillary line was not only reminiscent of Rafi's[1] original description but also of the lateral (*i.e.*, type 1) quadratus lumborum block described by Blanco.[23] This has prompted some authors to ponder whether the posterior transversus abdominis plane block is in fact a mislabeled quadratus lumborum block.[24] Such a parallel appears logical but remains unproven, as preliminary studies revealed that, contrarily to posterior transversus abdominis plane blocks,[16] local anesthetic injected in the setting of lateral quadratus lumborum blocks does not spread to the paravertebral spaces.[25,26]

In summary, the landmark-guided posterior approach constitutes the original method for transversus abdominis plane block. Over the last decade, technical difficulty and the search for more extensive (upper) abdominal wall anesthesia led to the subsequent development of ultrasound-guided lateral and subcostal approaches, respectively. In recent years, things seem to have come full circle with the (re)discovery of the (ultrasound-guided) posterior approach. The latter may share some similarities with the lateral quadratus lumborum block.

Approaches and Techniques for Transversus Abdominis Plane Blocks
Optimal Approach for Transversus Abdominis Plane Blocks

Before 2014, no randomized trial had compared the subcostal, lateral, and posterior approaches head-to-head in

clinical settings, and much of the knowledge was inferred from cadaveric[27] and volunteer[16,22] investigations as well as meta-analyses.[28] For instance, in a cadaveric study (n = 13), Milan *et al.*[27] injected 40 ml of dye under direct vision in the subcostal, lateral and posterior transversus abdominis plane compartments. These authors then traced the area of dye spread onto clear plastic, which was then photographed. Milan *et al.*[27] found that the spread was greatest for the subcostal approach (85 cm²) followed by its posterior (78 cm²) and lateral (59 cm²) counterparts, with statistical significance reached for the difference between subcostal and lateral approaches. These findings echoed the results of a 2010 volunteer study by Lee *et al.*,[22] who reported a higher number of dermatomes (4 *vs.* 3) anesthetized with the subcostal compared to the lateral approach.

Based on preliminary studies, the pattern of spread also seems to differ between approaches. In a volunteer study, Carney *et al.*[16] observed (with magnetic resonance imaging) that levobupivacaine-gadolinium injected with the lateral and subcostal approaches remained in the transversus abdominis plane compartment. In contrast, the mixture reached the quadratus lumborum muscle and T5–L1 paravertebral spaces with the posterior approach. Subsequently, in a 2013 meta-analysis, Abdallah *et al.*[28] reported that, compared with placebo, the posterior approach results in decreased pain and breakthrough opioid consumption during the first 48 h after lower abdominal surgery. Such benefits do not seem to occur with the lateral approach.[28] Abdallah *et al.*[28] speculated that the improved pain control seen with posterior transversus abdominis plane blocks stems from paravertebral local anesthetic spread, which results in sympathetic block and, consequently, improved visceral analgesia.

Since 2014, six randomized controlled trials have compared the different approaches for transversus abdominis plane blocks in the setting of laparoscopic cholecystectomy[29–32] Cesarean delivery[33] and laparoscopic gynecologic surgery.[34] Overall, these randomized controlled trials confirmed the knowledge derived from previous volunteer and cadaveric studies. For instance, in four trials, compared with their subcostal counterparts, lateral transversus abdominis plane blocks expectedly resulted in higher pain scores during the first 24 h after laparoscopic cholecystectomy.[29–32] Furthermore, lateral transversus abdominis plane blocks anesthetized fewer dermatomes[34] and also proved inferior to posterior transversus abdominis plane blocks for Cesarean delivery due to increased pain scores at rest (during the first 24 h), shorter analgesic duration and decreased patient satisfaction.[33]

In summary, based on the current knowledge, we suggest using the subcostal and posterior approaches instead of the lateral approach. Future randomized investigation is required to compare subcostal and posterior transversus abdominis plane blocks. In recent years, to circumvent the shortcomings associated with the lateral approach, some

operators have advocated combining the latter with the subcostal approach thereby creating a multiple quadrant injection method.[35–37] Although this strategy results in more widespread dermatomal anesthesia than the targeted lateral approach,[36] we suggest caution with local anesthetic dosing, especially in subsets of patients at risk for local anesthetic systemic toxicity (*e.g.*, elderly, children, individuals with low muscle masses). One possible strategy consists in retaining conventional volumes of local anesthetic but using more dilute concentrations (*e.g.*, bupivacaine 0.2%) with epinephrine.

Optimal Technique for Transversus Abdominis Plane Blocks

The transversus abdominis plane compartment can be located with landmarks or ultrasound guidance. Alternately, it can also be identified intraoperatively by surgeons.

To date, the landmark-guided technique has been used exclusively for the posterior approach.[1,8,17] Although still employed by some authors,[38] it can be fraught with technical challenges. The landmark-guided technique requires two fundamental steps: identification of the lumbar triangle of Petit and recognition of the intermuscular plane between the internal oblique and transversus abdominis muscles with tactile pops. Unfortunately, the triangle of Petit can be difficult to palpate in obese patients,[1,39] and its position varies significantly between individuals.[7,40] Furthermore, in 17.5% of patients, it can be absent because the external oblique overrides the latissimus dorsi muscle.[41] To complicate matters further, the technical endpoint (pop sensation) remains debated. Although some experts advocate the search of two distinct pops,[17] others use only a single pop, as the initial crossing of fascial extensions of the external oblique muscle (theoretically the first pop) may be too subtle to be felt.[38] In fact, in 36 patients undergoing bilateral transversus abdominis plane blocks, McDermott *et al.*[42] reported that the double pop endpoint resulted in correct needle position in only 24% of cases (as assessed by ultrasound guidance). Alarmingly, in 18% of the time, the needle tip inadvertently breached the peritoneum.[42] Thus, despite the lack of randomized controlled trials comparing landmark and ultrasound techniques, the potential for visceral injury[43] has led many authors to favor the use of ultrasound guidance for transversus abdominis plane blocks.[42,44,45]

To date, no randomized, controlled trial has investigated the optimal technique for ultrasound-guided transversus abdominis plane blocks in clinical settings. However, preliminary cadaveric studies suggest that, for the lateral approach, a minimal volume of 15 ml is required[46] and, for the subcostal approach, a multiple-injection technique (along the costal margin) provides more extensive coverage than its single injection counterpart.[47]

In 2010, West *et al.*[48] and Araco *et al.*[49] described for the first time the intraoperative performance of transversus abdominis plane blocks by surgeons. Since these initial

reports, multiple instances of surgical transversus abdominis plane blocks have been published. In fact, the number of permutations of transversus abdominis plane blocks is much greater with surgical than with (anesthesiologist–driven) percutaneous methods. For instance, like anesthesiologists, surgeons can target all three (subcostal, lateral, and posterior) transversus abdominis plane compartments[48,50] in the setting of open (laparotomy)[48] or laparoscopic[51] incisions. However, they can also perform transversus abdominis plane blocks by going through the abdominal wall[48,50,51] or through the peritoneum.[52,53] Furthermore, unlike anesthesiologists, they can identify the transversus abdominis plane compartment using conventional tactile feel,[48] intraoperative ultrasound,[54] or direct vision with actual dissection of the oblique muscles[49] or peritoneum.[55] Unfortunately, no trial has elucidated the optimal technique for surgical transversus abdominis plane blocks.

To date, four randomized controlled trials (Jadad scores of at least 3) have compared surgeon- and anesthesiologist–performed transversus abdominis plane blocks.[56–59] In three studies investigating subcostal transversus abdominis plane blocks performed for laparoscopic cholecystectomy,[56] and lateral transversus abdominis plane blocks performed for Cesarean delivery[58] or laparoscopic colorectal surgery,[57] surgical and anesthesiologist-driven transversus abdominis plane blocks provided similar postoperative analgesia and breakthrough opioid consumption. However the surgical technique resulted in a 60 to 80% decrease in performance time.[56,58] In contrast, in one trial investigating minimally invasive colorectal surgery, surgical lateral transversus abdominis plane blocks resulted in similar pain scores but 17.2-mg-lower intravenous morphine consumption at 48 h than their anesthesiologist–performed counterparts.[59]

In summary, based on the current knowledge, we suggest foregoing landmark guidance in favor of ultrasound for the performance transversus abdominis plane blocks. Surgical transversus abdominis plane blocks constitute an interesting alternative to their anesthesiologist-performed counterparts, as they result in comparable analgesia but require a shorter performance time. Further investigation is required to elucidate the optimal technique for ultrasound as well as surgical transversus abdominis plane blocks.

Pharmacology of Transversus Abdominis Plane Blocks

Transversus abdominis plane blocks display rapid first phase absorption kinetics,[60–63] and can lead to elevated plasmatic concentrations of total and unbound fractions of local anesthetic. Compared with other truncal blocks (*e.g.*, rectus sheath block), transversus abdominis plane blocks may result in a 50% shorter time to maximum serum concentration. The latter most likely stems from the large, highly vascularized absorptive surface area.[62,64] Furthermore, accidental intramuscular injection (inside the internal oblique or transversus abdominis muscle) could lead to even faster local anesthetic uptake.[65] All these factors may predispose to local anesthetic systemic toxicity.[66,67]

Pharmacokinetics of Single Dose of Local Anesthetic

Multiple trials have assessed local anesthetic plasmatic concentrations after boluses of ropivacaine,[36,60–63,68–74] levobupivacaine,[75–78] and bupivacaine.[79,80] The reported mean time to maximum serum concentration ranged from 10 to 35 min. However delayed absorption can occasionally occur with a time to maximum serum concentration as high as 240 min.[79] With increasing local anesthetic doses, a clear dose–dependent trend in maximum plasma concentration emerges. However the time to maximum serum concentration remains constant.[80]

Although local anesthetic plasmatic levels often exceed known toxic thresholds in many cases, only a minority of patients seems to display signs of local anesthetic toxicity. This could be explained by the fact that, in many trials, patients were under general anesthesia at the time of maximum plasma concentration. Interestingly, many reported cases of local anesthetic toxicity originate from two trials performed in obstetrical patients undergoing Cesarean delivery.[69,80] This strengthens the argument that the calculation of local anesthetic dosing should perhaps be based on lean rather than real (*i.e.*, pregnant) body weight.[66,69] Conversely, elevated plasma levels have been reported in patients receiving doses that would traditionally be considered safe (*e.g.*, 2.1 mg/kg of ropivacaine).[68] Therefore the discrepancy between local anesthetic dosage and (toxic) plasma levels underscores the complex interaction between bound and unbound local anesthetic concentrations.[68,80]

The impact of epinephrine (up to 5 μg/ml) on local anesthetic systemic absorption for transversus abdominis plane block has been addressed in two studies. These trials demonstrated 35% maximum plasma concentration decreases and time to maximum serum concentration prolongations ranging from 18.5 to 44 min with the addition of epinephrine to the local anesthetic mix.[62,75]

Pharmacokinetics of Continuous Infusion of Local Anesthetic

To date, only two trials have investigated local anesthetic pharmacokinetics in the setting of continuous transversus abdominis plane blocks.[81,82] In these studies, the time to maximum serum concentration for the infusion occurred at 48 h and 72 h for the subcostal and posterior approach, respectively. However, the unbound maximum plasma concentration peaked earlier (within 24 h) and remained steady thereafter.

Optimal Local Anesthetic Agent

To date, one randomized controlled trial (published in English) has investigated the optimal local anesthetic for transversus abdominis plane blocks. In 2016, Sinha *et al.*[83] compared bupivacaine 0.25% and ropivacaine 0.375% for transversus abdominis plane blocks in patients undergoing

laparoscopic cholecystectomy. Although the ropivacaine group displayed lower pain scores during the first postoperative hour, both drugs were equivalent in terms of the 24-h cumulative analgesic requirement.[83]

Optimal Local Anesthetic Dose

When it comes to the selection of an optimal local anesthetic dose for transversus abdominis plane blocks, little definitive information is available. A recent meta-analysis compared high dose (greater than 50 mg of bupivacaine equivalents) with low dose (less than or equal to 50 mg of bupivacaine equivalents) of long-acting local anesthetic and found no intergroup differences in terms of analgesia, 6-h or 24-h opioid consumption, time to first analgesic request, and patient satisfaction.[84] To date, very few dose-finding studies have investigated the ED50 for transversus abdominis plane blocks. This dearth of evidence may be attributed to the difficulty in carrying out such studies in light of the significant interindividual variability in analgesic effect. In one trial, the ED50 for ropivacaine in adults was 2.7 mg/kg, a dose for which toxicity has been previously reported.[85] In children, the ED50 of levobupivacaine was found to be 0.22 mg/kg,[86] and the EC50 of bupivacaine, 0.08239%.[87]

Local anesthetic dose constitutes the mathematical product of concentration and volume. In the literature, multiple trials have compared different local anesthetic concentrations (using constant volumes). Overall, these studies revealed minimal differences between low (0.125 to 0.25%) and high (0.5 to 0.75%) concentrations of bupivacaine, levobupivacaine, and ropivacaine in terms of postoperative pain and rescue analgesic consumption.[88–94] In terms of volume, large injectates (15 to 30 ml per side in adults; 0.1 to 1 ml/kg per side in children) are commonly used to ensure adequate local anesthetic spread for transversus abdominis plane blocks.[78,79,84,95] Although the minimal effective volume remains unknown, a trend toward superior analgesia was demonstrated with at least 15 ml per side in a meta-analysis conducted by Abdallah *et al.*[96] These findings concord with those of a subsequent cadaveric study, which reported that, compared with lower volumes, 15 ml can provide more extensive cephalo-caudal spread.[46] Finally, the overall local anesthetic dose seems to matter more than either concentration or volume alone, as differences in volumes carry minimal analgesic impact in the setting of a constant local anesthetic dose.[78,97,98]

Optimal Local Anesthetic Infusion Strategy

In the literature, two trials have compared continuous local anesthetic infusion with intermittent local anesthetic boluses (without a background infusion). In the first study, compared with a continuous infusion (8 ml/h of ropivacaine 0.2%), 20-ml aliquots every 8 h proved to be more economical because of lower local anesthetic daily requirement.[99] Furthermore, the intermittent bolus-group maintained similar block coverage on postoperative days 1 and 2, whereas its continuous infusion counterpart displayed a regression of two dermatomal segments.[99] A second trial simultaneously applied both strategies in volunteers[100]; one side received a continuous infusion of ropivacaine 0.2% at 8 ml/h, whereas the other side received intermittent boluses (24 ml of ropivacaine 0.2% every 3 h). The primary outcome, block extension at 6 h, was similar in both groups; however, there was a significant difference at various time points between 0 and 5 h favoring intermittent boluses.

Optimal Adjuvants

In the literature, several adjuvants (*i.e.*, dexamethasone, alpha-2 agonists, magnesium, opioids, liposomal formulation) have been investigated to prolong the duration of transversus abdominis plane blocks.

Dexamethasone is commonly used for peripheral nerve blocks.[101] A 2018 meta-analysis concluded that, compared with saline, perineural dexamethasone (4 to 8 mg) can increase the duration of transversus abdominis plane blocks by almost 3 h while reducing breakthrough analgesic consumption and postoperative nausea and vomiting.[102] To date, the optimal dose and mode of administration (intravenous *vs.* perineural) have not been investigated for transversus abdominis plane blocks.

Alpha-2 agonists (*i.e.*, clonidine and dexmedetomidine) have also been used as adjuvants for transversus abdominis plane blocks. Clonidine has been investigated in the setting of transversus abdominis plane blocks performed for Cesarean delivery. Compared with plain bupivacaine, analgesia was prolonged by 10 h with the simple addition of clonidine (1ug/kg per side); however, sedation occurred in almost one third of patients.[103] In the case of dexmedetomidine, studied doses include both weight-based regimens (*i.e.*, 0.5 to 1 µg/kg per side) and fixed dosing (*i.e.*, 100 µg per side). A 2018 meta-analysis reported significant reductions in pain scores at rest and on movement with the addition of dexmedetomidine for transversus abdominis plane blocks.[104] However, dexmedetomidine may result in increased sedation during the first postoperative hour as well as a lower heart rate during the first 4 h.[105] Future trials are required to investigate the optimal dose and route of administration (intravenous *vs.* perineural) of dexmedetomidine.

Since 2016, four randomized controlled trials have looked at the role of perineural magnesium for transversus abdominis plane blocks. Compared with control, doses between 0.15 and 0.5 g (per side) provide lower postoperative pain scores (for up to 12 h), longer analgesic duration, and lower morphine consumption.[106–109] Future trials are required to investigate the optimal dose and mode of administration (intravenous *vs.* perineural) of magnesium for transversus abdominis plane blocks.

Finally, two randomized controlled trials conducted by Hutchins *et al.* have investigated the benefits of liposomal bupivacaine.[110,111] Compared with bupivacaine with epinephrine, liposomal bupivacaine (130 mg) resulted in improved analgesia during the study period (72 h) as well as decreased opioid consumption and postoperative nausea or vomiting. To date, no trial has prospectively compared liposomal bupivacaine and continuous transversus abdominis plane blocks.

In summary, based on the current knowledge, we suggest the use of dilute concentrations of local anesthetic (*e.g.*, bupivacaine 0.2 to 0.25% or ropivacaine 0.2 to 0.25%) and injecte volumes of at least 15 ml (per side) for single-injection transversus abdominis plane blocks. For perineural transversus abdominis plane catheters, intermittent boluses (every 8 h) may provide more extensive blockade and higher cost efficiency than continuous local anesthetic infusion. Adjuvants such as dexamethasone, dexmedetomidine, and magnesium can increase the duration of transversus abdominis plane blocks. However, future investigation is required to elucidate their optimal dosing, mode of administration (intravenous *vs.* perineural), and combination. Furthermore, buprenorphine has been reported to prolong peripheral nerve blocks[112] and thus should also be investigated for transversus abdominis plane blocks in terms of efficacy as well as attendant emetic risk. Finally, patients undergoing transversus abdominis plane blocks remain at risk for local anesthetic systemic toxicity. In addition to careful local anesthetic dosing (based on lean weight), the operator should consider adding epinephrine to the local anesthetic mix, and providing patient monitoring for a period exceeding the time to maximum serum concentration (*e.g.*, 40 min).

Clinical Indications for Transversus Abdominis Plane Blocks

In the literature, transversus abdominis plane blocks have been used for a multitude of surgical interventions.[3] The current review article focuses on the most common ones (*i.e.*, Cesarean delivery, laparoscopic cholecystectomy, hysterectomy, colorectal resection, appendectomy, inguinal hernia repair, prostatectomy, and bariatric surgery). To highlight the contemporary evidence, we base our suggestions on systematic reviews or meta-analysis published in 2018 or 2019. In the absence of such recent reviews, we derive our conclusions from the cumulative findings of randomized controlled trials. However, only trials published in PubMed-indexed journals were retained for analysis. This precautionary step was undertaken to minimize the impact of weaker studies published in lower tiered journals. Furthermore, particular attention (discussion) was paid to the control arm of randomized controlled trials, as the validation of transversus abdominis plane blocks (or any block) requires that control subjects receive optimal standard treatment (*e.g.*, thoracic epidural and multimodal analgesia for open and laparoscopic abdominal surgery, respectively). For the purposes of the current review, the term *multimodal analgesia* was defined as the use of at least two nonopioid analgesic agents (*e.g.*, acetaminophen, nonsteroidal antiinflammatory drug, gabapentinoids, ketamine, local anesthetic wound infiltration)[113] in addition to *pro re nata* oral or parenteral opioids.

Cesarean Delivery

Cesarean delivery constitutes the ideal surgical setting to investigate transversus abdominis plane blocks because the conventional Pfannenstiel incision lies in a territory readily anesthetized by the commonly performed lateral approach. Furthermore, because Cesarean section involves uterine incision but not excision, postoperative visceral trauma and pain may (arguably) be less pronounced. To date, Cesarean delivery constitutes the most studied surgery for transversus abdominis plane blocks.[114–133] Unfortunately, the most recent systematic review article investigating the efficacy of transversus abdominis plane blocks for Cesarean section dates back to 2016[134] and thus did not include more recent trials.

Starting with the first trial investigating transversus abdominis plane blocks for Cesarean delivery (2008),[114] multiple randomized controlled trials have concluded that the addition of posterior or lateral transversus abdominis plane blocks to a pharmacologic regimen encompassing acetaminophen, nonsteroidal antiinflammatory drugs and parenteral opioids results in significant analgesic and opioid-sparing benefits.[115,118,120,121,130,132] However subsequent trials revealed that the efficacy of transversus abdominis plane blocks rivals at best that of wound infiltration[126–128,131] and is inferior to that of intrathecal morphine (100 to 200 μg).[117,122] Because the latter is commonly used to provide analgesia for Cesarean delivery, the issue became whether the addition of transversus abdominis plane blocks to multimodal regimens that include long-acting neuraxial opioids would result in supplemental analgesic benefits. Six randomized controlled trials have investigated the question.[116,119,123–125,129] Except for one study,[129] all trials (and the 2016 meta-analysis) concluded that posterior or lateral transversus abdominis plane blocks confer minimal advantages for Cesarean delivery in the setting of conventional doses of intrathecal morphine (*i.e.*, 100 to 250 μg).[116,119,123–125,134]

In summary, based on the current knowledge, we do not suggest the use of posterior or lateral transversus abdominis plane blocks for Cesarean delivery when long-acting intrathecal opioids are incorporated to the multimodal analgesic regimen. However, both approaches remain valuable analgesic options in patients who cannot receive intrathecal morphine or who undergo Cesarean section under general anesthesia.[135,136]

Laparoscopic Cholecystectomy

Laparoscopic cholecystectomy constitutes the second most studied surgery for transversus abdominis plane blocks.[15,29–31,50,88,137–144] The most recent meta-analysis investigating the efficacy of transversus abdominis plane blocks for laparoscopic cholecystectomy dates back to 2016.[145]

Over the last 10 yr, multiple trials have investigated the benefits of lateral transversus abdominis plane blocks in the setting of laparoscopic cholecystectomy with mixed results. Whereas initial trials by El Dalawlatly *et al.*[15] and Ra *et al.*[88] concluded that transversus abdominis plane blocks outperform intravenous morphine, a subsequent study detected no differences between the two analgesic strategies.[144] Furthermore, compared with local anesthetic infiltration of laparoscopic ports, lateral transversus abdominis plane blocks provide only marginal benefits in terms of postoperative pain scores[142] and analgesic duration.[137] In fact, in the context of a multimodal analgesic regimen that included acetaminophen and ibuprofen (as well as patient-controlled intravenous opioids), Petersen *et al.*[138] demonstrated that the benefits of transversus abdominis plane blocks are confined to a small (8 mm on a 0 to 100 mm scale) reduction in pain while coughing and a 2.5-mg decrease in opioid requirement only during the first two postoperative hours.

The contemporary evidence suggests that the subcostal approach consistently outperforms its lateral counterpart in the setting of laparoscopic cholecystectomy.[29–31] Therefore, from a methodologic standpoint, a critical analysis of potential benefits of transversus abdominis plane blocks for laparoscopic cholecystectomy should focus exclusively on the subcostal approach. In the literature, seven randomized controlled trials have compared subcostal transversus abdominis plane blocks with placebo (saline) or no treatment.[29–31,50,139,141,143] Except for one study that only detected a shorter extubation time with transversus abdominis plane blocks,[139] the six other trials unequivocally suggest that subcostal transversus abdominis plane blocks outperform the standard analgesic treatment[29–31,141,143] as well as periportal local anesthetic infiltration[50] with benefits extending up to 24 h postoperatively.[29,141] However, these findings should be interpreted with caution because in none of the six trials did the control group employ a multimodal analgesic regimen that included acetaminophen, nonsteroidal antiinflammatory drugs, and periportal local anesthetic infiltration.[146] Thus, it remains unclear whether, similarly to Cesarean delivery, the benefits of (subcostal) transversus abdominis plane blocks could be negated by multimodal analgesia.

In summary, based on the current knowledge, we suggest further investigation to determine whether (subcostal) transversus abdominis plane blocks provide clinical benefits in the context of a multimodal analgesic regimen that incorporates acetaminophen, nonsteroidal antiinflammatory drugs, and periportal local anesthetic infiltration. We suggest the subcostal approach (instead of its lateral counterpart) if operators elect to perform transversus abdominis plane blocks for laparoscopic cholecystectomy.

Hysterectomy

Transversus abdominis plane blocks have been extensively studied in the context of open[147–159] and laparoscopic hysterectomy.[160–169] In 2018, a meta-analysis authored by Zhou *et al.*[170] examined the benefits of transversus abdominis plane blocks for open and laparoscopic hysterectomy. Zhou *et al.*[170] concluded that, compared with placebo or no block, posterior/lateral transversus abdominis plane blocks result in reduced 24-hr morphine consumption, decreased pain scores at rest and on movement, lower incidences of nausea or vomiting, and increased analgesic duration after open hysterectomy. In contrast, transversus abdominis plane blocks seem to confer minimal benefits after laparoscopic hysterectomy.[170] The following year, Bacal *et al.*[171] decided to carry out a similar meta-analysis. However they limited the scope of investigation to benign disease. Similarly to Zhou *et al.*,[170] Bacal *et al.*[171] concluded that, compared with placebo or no block, posterior or lateral transversus abdominis plane blocks result in decreased early (2 h) and late (24 h) postoperative pain scores as well as a 10-mg lower morphine consumption at 24 h in patients undergoing open hysterectomy. Again, the benefits for laparoscopic hysterectomy seem marginal at best, as lateral transversus abdominis plane blocks can only decrease early postoperative pain.[171]

From a comparative standpoint, most published trials have used acetaminophen or nonsteroidal antiinflammatory drugs or periportal local anesthetic infiltration in the control group. However, none has employed a multimodal regimen that includes gabapentinoids,[172] ketamine, and possibly intrathecal opioids.[173] Thus, future investigation is required to determine whether the benefits of transversus abdominis plane blocks (for open hysterectomy) would survive the implementation of such a multimodal analgesic regimen.

In summary, based on the current knowledge, we do not suggest the use of lateral transversus abdominis plane blocks for laparoscopic hysterectomy. Future trials are needed to determine the benefits of posterior transversus abdominis plane blocks for the latter. Although the current evidence supports the use of posterior and lateral transversus abdominis plane blocks for open hysterectomy, the authors suggest further investigation to determine whether these benefits would still be present in the context of a multimodal analgesic regimen that includes gabapentinoids, ketamine, or intrathecal opioids.

Colorectal Surgery

Transversus abdominis plane blocks have been extensively studied in the settings of open[53,174,175] and laparoscopic colorectal surgery.[176–182] Although a recent meta-analysis has summarized the benefits of transversus abdominis plane blocks for colorectal surgery,[183] the inclusion of both open and laparoscopic procedures constitutes a methodologic limitation, as the two types of interventions display inherently different patterns of postoperative pain and thus should be analyzed separately.

In 2018, Oh *et al.*[184] restricted the scope of their review article to laparoscopic colorectal surgery to investigate the potential benefits of transversus abdominis plane blocks. These authors reported that (lateral) transversus abdominis

plane blocks decrease early and late dynamic pain on movement after laparoscopic colorectal surgery compared with placebo or no treatment, despite similar pain at rest and breakthrough opioid consumption. Although statistically significant, these differences may not be clinically meaningful (0.2 to 0.7 on a 0 to 10 scale).[184] Furthermore, Oh *et al.*'s results should be interpreted with caution, as only two[177,178] of the five trials[176–178,180,181] included for analysis used multimodal analgesia.

For open colorectal surgery, multiple trials have previously demonstrated that, compared with placebo, transversus abdominis plane blocks result in decreased postoperative pain and morphine consumption.[53,174] However, thoracic epidural analgesia is still considered by many to be the criterion analgesic standard for laparotomy: thus the more pertinent clinical question resides in the comparison of transversus abdominis plane and thoracic epidural blocks.[185,186] Randomized trials investigating single-injection transversus abdominis plane blocks and continuous epidural catheters also highlight a second important methodologic issue pertaining to study design. For instance, for upper abdominal surgery (gastrectomy), Wu *et al.*[187] found that low thoracic epidural analgesia proved superior to bilateral subcostal transversus abdominis plane blocks in terms of breakthrough opioid consumption. However, the benefits associated with epidural analgesia seem to become less pronounced in recent years, as authors started using longer acting (liposomal) formulations of bupivacaine.[188–190] Furthermore, preliminary evidence seems to indicate that continuous (subcostal) transversus abdominis plane blocks outperform their single-injection counterparts.[191] These cumulative findings suggest that block duration constitutes an important confounding variable. Therefore, to properly investigate the benefits of transversus abdominis plane blocks for open colorectal surgery, one must compare continuous thoracic epidural blocks with continuous transversus abdominis plane blocks.

To date, three randomized controlled trials have tackled the issue with mixed findings.[192–194] Niraj *et al.*[192] and Ganapathy *et al.*[194] concluded that, compared with their thoracic epidural counterparts, subcostal transversus abdominis plane catheters (with or without concomitant lateral transversus abdominis plane catheters) result in similar analgesia but an increased need for breakthrough analgesics. In contrast, Wahba *et al.*[193] reported that, in patients with ischemic heart disease, thoracic epidural blocks resulted in decreased pain scores, 100-min longer analgesic duration, and 10.5-mg lower intravenous morphine consumption during the first 48 h as well as decreased sedation (during the first 24 h) and improved patient satisfaction (2 points on a 0 to 10 scale). Nonetheless, despite their findings favoring the use of thoracic epidural analgesia, Wahba *et al.*[193] opined that transversus abdominis plane blocks remain a valid analgesic option if thoracic epidural analgesia is contraindicated.

Based on the current understanding, we suggest the use of thoracic epidural analgesia for open colorectal surgery. However, subcostal transversus abdominis plane blocks remain a valid alternative for patients undergoing laparotomy in whom neuraxial blocks are contraindicated. We suggest further investigation to determine whether the benefits of (lateral) transversus abdominis plane blocks for laparoscopic colorectal surgery would persist in the setting of multimodal analgesia.

Appendectomy

To date, three randomized trials have investigated the benefits of transversus abdominis plane blocks for open appendectomy[195–197] and three studies have done the same for laparoscopic appendectomy.[198–200] Overall the findings have been fairly consistent. For open appendectomy, both lateral and posterior transversus abdominis plane blocks have been shown to result in decreased postoperative pain scores at rest and on movement as well as significant reductions in consumption of intravenous morphine (22 mg) and tramadol (78 mg) at 24 h and intravenous morphine (12.3 mg) at 48 h[195–197] despite the use of acetaminophen and diclofenac.[195,196] In contrast, for laparoscopic appendectomy, two trials have concluded that lateral transversus abdominis plane blocks provide no benefit in the setting of multimodal analgesia.[198,200] However, Tanngaard *et al.*[199] were able to obtain a cumulative decrease in static and dynamic pain during the first 12 h by supplementing lateral transversus abdominis plane blocks with subcostal transversus abdominis plane blocks. Because the lateral approach provides minimal benefits for laparoscopic appendectomy,[198,200] Tanngaard *et al.*'s encouraging results could perhaps be attributed to subcostal transversus abdominis plane blocks. Therefore, future trials should assess isolated subcostal as well as posterior transversus abdominis plane blocks in the setting of laparoscopic appendectomy.

Based on the current knowledge, we suggest the use of posterior or lateral transversus abdominis plane blocks for open appendectomy. The current evidence does not support the role of lateral transversus abdominis plane blocks for laparoscopic appendectomy. Further investigation is required to elucidate the potential benefits of subcostal or posterior transversus abdominis plane blocks for laparoscopic appendectomy.

Inguinal Hernia Repair

Transversus abdominis plane blocks have been extensively studied in the context of open[201–206] and laparoscopic inguinal hernia repair.[207–209] The most recent meta-analysis investigating the benefits of transversus abdominis plane blocks for hernia repair dates back to 2017.[210]

For open inguinal repair, lateral or posterior transversus abdominis plane blocks result in lower pain scores and opioid consumption compared with no block[203,206] or wound

infiltration.[201,205] In fact, Petersen *et al.*[202] have shown that lateral transversus abdominis plane blocks provide similar efficacy to ilioinguinal blocks combined with wound infiltration. For laparoscopic inguinal hernia repair, two trials have also found decreased pain and opioid requirement with lateral transversus abdominis plane blocks compared with no block[207] and portal local anesthetic infiltration.[208] However, for bilateral laparoscopic inguinal hernia repair, preperitoneal instillation of local anesthetic outperforms transversus abdominis plane blocks.[209] To date, of all published trials for open and laparoscopic inguinal hernia repair, only one[208] has employed the recommended multimodal regimen, which includes acetaminophen, nonsteroidal antiinflammatory drugs, as well as local anesthetic infiltration.[211] Thus, further investigation is required to determine whether the benefits of transversus abdominis plane blocks would persist despite the implementation of such multimodal analgesia.

In summary, based on the current knowledge, we suggest further investigation to determine whether the benefits of posterior and lateral transversus abdominis plane blocks for open and laparoscopic inguinal hernia repair would persist in the context of a multimodal analgesic regimen that includes acetaminophen, nonsteroidal antiinflammatory drugs, and local anesthetic infiltration.

Prostactectomy

To date, three studies have investigated the efficacy of transversus abdominis plane blocks for open prostatectomy.[212–214] Two of the three trials could not detect significant benefit associated with lateral transversus abdominis plane blocks. Thus, we do not suggest their use for open prostatectomy. However future studies are required to investigate posterior transversus abdominis plane blocks for the latter as well as the potential benefits of transversus abdominis plane blocks for laparoscopic prostatectomy.

Bariatric Surgery

To date, three studies have investigated the efficacy of transversus abdominis plane blocks for (laparoscopic) bariatric surgery.[55,215,216] Two studies reported that transversus abdominis plane blocks result in lower postoperative pain scores and analgesic consumption as well as quicker ambulation and oral intake.[55,215] Although statistically significant, the differences in pain scores (less than 2 on a 0 to 10 scale), intravenous morphine consumption at 24 h (3.1 mg), ambulation (less than or equal to 1.7 h), and oral intake (2.4 h) may not be clinically relevant.[55,215] Interestingly, in the only trial that used a multimodal analgesic regimen (*i.e.*, acetaminophen, ketorolac at the end of surgery, and periportal local anesthetic infiltration), Albrecht *et al.*[216] reported no clinical benefits associated with (subcostal) transversus abdominis plane blocks. Thus, further confirmatory investigation is required to elucidate the benefits of transversus abdominis plane blocks in the setting of multimodal analgesia.

Complications of Transversus Abdominis Plane Blocks

Complications related to transversus abdominis plane blocks can be attributed to the needle or the local anesthetic agent.

In terms of needle-related adverse events, the abdominal wall is sufficiently vascularized to sustain needle trauma, as evidenced by the recent report of a (self-resolving) abdominal wall hematoma in an obstetrical patient with HELLP syndrome.[217] Furthermore, during the performance of transversus abdominis plane blocks, the needle tip can inadvertently traverse the transversus abdominis muscle (and peritoneum) thereby resulting in peritoneal breach and visceral injury.[43,218–220] Interestingly, if the needle tip is positioned just between the transversus abdominis muscle and the transversalis fascia (without puncturing the peritoneum), local anesthetic injection could result in transient femoral nerve blockade because the fascia iliaca constitutes the posterolateral continuation of the transversalis fascia.[221–223] The preceding complications underscore the importance of visualizing the entire length of the needle during the performance of ultrasound–guided transversus abdominis plane blocks.[224]

Because transversus abdominis plane blocks require relatively large injectates and are often carried out bilaterally, local anesthetic systemic toxicity remains a concern especially in elderly patients or those with decreased muscle mass. There exist multiple reports of local anesthetic systemic toxicity after the administration of (levo) bupivacaine (2.7 to 2.9 mg/kg)[225,226] as well as ropivacaine (4.9 to 7.9 mg/kg)[226,227] for transversus abdominis plane blocks. In none of these cases did the operators use adjunctive epinephrine to curtail local anesthetic plasmatic absorption.[228] Furthermore, in one report,[225] the 2.9-mg/kg dose of bupivacaine was administered to a patient experiencing acute fatty liver of pregnancy, a condition known to increase the free fraction of plasma bupivacaine (attributable to a decreased production of local anesthetic-binding serum proteins).[225] The prohibitively supratoxic dose (7.9 mg/kg) of ropivacaine reported by Sherrer *et al.*[227] stemmed from a lack of communication between surgeon and anesthesiologist, as the former carried out intraperitoneal local anesthetic infiltration (using 20 ml of ropivacaine 0.75%) before the latter's performance of transversus abdominis plane blocks (using 40 ml of ropivacaine 0.75%). Finally, local anesthetic injection in the transversus abdominis plane compartment may result in motor block of the thoracolumbar nerves. In turn, this could result in paresis of the abdominal muscles as evidenced by a bulge in the abdominal wall when the patient coughs or bears down.[229,230] In both reported cases, the bulge subsided uneventfully as the transversus abdominis plane block wore off.[229,230]

In summary, based on the current knowledge, care must be taken to visualize the entire length of the needle during the performance of transversus abdominis plane blocks to prevent breaching the transversus abdominis muscle and the

peritoneum thereby minimizing the risk of femoral blockade and visceral injury. Furthermore, a thorough analysis of risks and benefits must be undertaken before the performance of transversus abdominis plane blocks in coagulopathic patients. Finally, in addition to respecting ceiling doses of local anesthetic, the prudent anesthesiologist should consider using dilute local anesthetic concentrations as well as adjunctive epinephrine to delay local anesthetic plasmatic absorption, especially in subsets of patients at risk for local anesthetic systemic toxicity. Moreover, communication between surgeon and anesthesiologist is paramount to avoid supratoxic cumulative doses resulting from concomitant local anesthetic infiltration and transversus abdominis plane blocks.

Alternative Truncal Blocks

From an anatomical standpoint, abdominal truncal blocks can performed anywhere from neuraxial (*i.e.*, caudal block) and paraneuraxial (*e.g.*, thoracic paravertebral block, erector spinae plane block, retrolaminar, transmuscular quadratus lumborum blocks) locations to terminal compartments (*e.g.*, rectus sheath block) or terminal neural targets (*i.e.*, ilioinguinal and iliohypgastric nerve). To date, transversus abdominis plane blocks have been compared with a plethora of alternatives such as caudal blocks,[231–233] thoracic paravertebral blocks,[234] quadratus lumborum blocks,[235–238] rectus sheath blocks,[63,64,159,239] and ilioinguinal and iliohypogastric nerve blocks.[233,240–244] To highlight the best evidence, only randomized controlled trials published in PubMed-indexed journals were retained for analysis.

Transversus Abdominis Plane Block *versus* Caudal Block (in Pediatric Patients)

Three randomized trials have compared ultrasound-guided (lateral or posterior) transversus abdominis plane blocks and (landmark- or ultrasound-guided) caudal blocks in children undergoing lower abdominal surgery (*i.e.*, ureteroneocystostomy, herniorrahphy, orchidopexy, hydrocelectomy, testicular detorsion).[231–233] In two trials, transversus abdominis plane blocks resulted in significant advantages compared with caudal blocks, as patients required less breakthrough intravenous morphine (0.05 mg/kg *vs.* 0.09 mg/kg) at 24 h.[231] Furthermore, fewer children reported pain during the 6-h to 24-h postoperative interval (44% *vs.* 75%).[232] However, one trial failed to detect significant differences between transversus abdominis plane and caudal blocks in terms of pain and analgesic consumption.[233]

Transversus Abdominis Plane Block *versus* Thoracic Paravertebral Block

To date, only one trial has compared lateral transversus abdominis plane and thoracic paravertebral blocks. In 2012, Melnikov *et al.*[234] compared bilateral ultrasound-guided lateral transversus abdominis plane blocks with bilateral T10 thoracic paravertebral blocks in patients undergoing vertical laparotomy for total hysterectomy with salpingo-oophorectomy. Although the transversus abdominis plane group displayed a significant higher cumulative opioid (ketomebidon) consumption at 24 and 48 h, there were no intergroup differences in terms of pain scores and patient satisfaction.[234]

Transversus Abdominis Plane Block *versus* Quadratus Lumborum Block

Four randomized trials have compared ultrasound-guided lateral transversus abdominis plane blocks and quadratus lumborum blocks (with local anesthetic injection on the anterolateral aspect of the quadratus lumborum muscle) with similar results.[235–238] In the setting of Cesarean section, open hysterectomy, inguinal hernia, orchiopexy, and lower abdominal surgery, quadratus lumborum blocks result in 2.5- to 7.5-mg decreases in morphine consumption at 24 h compared with their transversus abdominis plane counterparts.[235,237,238] Furthermore, three of the four trials also found lower pain scores[236–238] and two studies reported 80% longer analgesic duration with quadratus lumborum blocks.[237,238]

Transversus Abdominis Plane Block *versus* Rectus Sheath Block

To date, two small pharmacologic trials (combined n = 72) have compared lateral ultrasound-guided transversus abdominis plane blocks and rectus sheath blocks in patients undergoing laparoscopic gynecologic surgery.[63,64] Both studies reported that transversus abdominis plane blocks displayed a 34% to 47% earlier peak of local anesthetic plasma levels. Whereas one trial found no differences in postoperative analgesia,[64] the other one observed longer postoperative analgesia in the transversus abdominis plane group.[63] In a recent trial, Abo-Zeid *et al.*[239] also reported longer analgesic duration (and lower breakthrough opioid consumption) after abdominoplasty with lateral transversus abdominis plane blocks compared with rectus sheath blocks. In 2017, Cowlishaw *et al.*[159] compared continuous subcostal transversus abdominis plane blocks (inserted with ultrasound guidance by anesthesiologists) and rectus sheath blocks (inserted under direct vision by surgeons) in patients undergoing midline laparotomy for gynecologic oncologic surgery. These authors found no intergroup differences in terms of postoperative pain and breakthrough opioid consumption.[159]

The analgesic difference between transversus abdominis plane and rectus sheath blocks could be partly ascribed to the site of surgical incision. Because rectus sheath blocks anesthetize somatic structures confined to the territory of the rectus abdominis muscle, their clinical usefulness may be highest in the setting of midline laparotomy (as evidenced by Cowlishaw *et al.*'s results[159]). In contrast, when the surgical incision exceeds the confines of the rectus abdominis muscle (*e.g.*, laparoscopic gynecologic surgery[63] or abdominoplasty,[239]) transversus abdominis plane blocks may offer improved versatility.

Transversus Abdominis Plane Blocks *versus* Ilioinguinal and Iliohypgastric Nerve Block

To date, five trials have compared lateral transversus abdominis plane blocks and ilioinguinal and iliohypogastric nerve blocks in the setting of inguinal hernia repair with mixed results.[233,240–242,244] In two randomized controlled trials, transversus abdominis plane blocks provided better postoperative analgesia than ilioinguinal and iliohypogastric nerve blocks.[233,241] However, the results of these two studies should be interpreted with caution, as one trial carried out ilioinguinal and iliohypogastric blocks with a blind technique,[240] whereas the other attributed its findings to the operators' lack of experience with ilioinguinal and iliohypogastric nerve blocks.[233] In fact, studies by Fredrickson *et al.*[240] and Kamal *et al.*[244] concluded that, when performed with ultrasound guidance, ilioinguinal and iliohypogastric nerve blocks result in superior postoperative analgesia[240] as well as 28% longer analgesic duration[244] and lower breakthrough analgesic consumption[240,244] than lateral transversus abdominis plane blocks. To complicate matters further, Okur *et al.*[242] recently found minimal differences between the two blocks.

In 2017, one trial compared bilateral lateral transversus abdominis plane blocks with bilateral ilioinguinal and iliohypogastric nerve blocks for patients undergoing Cesarean delivery.[243] Although postoperative pain scores were similar in both groups, patients allocated to transversus abdominis plane blocks required 1,000 mg less breakthrough tramadol during the first 24 h.

In summary, based on the current knowledge, we suggest the use of lateral quadratus lumborum blocks over lateral transversus abdominis plane blocks for lower abdominal surgery (*e.g.*, Cesarean delivery, hysterectomy, inguinal herniorraphy and orichiopexy surgery). In light of contradictory or preliminary findings, further trials are required to compare lateral and posterior transversus abdominis plane blocks with caudal blocks, thoracic paravertebral blocks, rectus sheath blocks, and ilioinguinal and iliohypogastric nerve blocks.

Current Knowledge and Future Research

In summary, over the last 18 yr, transversus abdominis plane blocks have been the topic of considerable research. At times, the collective results of published trials can be difficult to interpret in light of two important confounding variables. First, the term *transversus abdominis plane block* encompasses various approaches that result in radically different somatic and visceral coverage. For instance, whereas the lateral approach can be used for infraumbilical surgery, its subcostal counterpart should be preferred for procedures involving the upper abdomen. Furthermore, of the three described approaches, only the posterior one can achieve local anesthetic spread to the paravertebral spaces, thereby providing sympathetic blockade and visceral analgesia. Therefore any positive (analgesic) outcome related to transversus abdominis plane block should be viewed as approach-specific. Conversely, even if a given approach fails to provide benefits for a surgical intervention, it should not deter operators (and researchers) from exploring an alternative approach. Second, the findings of any comparative trial axiomatically depend on the control group. Because multimodal analgesia has become normative in clinical practice, trials involving transversus abdominis plane blocks that omitted its use in their control group leave many questions unanswered. One need only think of the fact that the initial benefits reported with transversus abdominis plane blocks after Cesarean delivery quickly dissipated when intrathecal morphine was incorporated to the standard analgesic regimen. Unfortunately, to date, many published trials have either compared transversus abdominis plane blocks with no treatment or failed to provide adequate multimodal analgesia to their control groups.

Despite the contradictory findings, scarcity of evidence, and shortcomings afflicting some randomized controlled trials, certain clinical suggestions can nonetheless be made (table 1). Overall, transversus abdominis plane blocks appear most beneficial in the setting of open appendectomy (posterior or lateral approach). Lateral transversus abdominis plane blocks are not suggested for laparoscopic hysterectomy, laparoscopic appendectomy, and open prostatectomy. However, transversus abdominis plane blocks could serve as an analgesic option for Cesarean delivery (posterior or lateral approach) and open colorectal section (subcostal or lateral approach) if there exist contraindications to intrathecal morphine and thoracic epidural analgesia, respectively.

Table 1. Authors' Suggestions Pertaining to Clinical Indications and Alternatives for TAP Blocks

Clinical indications	• Cesarean delivery: posterior and lateral TAP block not recommended if long-acting intrathecal opioids are used (1) • Laparoscopic cholecystectomy: subcostal approach recommended over lateral approach if the operator elects to perform a TAP block (2) • Laparoscopic hysterectomy: lateral TAP block not recommended (1) • Open colorectal surgery: thoracic epidural block recommended (lateral or subcostal continuous TAP blocks recommended if contraindication to neuraxial block) (2) • Open appendectomy: posterior or lateral TAP block recommended (2) • Laparoscopic appendectomy: lateral TAP block not recommended (2) • Open prostatectomy: lateral TAP block not recommended (2)
Alternative truncal blocks	• Quadratus lumborum block (lateral or Type 1): recommended over lateral TAP block (2)

Levels of evidence are indicated in parentheses. Based on the Oxford Levels of Evidence (Level 1 = systematic review of randomized trials or n-of 1 trials; Level 2 = randomized trial or observational study with dramatic effect; Level 3 = nonrandomized controlled cohort or follow-up study; Level 4 = case–series or case–control studies, or historically controlled studies; Level 5 = mechanism-based reasoning). TAP, transversus abdominis plane.

Table 2. Clinical Areas Pertaining to TAP Blocks Warranting Further Investigation

- Comparison between subcostal and posterior TAP block
- Comparison between posterior TAP block and lateral (type 1) quadratus lumborum block
- Optimal technique for surgical TAP block
- Optimal single dose and mode of administration for either dexamethasone, dexmedetomidine, magnesium, or buprenorphine for TAP block
- Optimal combination of multimodal regional analgesia (dexamethasone ± dexmedetomidine ± magnesium ± buprenorphine) for TAP block
- Comparison between continuous TAP block and single-injection TAP block using liposomal bupivacaine
- Benefits of TAP blocks in the setting of multimodal analgesia for laparoscopic cholecystectomy (subcostal approach), hysterectomy (posterior/lateral approach), laparoscopic colorectal surgery (lateral approach), open/laparoscopic inguinal hernia repair (posterior/lateral approach), laparoscopic prostatectomy (posterior/lateral approach)
- Posterior TAP block for laparoscopic hysterectomy, laparoscopic appendectomy, and open prostatectomy
- Posterior or lateral TAP block for laparoscopic prostatectomy
- Comparison between posterior TAP block and thoracic epidural block for open colorectal surgery with emphasis on respiratory and gastrointestinal (*i.e.*, return of bowel function) outcomes
- Further comparison between (posterior/lateral) TAP block and caudal, thoracic paravertebral, rectus sheath, and ilioinguinal/iliohypogastric block
- Comparison between (posterior) TAP block and newer truncal block (*e.g.*, anterior quadratus lumborum block, retrolaminar block, erector spinae plane block)

TAP, transversus abdominis plane.

Currently, knowledge gaps remain that require further investigation (table 2). For instance, posterior and subcostal transversus abdominis plane blocks should be compared in clinical settings (upper abdominal surgery); differences between posterior transversus abdominis plane blocks and lateral (*i.e.*, type 1) quadratus lumborum blocks should be elucidated; the optimal dose, mode of administration, and combination of adjuvants to prolong transversus abdominis plane blocks requires future investigation. Furthermore, posterior transversus abdominis plane blocks should be investigated for surgical interventions in which their lateral counterparts have proven not to be beneficial (*i.e.*, laparoscopic hysterectomy, laparoscopic appendectomy, open prostatectomy). For such trials, it will be paramount that the control group receive adequate multimodal analgesia. More importantly, because posterior transversus abdominis plane blocks can purportedly provide sympathetic blockade and visceral analgesia, they should be compared with thoracic epidural analgesia for open colorectal surgery with emphasis on respiratory and gastrointestinal (*i.e.*, return of bowel function) outcomes as well as adverse events such as hypotension. Moreover, in the context of an ever-expanding array of ultrasound-guided truncal blocks,[2] the benefits (if any) of transversus abdominis plane blocks over newer and more proximal interfascial plane blocks (*i.e.*, retrolaminar, erector spinae plane, and anterior quadratus lumborum blocks) need to be investigated with well-designed and adequately powered trials.[2,4,5] Finally, in addition to postoperative pain scores and breakthrough opioid consumption, future randomized controlled trials should consider including cost analyses as well as hard outcomes such as length of stay.

Research Support

Competing Interests

The authors declare no competing interests.

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MIND TO MIND

Creative writing that explores the abstract side of our profession and our lives

Stephen T. Harvey, M.D., Editor

My Brother's Ghost

Regine Cabato, A.B.

In my grandfather's hospital, the ghosts are transient.
If only because they are retracing their steps—they were alive
here last, or lived here first. So much of his life was spent
here: peering into the intimate clockwork of each person.
As a child I would watch my father reset the clocks
inside of people, as *Papang* did. The job requires the sureness
of hands. I have no such certitude, and I am almost never
on time. But who is always so sure? The day after my brother
was born, *Papang*'s heart gave—he collapsed on the bathroom floor.
I imagine how my father must have fumbled for the vital
signs, watching the seconds run out of him too quickly, standing witness
to how life beckoned in the birthing room next door and death
passed in this one, leaving my father to become a father
on his own. Everybody trusts doctors, nobody trusts them
to die. I will never know *Papang*, except in memorial
parks and hospitals, the portrait of him in our living
room, and his name. A thief tried to steal the brass lettering of it
at the clinic entrance, but it is safely kept
in my father's own name, and my brother's after him.
In the office I am only an observer; words are no good
for saving lives. Only, sometimes, for comfort.
I imagine *Papang* must visit the child born
in the other room, and so the rest of us. Nowadays,
the clocks in the hospital all work. My brother reads
x-rays, raises his own son. My father's hair is white.
When I am called, late as usual, I squeeze through the patience
of patients filing across the waiting room, which is sometimes fuller
when people bring their ghosts with them. Sometimes I find myself
checking my watch too frequently, hoping there is no emergency.

MIND TO MIND

Creative writing that explores the abstract side of our profession and our lives

Stephen T. Harvey, M.D., Editor

Bacchus Listed for a Liver Transplant

Douglas L. Hester, M.D., M.F.A.

He staggers in his toga, jovial
but jaundiced. His flagging liver struggles under
the load. His hands twitch, palms redder
than wine. But Bacchus' cup remains full.
He toasts MELD scores, organ transplants.
"Glorious, glorious! What surgeons have—hic—
done! Here's to hepatocytes, which
happily relocate. Such advances
demand another round." Tonight Bacchus
drowns the fact a life must soon be taken
early: some family will gift him the liver
from a brain-dead body playing host
to second chances. Vats of crushed grapes
make this batch full-bodied, but bitter.

Driving Pressure–guided Ventilation: Comment

To the Editor:

I read with interest Park *et al.*'s article "Driving Pressure during Thoracic Surgery: A Randomized Clinical Trial" which appeared in March's edition of ANESTHESIOLOGY.[1] In this double-blinded, prospective study, the intraoperative utilization of driving pressure guided ventilation, in which positive end–expiratory pressure (PEEP) was incrementally titrated to achieve the lowest plateau pressure minus PEEP value at 6 ml/kg tidal volume (V_T), reduced the incidence of postoperative pneumonia and acute respiratory distress syndrome following thoracic surgery.[1] Despite the success of this study, a potentially important concept which was not evaluated was the optimization of delivered tidal volume (V_T) during the transition from two-lung to one-lung ventilation. Per the study protocol, subjects from both arms were ventilated with a fixed V_T of 6 ml/kg, throughout all stages of the procedure. As the authors mention, a 6 ml/kg predicted body weight V_T target is central to intensive care unit lung-protective ventilation, but the supporting data and practice itself may not be extrapolatable to one-lung ventilation. It is certainly possible that utilization of 6 ml/kg predicted body weight V_T during one-lung ventilation could lead to more volutrauma and barotrauma than it would during two-lung ventilation.

Currently there is only sparse literature to guide ventilation, and particularly V_T, during one-lung ventilation. Maret *et al.* found that utilization of V_T of 5 ml/kg ideal body weight and 5 to 8 cm H_2O of PEEP during one-lung ventilation compared to 10 ml/kg without PEEP resulted in reductions in major postthoracic surgical complications (pneumonia, acute lung injury, acute respiratory distress syndrome, pulmonary embolism, shock, myocardial infarction, or death) and hospital length of stay.[2] Similarly, a retrospective study of pneumonectomy patients identified an increasing incidence of postoperative respiratory failure with each 1 ml/kg predicted body weight increase in V_T.[3] At this time, it is unclear if the outcome benefits of minimization of driving pressure during thoracic surgery would increase, decrease, or remain the same if smaller V_T targets were incorporated into the ventilation strategy. For example, targeting a V_T of 3 to 4 ml/kg predicted body weight during one-lung ventilation, representing a 50% reduction of V_T goals from two-lung ventilation, would be an intuitive approach to maintaining lung-protective ventilation throughout thoracic procedures, but this range has not been studied and could result in undesirable increases in driving pressure as

respiratory rate is increased and inspiratory time is decreased to maintain adequate minute ventilation. Further research is needed to determine the optimal V_T for one-lung ventilation, with a focus on patient-oriented perioperative outcomes.

Nonetheless, the study group should be applauded for contributing to the growing body of evidence-based medicine which supports utilization of intensive care unit–based lung-protective ventilation strategies in the operating room, and their results certainly support the utilization of driving-pressure guided ventilation during thoracic surgery.

Competing Interests

The author declares no competing interests.

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Driving Pressure–guided Ventilation: Comment

To the Editor:

I read with interest the randomized clinical trial by Park *et al.*[1] on the novel approach of using driving pressure–guided

ventilation during one-lung ventilation as a method to reduce postoperative pulmonary complications in comparison to conventional protective ventilation. There are several significant limitations to the study that impact interpretation of the results and conclusions. First, the inclusion of lung resection and esophagectomy patients in assessing the effects of driving pressure manipulations on combined pulmonary outcomes is the most important limitation of this study. In comparison to lung resections, esophagectomies are different in that preoperative chemoradiation is standard, the operation typically involves an abdominal and/or neck incision in addition to the thoracic approach, intraoperative ventilation includes a significant period of two-lung ventilation, and there are greater fluid requirements, as well as higher risks of aspiration and greater postoperative morbidity and mortality.[2,3] The Society of Thoracic Surgeons (Chicago, Illinois) maintains two separate databases for these operations. For example, the reported incidence of pneumonia within 30 days of lung resection is 4.8% (1,116 of 27,844)[2] and 12.2% (529 of 4,321)[3] after esophagectomy. Similar to the authors' efforts to focus on the effects of intraoperative ventilatory parameters during one-lung ventilation on the combined incidence of postoperative pneumonia and/or acute respiratory distress syndrome (ARDS), we reported an overall incidence of 4.0% (24 of 608) following anatomic lung resection.[4] It would be more informative if the authors could share their outcome data by the type of surgery and not combined as presented even if the results were negative.

Second, in the third paragraph of the results and in figure 2, a chi-square test was incorrectly used for analyzing the incidence of ARDS between the driving pressure group and protective ventilation group (0 of 145 *vs*. 5 of 147; $P = 0.025$) where it would have been more appropriate to use the Fisher exact test of this result which would yield a nonsignificant $P = 0.060$ value. Third, the authors discuss the importance of finding a median difference of 1 cm H_2O lower in the driving pressure group *versus* the protective ventilation group as being associated with a lower incidence of pulmonary complications (fig. 2),[1] however, with the exception of the incidence of ARDS which was not statistically significant (as mentioned previously), pneumonia occurred more frequently in the operated (nonventilated) lung compared to that of the ventilated lung in either group, theoretically protected by a lower driving pressure. Finally, although the two patient groups were well matched with respect to preoperative baseline characteristics, it would be interesting to know, regardless of group assignment, whether major pulmonary complications after lung resection only were associated with proven and independent negative prognostic factors[5,6] such as reduced preoperative diffusion capacity of carbon monoxide, preoperative chemotherapy, and increasing intraoperative fluid administration.

Competing Interests

The author declares no competing interests.

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Driving Pressure–guided Ventilation: Reply

In Reply:

We thank Dr. Fierro for his emphasis on tidal volume reduction in response to our recent article "Driving Pressure during Thoracic Surgery: A Randomized Clinical Trial."[1] The definition of driving pressure is: plateau pressure − positive end expiratory pressure. Another formula of driving pressure is: tidal volume / static lung compliance. Therefore, reduction of tidal volume can also reduce driving pressure. However, the key point is that reduction of tidal volume can

increase driving pressure if it deceases lung compliance (as in atelectasis), or increased tidal volume can decrease driving pressure if it increases lung compliance (as in recruitment). Therefore, reduction of tidal volume would decrease driving pressure until it reaches to the point where lung compliance starts to decrease. No study ever tested tidal volume in terms of driving pressure and it would be another interesting study subject. We think optimal tidal volume would be different in each individual if it is based on the lowest driving pressure.

We thank Dr. Amar for his careful review of our study.[1] As he said, lung resection and esophagectomy are two different surgeries. However, our hospital has many esophageal cancer surgeries (more than 300 cases per year). All included patients underwent the Ivor Lewis operation which usually takes only 4 to 5h. All patients had no preoperative adjuvant chemora-dio therapy. We only studied complications until postoperative day 3, thus a lot of delayed complications (graft failure, aspiration pneumonia, among others) were not included. For this reason, we did not see inclusion of esophageal cancer surgery as a problem. The number of esophageal surgeries was small (control group n = 12 vs. driving pressure group n = 16) and the incidence of pulmonary complications diagnosed by Melbourne Group Scale was control group n = 3 and driving pressure group n = 4. Dr. Amar's other concern was the use of statistics. As he said, it is correct to use the Fisher exact test when expected frequencies are less than 5. Our concern was that the Fisher exact test runs an exact procedure especially for small-sized samples and is more conservative than the chi-square test. Our institutional statistician advised that acute respiratory distress syndrome (ARDS) is a small part of our primary outcome (pulmonary complications); therefore, showing the incidence itself is enough (ARDS: control group n = 5, driving pressure group n = 0). $P = 0.05$ cut is a consensus, some argue $P = 0.10$, or $P = 0.001$ is meaningful. Our P value by two different statistics was 0.025 versus 0.060, and the difference mostly came from small incidence of ARDS. Dr. Amar questioned why pneumonia occurred more frequently in both operated and nonoperated lungs in the control group. We think direct surgical injury and one-lung ventilation are associated with a profound inflammatory cytokine release because of abundant immune cells on the lung endothelium and alveolus.[2] Excessive neutrophils recruited in response to the proinflammatory cytokines increase pulmonary vascular permeability in both dependent and nondependent lungs.[3] These reactions often precede systemic inflammatory response syndrome, ARDS, and pneumonia.[4–6]

Competing Interests

The authors report no conflicts of interest.

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Extracorporeal Membrane Oxygenation 1-yr Outcome: Comment

To the Editor:

Current trials published in medical literature, and especially the critical care literature, measure similar primary endpoints, namely, mortality. This measure is often an appropriate way of examining the effectiveness of some of our most novel and innovative treatments. Many trials also measure a number of other secondary endpoints, including time free from a ventilator or time spent in the hospital. But often these trials do not describe a patient's neurologic status or functional status after these interventions. Treatments for medical conditions once thought nonsurvivable have advanced rapidly in recent years. Patients can be kept alive in the face of complete failure of multiple organs, often for

extended periods of time. While mortality is an important endpoint, we applaud the recent publication by Grasselli *et al.*[1] for examining endpoints specifically related to a patient's quality of life.

An excellent example of quality of life–related outcomes research is in the cardiac arrest literature and the use of the modified Rankin scale to show neurologic outcomes after interventions.[2] Given that the incidence of the post–intensive care syndrome, or one of its three components, can be 25% or higher for patients *and* families or caregivers,[3] we think the time is right to expand outcomes to examine a patient's functional status and quality of life after discharge from the intensive care unit. In a recent meta-analysis, only 48 studies out of 11,927 (0.4%) included health-related quality of life after discharge from the intensive care unit as an outcome measure.[4]

In a recent large trial of extracorporeal membrane oxygenation (extracorporeal membrane oxygenation for acute respiratory distress syndrome),[5] 60-day mortality was not different between extracorporeal membrane oxygenation and conventional mechanical ventilation, but there was no information gathered on patients' quality of life after these interventions. Therefore, we were delighted to see Grasselli *et al.*'s[1] publication related to quality of life after extracorporeal membrane oxygenation and applaud them for including these measures in those who survived a very severe illness. The finding that those who underwent treatment with extracorporeal membrane oxygenation had less of an impact on health-related quality of life is especially important for such an invasive intervention. Could extracorporeal membrane oxygenation be a mechanism for helping people recover closer to their baseline functional status? Also, the fact that this intervention is often offered to a younger patient population (in this study, an average age of 54 yr)[1] makes us more hopeful that survivors of extracorporeal membrane oxygenation can have an acceptable quality of life for many years into the future.

We are hopeful that publications such as Grasselli *et al.*'s[1] are the beginning of a trend to new measures in the medical literature. Since "the ultimate goal of health care is to restore or preserve functioning and well-being related to health,"[6] measures such as these may shed new light on treatments that allow our patients to be happier and more satisfied with their medical care.

Competing Interests

The authors declare no competing interests.

Thomas Phillips, D.O., Ryan J. Fink, M.D. Oregon Health and Science University, Portland, Oregon. phillith@ohsu.edu

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Extracorporeal Membrane Oxygenation 1-yr Outcome: Reply

In Reply:

We thank Drs. Phillips and Fink for their interest in our work[1] and we completely agree with all their observations. Through the years, we have assisted with continuous

significant advancements in the care of critically ill patients. Better management of mechanical ventilation and sepsis, advanced monitoring techniques, and more recently, extracorporeal life support techniques, all contributed to improve the rate of survival of critical illness. There is also a tendency to admit older patients with more comorbidities to intensive care units (ICU). At the same time, however, we are realizing that being discharged alive from ICU might not be "the last stage of the journey," but rather, the beginning of an even longer and potentially more painful ordeal. Indeed, ICU survivors experience not only the direct consequences of the critical illness, but also significant long-term outcomes including physical weakness, neurocognitive impairment, and psychiatric disorders that, in turn, significantly affect their quality of life.[2] Moreover, families and caregivers are also at increased risk for psychologic sequelae, particularly posttraumatic stress disorder. Hence, in critical patients, long-term mortality, morbidity, and quality of life may be considered more meaningful outcomes than short-term mortality.[3]

In our study,[1] acute respiratory distress syndrome survivors had almost full recovery of lung function, but severe impairment of quality of life, and stress, anxiety, depression, and posttraumatic stress disorder occurred with alarming frequency. Interestingly, patients treated with extracorporeal membrane oxygenation had a better health-related quality of life then those receiving conventional treatment. We acknowledge that the generalizability of our results is limited, since they come from a single-center study with significant methodologic limitations,[5] conducted in a highly specialized tertiary referral center. For these reasons, our data do not prove that extracorporeal membrane oxygenation is "a mechanism for helping people recover closer to their baseline functional status," but they provide a hypothesis for future research. We strongly believe that larger, multicenter, well-designed trials are necessary to understand the actual impact of extracorporeal membrane oxygenation support upon long-term outcomes. Moreover, from a clinical perspective, we believe that specialized multidisciplinary follow-up programs[5] may allow the early recognition and treatment of physical and/or psychologic sequelae and can play a crucial role to improve the quality of life of patients recovering from critical illnesses.

Competing Interests

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