Volume 127—No. 1—Pp. 1–208

ANESTHESIOLOGY

JULY 2017

July 2017
Volume 127, Number 1
ISSN 0003-3022

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Saturday, October 21
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Atul Gawande, M.D.*
Professor, Harvard Medical School,
Renowned surgeon and researcher,
Author, *Being Mortal* and *The Checklist Manifesto*

*Dr. Gawande will not receive any personal reward for speaking at this event.

Ellison C. Pierce Lecture: Anesthesia Patient Safety: Closing the Gap Between Perception and Reality Presented by: **Robert K. Stoelting, M.D.**

SOAP/FAER Gertie Marx Plenary Lecture: Links to Improving Anesthesia Outcomes Presented by: **Robert D'Angelo, M.D.**

WLM Lewis H. Wright Lecture: A History of Neuroscience Research in Anesthesiology Presented by: **Emery N. Brown, M.D., Ph.D.**

Emery A. Rovenstine Memorial Lecture: Quality Anesthesia: Medicine Measures – Patient's Decide Presented by: **Lee A. Fleisher, M.D.**

John W. Severinghaus Lecture on Translational Science: Rethinking the Concepts of Balanced, Multimodal and Opioid-Free General Anesthesia Presented by: **Emery N. Brown, M.D., Ph.D.**

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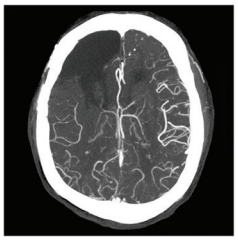
THIS MONTH IN

ANESTHESIOLOGY

36 Goal-directed Fluid Therapy Does Not Reduce Primary Postoperative Ileus after Elective Laparoscopic Colorectal Surgery: A Randomized Controlled Trial

Primary postoperative ileus is a major determinant of in-hospital recovery after colorectal surgery. Both fluid overload and hypovolemia can affect recovery of bowel function. To test the hypothesis that patients treated with fluid therapy based on objective measures of hypovolemia (goal-directed fluid therapy, GDFT) would experience less primary postoperative ileus than those receiving fluid therapy based on traditional principles, 128 patients undergoing laparoscopic colorectal surgery were randomly assigned to receive GDFT based on near-maximal stroke volume optimization or fluid therapy based on traditional principles. Intraoperative GDFT did not reduce the incidence of primary postoperative ileus in the context of a well-established Enhanced Recovery After Surgery program. GDFT had been shown to accelerate the recovery of bowel function mainly when compared to liberal fluid administration. These previously demonstrated benefits may have been offset by advances in perioperative and surgical care. *(Summary: M. J. Avram. Illustration: S. Jarret, C.M.I. Photo: J. P. Rathmell.)*



9 Risks of Cardiovascular Adverse Events and Death in Patients with Previous Stroke Undergoing Emergency Noncardiac, Nonintracranial Surgery: The Importance of Operative Timing

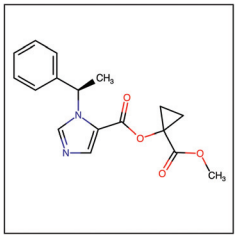
There is a steep decline and subsequent stabilization of risks of adverse perioperative outcomes within the first 9 months after stroke among patients undergoing elective surgery. This is thought to be due to deteriorating cerebral autoregulation within the first 5 days after stroke and impaired autoregulation for up to 3 months. The hypothesis that very early or more delayed surgery would be associated with better outcomes than surgery conducted at an intermediate time point when autoregulation may be maximally dysregulated was tested in a retrospective review of 146,694 emergency noncardiac, nonintracranial surgeries between 2005 and 2011, including 7,861 patients who had a previous stroke. There was a time-dependent increased risk of 30-day major adverse cardiovascular events and all-cause mortality associated with previous stroke. Elderly patients with comorbidities in addition to stroke were at especially high risk of perioperative major adverse cardiovascular events. *See the accompanying Editorial View on* **page 3**. *(Summary: M. J. Avram. Image: Thabele M. Leslie-Mazwi, M.D., Massachusetts General Hospital.)*

50 Epidural Neostigmine *versus* Fentanyl to Decrease Bupivacaine Use in Patient-controlled Epidural Analgesia during Labor: A Randomized, Double-blind, Controlled Study

Epidural neostigmine has been shown to reduce the epidural local anesthetic requirement for labor analgesia to a degree similar to that of opioids in small, single-dose studies. The hypothesis that epidural bupivacaine with neostigmine will provide more clinical effect by decreasing total hourly local anesthetic consumption compared to epidural bupivacaine with fentanyl was tested in 151 parturients randomized to receive 15 ml of 1.25 mg/ml bupivacaine mixed with 2 μg/ml fentanyl or 2, 4, or 8 μg/ml neostigmine. Hourly patient-controlled epidural analgesia bupivacaine requirements for labor in parturients administered study solutions of epidural bupivacaine with 2 to 8 μg/ml neostigmine were similar to those of patients receiving solutions of epidural bupivacaine with 2 μg/ml fentanyl. Neostigmine for epidural use is classified as an investigational drug by the U.S. Food and Drug Administration. *(Summary: M. J. Avram. Image: J. P. Rathmell.)*

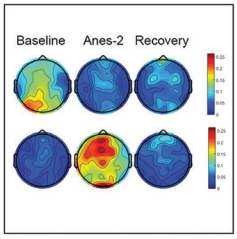
136 Pain Catastrophizing Moderates Relationships between Pain Intensity and Opioid Prescription: Nonlinear Sex Differences Revealed Using a Learning Health System

Pain catastrophizing is a cascade of negative thoughts and emotions in response to actual or anticipated pain. It may explain up to 20% of the variance in chronic pain intensity and may, as a result, influence pain treatment. The relationship between existing opioid prescription, pain intensity, and pain catastrophizing was characterized in a retrospective observational study of 1,794 patients with chronic pain presenting for initial evaluation at a multidisciplinary pain treatment center. Using an advanced analytical approach, a significant relationship between pain intensity and opioid prescription was found that was much stronger in females, especially those with high levels of pain catastrophizing. Although males and females had similar levels of catastrophizing and opioid prescription, opioid prescriptions were more common at lower levels of catastrophizing for females. *(Summary: M. J. Avram. Photo: J. P. Rathmell.)*



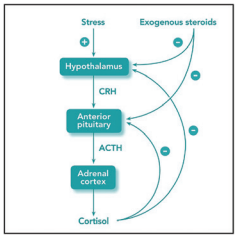
20 A Phase 1, Single-center, Double-blind, Placebo-controlled Study in Healthy Subjects to Assess the Safety, Tolerability, Clinical Effects, and Pharmacokinetics–Pharmacodynamics of Intravenous Cyclopropyl-methoxycarbonylmetomidate (ABP-700) after a Single Ascending Bolus Dose

The clinical use of etomidate is limited by variability in recovery times and inhibition of adrenocortical steroid synthesis. Cyclopropyl-methoxycarbonylmetomidate (ABP-700) is an etomidate analog that undergoes rapid hydrolysis by nonspecific tissue esterases and did not produce prolonged inhibition of steroid synthesis in animals. The safety and efficacy of ABP-700 were assessed and its maximum tolerated dose was determined in a placebo-controlled single ascending dose first-in-human study conducted in 60 volunteers divided into 10 cohorts. ABP-700 was safe up to a maximum tolerated bolus dose of 1.0 mg/kg. Onset of hypnosis after bolus administration was rapid as was recovery. ABP-700 did not cause cardiovascular depression, centrally induced respiratory depression, or suppression of the physiologic response of the adrenal axis to adrenocorticotropic hormone stimulation. Involuntary muscle movements were observed at doses of 0.175 mg/kg and above. *(Summary: M. J. Avram. Image: Chemical structure of cyclopropyl-methoxycarbonylmetomidate (ABP-700), available at:* https://chem.nlm.nih.gov/chemidplus/rn/1446482-29-6 *[public domain].)*



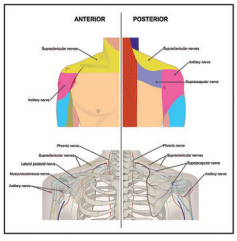
58 Neurophysiologic Correlates of Ketamine Sedation and Anesthesia: A High-density Electroencephalography Study in Healthy Volunteers

Electroencephalographic characteristics of ketamine anesthesia are distinct from those associated with anesthetics that act primarily *via* the γ-aminobutyric acid receptor. Spectral and connectivity analyses of high-density electroencephalographic recordings were used to characterize neurophysiologic changes associated with ketamine as a single agent during subanesthetic administration, anesthetic dosing, and a recovery period in 10 healthy volunteers. During subanesthetic ketamine administration, spectral power gradually shifted from the alpha bandwidth to the theta bandwidth, with maintenance of anterior-to-posterior connectivity (as measured by alpha-directed phase lag index). During ketamine anesthesia, however, there was a marked increase in theta power, theta-weighted phase lag index increased in anterior and posterior regions, and anterior-to-posterior alpha connectivity (as measured by theta-directed phase lag index) was significantly reduced. These connectivity patterns returned to near baseline levels in each bandwidth upon recovery. *(Summary: M. J. Avram. Illustration: Original to article.)*



166 Perioperative Steroid Management: Approaches Based on Current Evidence (Clinical Concepts and Commentary)

Chronic steroid therapy is a cornerstone treatment for many common conditions. When a patient on chronic steroid therapy presents for surgery, the anesthesiologist must decide whether to administer perioperative stress dose steroids to mitigate secondary adrenal insufficiency, a rare but potentially fatal complication of chronic steroid use. This Clinical Concepts and Commentary begins with a review of the physiology of the hypothalamic–pituitary–adrenal axis and its suppression in patients on chronic steroid therapy. It then reviews the historical basis for administering perioperative stress dose steroids as well as the current evidence for doing so. In the absence of class A and B evidence for determining an agreed-upon standard of care, the authors conclude by describing their practical approach to perioperative management of patients on chronic steroid therapy that involves categorizing them into one of four groups based on available evidence. *(Summary: M. J. Avram. Illustration: Original to article.)*



173 Phrenic Nerve Palsy and Regional Anesthesia for Shoulder Surgery: Anatomical, Physiologic, and Clinical Considerations (Review Article)

Regional anesthesia to provide perioperative analgesia for shoulder surgery has been achieved by performing an interscalene block, which targets the C5 and C6 roots of the brachial plexus in the interscalene region. Conventional interscalene block is associated with complications, the most common of which is phrenic nerve palsy with ensuing hemidiaphragmatic paresis. Transient phrenic nerve palsy after regional anesthesia for shoulder surgery results from a direct inhibitory effect of local anesthetic on the phrenic nerve or its roots, hence minimizing its occurrence depends on reducing the dose of local anesthetic reaching these neural structures. This can be achieved by modifying the local anesthetic dose, the interscalene block injection site and technique, or the location of local anesthetic injection and using a different regional anesthetic technique altogether. Strategies for reducing phrenic nerve palsy while ensuring adequate analgesia are reviewed. *(Summary: M. J. Avram. Illustration: Original to article.)*

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ON THE COVER:

Management of perioperative fluid impacts gastrointestinal function. In this issue of ANESTHESIOLOGY, Gómez-Izquierdo *et al.* randomized patients undergoing laparoscopic colorectal surgery within an Enhanced Recovery After Surgery program to receive intraoperative goal-directed fluid therapy or fluid therapy based on traditional principles and assessed the impact on postoperative ileus. Intraoperative goal-directed fluid therapy did not reduce postoperative ileus, suggesting that previously demonstrated benefits might have been offset by advancements in perioperative care.

- Gómez-Izquierdo *et al.*: Goal-directed Fluid Therapy Does Not Reduce Primary Postoperative Ileus after Elective Laparoscopic Colorectal Surgery: A Randomized Controlled Trial, p. 36

After emergency noncardiac nonintracranial surgery, risks of 30-day major adverse cardiovascular events (acute myocardial infarction, ischemic stroke, or cardiovascular death) were high for patients with stroke less than 3 months before surgery (odds ratio [OR] = 4.7), 3 to 9 months (OR = 1.9), and more than 9 months (OR = 1.6) compared with no previous stroke. Risks of death (1.6, 1.2, and 1.2) in the same period were also increased. Risk of major adverse cardiovascular events was significantly lower after immediate (1 to 3 days after stroke) compared with early surgery (4 to 14 days). These patterns were similar to that observed in poststroke patients having elective surgery.

◇ Refers to This Month in Anesthesiology
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This Month's ANESTHESIOLOGY Journal-based CME Article

Read the article by Albaladejo *et al.* entitled "Management of Severe Bleeding in Patients Treated with Direct Oral Anticoagulants: An Observational Registry Analysis" on page 111 of this issue.

Learning Objectives

After successfully completing this activity, the learner will be able to recognize patient characteristics that increase the likelihood that direct oral anticoagulants (DOACs) will be prescribed, develop a therapeutic plan to manage major hemorrhage in patients taking DOACs, and advise patients of future health risks following major hemorrhage resulting from DOACs.

Disclosures

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Authors: Pierre Albaladejo, M.D., Ph.D., Pierre Sié, M.D., Ph.D., Sophie Kauffmann, M.D., Vincent Mémier, M.D., Pierre Suchon, M.D., Ph.D., Alain Viallon, M.D., Ph.D., Yves Gruel, M.D., Ph.D., Lorenn Bellamy, M.D., Emmanuel de Maistre, M.D., Ph.D., Pauline Romegoux, B.Sc., Sophie Thoret, Ph.D., Gilles Pernod, M.D., Ph.D., and Jean-Luc Bosson, M.D., Ph.D., report no relevant financial relationships with commercial interests. Jean Stéphane David, M.D., Ph.D., reports receiving honoraria from LFB Laboratory. Charles-Marc Samama, M.D., Ph.D., F.C.C.P., has received honoraria from Octapharma, Daiichi-Sankyo, and Portola.

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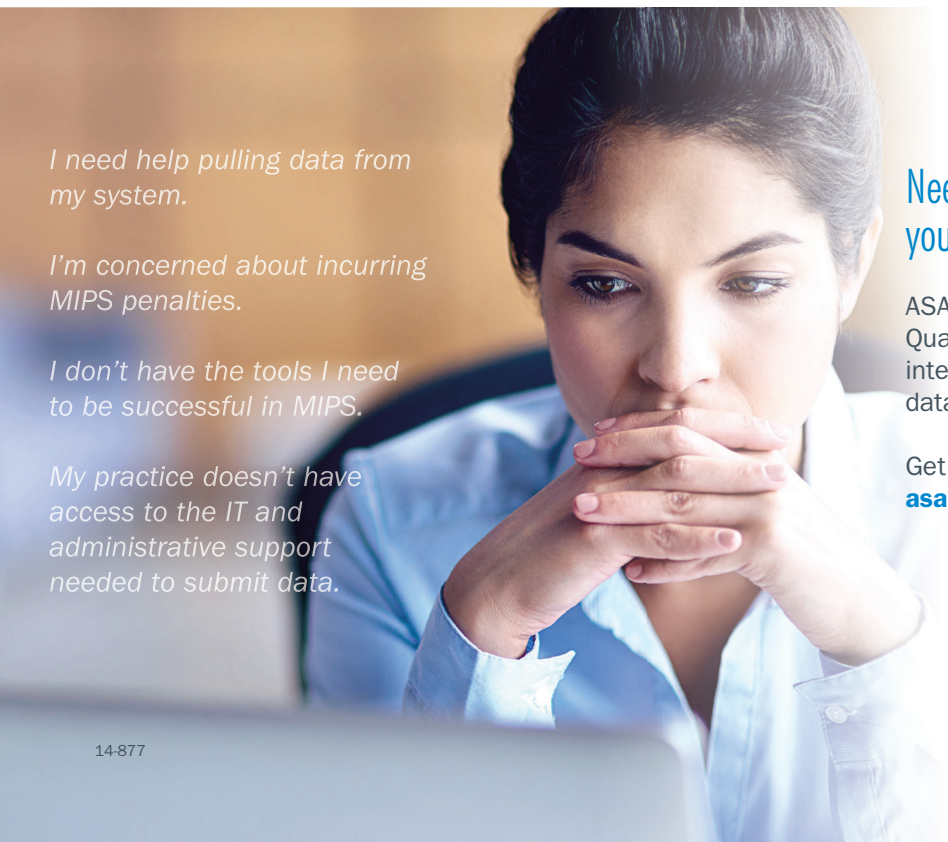

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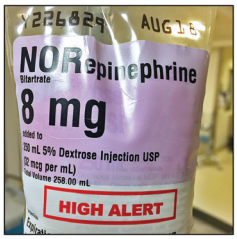
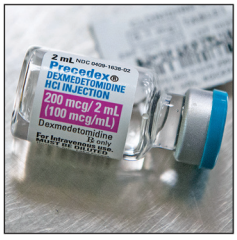


Association between US norepinephrine shortage and mortality among patients with septic shock. JAMA 2017; 317:1433–42.

In 2011 the United States experienced a widespread shortage of drugs, including norepinephrine (noradrenaline). Norepinephrine is considered a standard vasopressor in the treatment of septic shock. This investigation aimed to determine if the 2011 norepinephrine shortage had an effect on in-hospital mortality in patients with septic shock. The investigators included more than 27,000 patients from 26 U.S. hospitals with shortages of norepinephrine in this retrospective cohort study. During the drug shortage period, the use of norepinephrine as first-line vasopressor to treat septic shock in hospitals with shortages of norepinephrine declined from 77 to 56% and this decrease was associated with an increased in-hospital mortality from 36 to 40% with an absolute mortality difference of 3.7% (95% CI, 1.5 to 6.0%, $P = 0.03$). The authors concluded that hospitals affected by the U.S. norepinephrine shortage had higher in-hospital mortality rates among patients treated for septic shock. *(Summary: Peter Nagele and Deborah J. Culley. Image: J. A. Fox, Brigham Health.)*

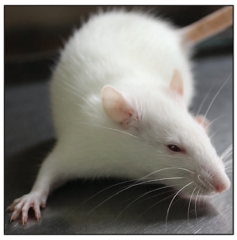
Take home message: Drug shortages can lead to a decrease in the use of preferred drugs in severely ill patients, resulting in increased mortality.



Effect of dexmedetomidine on mortality and ventilator-free days in patients requiring mechanical ventilation with sepsis: A randomized clinical trial. JAMA 2017; 317:1321–8.

Dexmedetomidine has been proven to be effective in providing sedation among intubated patients, although little is known about the effect of dexmedetomidine on 28-day mortality or duration of ventilation in patients with sepsis. The study by Kawazoe *et al.* randomized 201 patients with sepsis to sedation with dexmedetomidine or a control group where sedation was provided without dexmedetomidine. Interestingly, there were no differences in 28-day mortality ($P = 0.20$), the number of ventilator-free days ($P = 0.20$), or length of intensive care unit stay ($P = 0.43$) between the two groups despite the finding that the dexmedetomidine group had better sedation control ($P = 0.01$). Subgroup analysis identified that patients with an Acute Physiology and Chronic Health Evaluation (APACHE II) score of 23 or higher randomized to the dexmedetomidine group had lower hospital mortality when compared to the control group ($P = 0.03$). The authors note that the study may have been underpowered to detect differences in 28-day mortality between the two treatment groups and suggest that further investigations are warranted. *(Summary: Deborah J. Culley. Image: J. P. Rathmell.)*

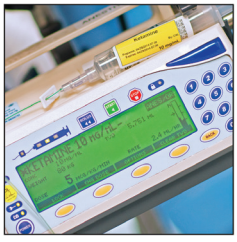
Take home message: Dexmedetomidine administration in the setting of sepsis may not decrease the number of ventilator-free days or 28-day mortality.



A nontoxic pain killer designed by modeling of pathological receptor conformations. Science 2017; 355:966–9.

Toxicity and side effects of μ-opioids such as respiratory depression, use reinforcement, and constipation often limit the clinical utility of opioids. Recently, Spahn *et al.* attempted to address this issue by designing an opioid ligand that preferentially binds to the μ-opioid receptor under acidic conditions such as those that exist after tissue incision or during inflammation. Their ligand, (±)-N-(3-fluoro-1-phenethylpiperidin-4-yl)-N-phenyl propionamide (NFEPP), has a pKa of 6.8 supporting protonation and μ-opioid receptor activation only at subphysiologic pH. Biochemical studies confirmed that low pH was required for NFEPP to inhibit adenosine 3',5'-cyclic monophosphate accumulation *in vitro*. Using rodent models of incisional and inflammatory pain, the authors demonstrate that analgesia was only observed after injury. Importantly, NFEPP did not promote place preference indicating low abuse liability, and it did not slow gastrointestinal motility. This new approach of designing opioid molecules that are active only in injured tissues may lead to the availability of safe and powerful drugs with a reduced side effect profile. *(Summary: David Clark and Deborah J. Culley. Image: ©ThinkStock.)*

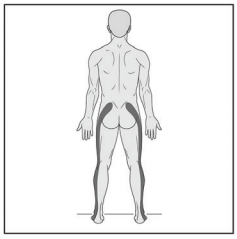
Take home message: In animal models, newer opioids designed to activate μ-opioid receptors only in the setting of pain may lead to pain relief from opioids with a reduced side effect profile.



A consensus statement on the use of ketamine in the treatment of mood disorders. JAMA Psychiatry 2017; 74:399–405.

Ketamine has been used as an intravenous anesthetic agent for several decades. Recently, several groundbreaking studies have demonstrated that a subanesthetic dose of ketamine (typically 0.5 mg/kg IV given over 40 min) has immediate antidepressant effects in patients with treatment-resistant major depression. These discoveries have led to substantial excitement among the general public and mental health professionals alike. However, the use of ketamine for the treatment of depression is largely off-label and inadequately studied. In this special communication, the Council of Research Task Force on Novel Biomarkers and Treatments of the American Psychiatric Association released a timely consensus statement on the safety and effectiveness of using ketamine in the treatment of mood disorders. The statement covers patient selection, clinician experience and training (with an important corollary for anesthesiology), appropriate treatment setting, ketamine delivery, and adequate safety measures. This is an important document for any anesthesiologist who is actively involved in the care of psychiatric patients. *(Summary: Peter Nagele and Deborah J. Culley. Image: J. P. Rathmell.)*

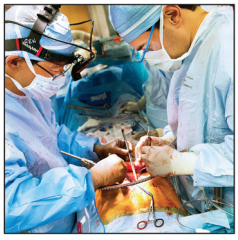
Take home message: Interest in the use of ketamine as a treatment for depression has led to this consensus statement on the use of ketamine in the treatment of depression.



Trial of pregabalin for acute and chronic sciatica. N Engl J Med 2017; 376:1111–20.

Pregabalin is an approved treatment for several neuropathic pain conditions. This randomized, controlled trial investigated the effectiveness of pregabalin in the setting of acute and chronic sciatica. A total of 209 patients were randomized to receive 150 mg pregabalin per day (adjusted to a maximum of 600 mg) or placebo for 8 weeks. The primary outcome was the leg-pain intensity score where a score of 0 represents no pain and a score of 10 represents the worst possible pain. At week eight, there were no significant differences in leg-pain intensity scores between the pregabalin and placebo groups (mean difference 0.5; 95% CI, –0.2 to 1.2; $P = 0.19$). Interestingly, there were more adverse events reported in the pregabalin group when compared with that of the placebo group ($P = 0.002$). The authors conclude that pregabalin did not improve leg-pain scores associated with sciatica but resulted in significantly more adverse events. *(Summary: Peter Nagele and Deborah J. Culley. Image: G. Nelson.)*

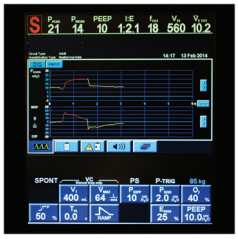
Take home message: Pregabalin may not be effective in treating sciatic pain and may result in more adverse events, although the study may have been underpowered to detect a difference.



Prevalence and causes of attrition among surgical residents: A systematic review and meta-analysis. JAMA Surg 2017; 152:265–72.

The attrition of general surgery residents is thought to be a significant issue for surgical training programs. This systematic review and meta-analysis was designed to determine the prevalence of attrition of general surgery residents, the drivers for the attrition, and where these residents went after they left their surgical residency program. Overall the authors noted that 18% of general surgery residents left their general surgery residency program ($P < 0.001$). The two most common reasons for the high attrition rate included lifestyle issues and choosing to join another specialty. Interestingly, female residents were more likely to leave their general surgery programs when compared to male residents ($P = 0.008$). Among residents who left a general surgery program, 20% moved on to another general surgical program. Anesthesiology was the second most popular medical specialty when residents choose to leave general surgery ($P < 0.001$). The authors note that there is need for interventions to decrease the rate of resident attrition from general surgery residency programs. *(Summary: Deborah J. Culley. Image: J. P. Rathmell.)*

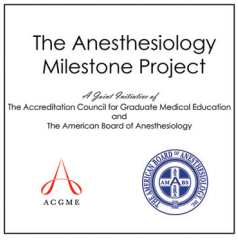
Take home message: There is a high prevalence of attrition among general surgery residents from their initial training program.



Effect of intensive vs moderate alveolar recruitment strategies added to lung-protective ventilation on postoperative pulmonary complications: A randomized clinical trial. JAMA 2017; 317:1422–32.

Pulmonary complications are common after cardiac surgery. Although lung-protective ventilation has now become the standard for perioperative mechanical ventilation (small tidal volume of 6 ml/kg of predicted body weight), it is unclear whether an intensive or moderate alveolar recruitment strategy influences the risk of postoperative pulmonary complications. In this trial, moderate recruitment consisted of three sustained lung inflations (30 s each) with 20 cm H_2O continuous positive airway pressure and an inspired oxygen fraction of 0.60. Intensive recruitment consisted of three cycles of lung inflation (60 s each) at a positive end-expiratory pressure of 30 cm H_2O, driving pressure of 15 cm H_2O, and inspired oxygen fraction of 0.40. In this single-center randomized controlled trial involving 320 patients undergoing cardiac surgery, the use of intensive alveolar recruitment was associated with a reduction in postoperative pulmonary complications: 15% *versus* 26% in patients receiving moderate recruitment (odds ratio, 1.86; 95% CI, 1.2 to 2.83; $P = 0.003$). There were no significant differences in reduction in hospital mortality and hospital length of stay with intensive alveolar recruitment on univariate analysis. These results should be confirmed in a large multicenter trial before implementing these findings in practice. *(Summary: Peter Nagele and Deborah J. Culley. Image: J. P. Rathmell.)*

Take home message: Intensive alveolar recruitment may decrease pulmonary complications in patients undergoing cardiac surgery.



Comparison of male vs female resident milestone evaluations by faculty during emergency medicine residency training. JAMA Intern Med 2017; 177:651–7.

Although many believe that gender bias is prominent in academic medicine, few studies have evaluated whether gender is associated with differences in resident evaluations. This longitudinal retrospective study examined the results of electronic, milestone-based evaluations for emergency medicine residents in eight residency programs. A total of 359 residents were evaluated with 285 faculty members contributing to the 33,456 direct observation evaluations. At the beginning of residency, female residents scored slightly higher than male residents; however, male residents achieved milestones at a rate of 13% faster throughout the remainder of their training when compared with their female counterparts. Interestingly, there were no differences in milestone scores based on the gender of the evaluator. The authors conclude that the difference is due to unconscious gender bias by the faculty independent of their own gender. Another explanation could be that implicit bias extends beyond the evaluation system to the program for training, which could result in females needing an additional 3 to 4 months of training to achieve the same milestone level. It is important that residency programs develop methods to prevent gender bias in both their evaluation systems and residency training programs. *(Summary: Cathleen Peterson-Layne and Deborah J. Culley. Image: ©Accreditation Council for Graduate Medical Education/American Board of Anesthesiology, reproduced with permission.)*

Take home message: Implicit gender bias may exist in residency programs and programs should adopt practices to prevent gender bias in all areas of the program.

ANESTHESIOLOGY

Unwrapping an ERAS Bundle

Goal-directed Fluid Therapy & Ileus

Gómez-Izquierdo *et al*.[1] evaluated the impact of fluid strategy on postoperative ileus in an established laparoscopic colorectal **Enhanced Recovery After Surgery** (ERAS) program.

128 patients were randomized to

- **Goal-directed fluid therapy**
- ***vs.***
- **Traditional fluid therapy**

Preop Prep

- **Patient education:** 100% *vs.* 100%
- **Carbohydrate preop drink:** 73% *vs.* 71%
- **Selective bowel prep:** 56% *vs.* 36%

Goal-directed fluid therapy resulted in less overall fluid given, but more colloid, increased stroke volume, and higher cardiac output.

- **Brief NPO:** 4h *vs.* 4h
- **Intraop fluid therapy:** Colloid / Crystalloid — 2.4L *P*<0.001 *vs.* 1.5L
- **PONV prophylaxis:** 100% *vs.* 100%
- **Epidural:** 100% *vs.* 100%
- *Intraop Management*

Despite the difference in fluid administration, the postoperative ileus rate was the same (RR 1, 95% CI 0.5 to 1.9).

- **Ketorolac:** 78% *vs.* 77%
- **Multimodal analgesia:** 100% *vs.* 100%
- **Early clears and food:** 100% *vs.* 100%
- **Early mobilization:** 100% *vs.* 100%

Postop Care

- **Ileus:** 22% *P* = 1.00
- 22%

Improvement in ileus may not have been seen due to the inclusion of other ERAS components focused on early gastrointestinal recovery.

Intraop = intraoperative; NPO = *non per os*; PONV = postoperative nausea and vomiting; Postop = postoperative; Preop = preoperative; RR = risk ratio.

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.

1. Gómez-Izquierdo JC, Trainito A, Mirzakandov D, Stein BL, Liberman S, Charlebois P, Pecorelli N, Feldman LS, Carli F, Baldini G: Goal-directed fluid therapy does not reduce primary postoperative ileus after elective laparoscopic colorectal surgery: A randomized controlled trial. Anesthesiology 2017; 127:36–49

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SYMPOSIUM

"Frontiers in Opioid Pharmacotherapy"

Sunday, October 22, 2017
9:00 am – 12:00 pm
Boston Convention & Exhibition Center | Boston, MA

FEATURED SPEAKERS:

Laura M. Bohn, Ph.D.
Professor, Molecular Medicine and Neuroscience, The Scripps Research Institute
"Refining opioid receptor signaling to improve therapeutic outcomes"

Nora D. Volkow, M.D.
Director, National Institute on Drug Abuse (NIDA)
"The Current Opioid Epidemic: Intersection Between Pain and Addiction"

The latest science on an issue of significant clinical importance

Recent decades have seen tidal shifts in the therapeutic application of opioids for pain, yet opioid-related problems of addiction, diversion, and overdose have grown with their increased clinical use. New concepts in opioid pharmacology have led to drug discovery efforts aimed at clinical effectiveness with diminished side effects.

Join us in Boston for a one-of-a-kind symposium

At the ANESTHESIOLOGY® 2017 conference, two notable experts will offer the latest science and perspectives on Opioid Pharmacotherapy, with focused presentations followed by discussion. These presentations will be followed by oral presentations of the top meeting abstracts on this topic.

Register for ANESTHESIOLOGY® 2017 at asahq.org.
One-day registrations are available.

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Editor's Note: Anesthesiology 2017

Expanding the Richness and Reach

Evan D. Kharasch, M.D., Ph.D.

ONE year ago, I began as Editor-in-Chief of Anesthesiology with the goal to attract and publish the highest quality possible, peer-reviewed, specialty-relevant science—basic *and* clinical—that asks and addresses important questions.[1] Beyond that, the future was envisioned in terms of journal richness and reach, article quality and impact, content delivery, communication and comprehension, and service to authors, readers, and the many constituencies that we serve. A great deal has been accomplished and changed in this past year, and this message serves to inform our authors and our readers.

Anesthesiology publishes both clinical research and clinically relevant basic science. Between July 2016 and June 2017, the Journal published more than 150 original investigations, two thirds of which were clinical investigations. We published 100 articles in the category of Perioperative Medicine, 75% of which were clinical studies, as well as 27 Critical Care Medicine and 25 Pain Medicine articles. These were accompanied by 55 Editorial View articles and 23 articles in the realm of synthesis and recapitulation (Review Articles, Clinical Concepts and Commentary, Special Articles, and Classic Papers Revisited).

Considerable effort has been made to expand the reach of the Journal, meaning both increased numbers of users (readers and viewers) and increased readability and comprehension. Our Electronic Alerts, which previously were accessible only to members of the American Society of Anesthesiologists (ASA), are now freely available to all. Anyone can sign up to receive these Electronic Alerts by email, including the weekly electronic table of contents (eToC), a weekly Online First alert highlighting articles published online prior to print publication, and topic collection alerts based on areas of interest.

"[We] have expanded our electronic multimedia content and library to engage Anesthesiology consumers in new ways."

This availability is prominently featured at the very top of the online journal (http://anesthesiology.pubs.asahq.org). We have also made the Anesthesiology Web site part of the ASA Single Sign-on system, which allows ASA members easier access to multiple ASA publications and content with a single log-in, without having to sign in to each publication individually. Anesthesiology Web viewership has increased more than 40%. To further enhance ease of use, for those who wish to download articles and read offline or save articles electronically, we have created the ability to download an entire month's issue as a single PDF rather than requiring the downloading of every article individually. And we have retained that single article download ability as well. Commensurate with these enhancements and increased use of the journal Web site, use of the iPad app, first launched in 2010, has dwindled, and it has now been retired.

Several new offerings have expanded our electronic multimedia content and library to engage Anesthesiology consumers in new ways. The monthly Editor-in-Chief podcast has been a convenient hands-free way to hear the summaries of several highlighted articles each month. Through the leadership of Associate Editor Yandong Jiang, M.D., Ph.D., and a team of editors and translators, the podcast is now also newly available in Mandarin. This new feature has seen rapid acceptance, and our expanded reach to Chinese listeners is more than twice that of our English podcast. Another offering is our new monthly audio podcast—an extended audio interview with authors and editorialists about the content and additional aspects of a featured research article. James Rathmell, M.D., and BobbieJean Sweitzer, M.D., are leading that effort. And a new monthly video abstract provides a 3- to 5-min narrated and illustrated summary

Timothy J. Brennan, Ph.D., M.D., served as Handling Editor for this article.

Accepted for publication April 24, 2017. From the Departments of Anesthesiology and Biochemistry and Molecular Biophysics, Washington University in St. Louis, St. Louis, Missouri, and Center for Clinical Pharmacology, St. Louis College of Pharmacy and Washington University in St. Louis, St. Louis, Missouri.

 Anesthesiology 2017; 127:1-2

of a featured research article. Multimedia content is available on iTunes, Google Play, YouTube, and iHeartRadio. Podcasts can be found on the podcast page of the Anesthesiology Web site, or by subscription *via* iTunes for iOS devices or Google Play for Android devices (these devices will receive automatic updates when a new podcast is available).

We are also pleased to announce that, going forward, Anesthesiology will be expanding video content offerings to other article types, including Review Articles and Clinical Concepts and Commentary. These videos will allow readers to visualize aspects of clinical care and techniques, such as regional block placement or ultrasound, further enriching the article content. This new capability will be immediately available to authors of these article types.

Complementing these new content offerings is enhanced engagement through social media—Facebook and Twitter, whose reach has increased 50 to 100% in the past year through the efforts of our social media Associate Editors Jorge Galvez, M.D., and Allan Simpao, M.D., M.B.I. They have also initiated a periodic Twitter journal club, working with a host anesthesiology department to focus on a featured Anesthesiology article and engage in an online discussion. Twitter journal club discussions are archived on the Anesthesiology Web site. The Journal has also partnered with Kudos, a Web-based system that helps investigators maximize the visibility and impact of their published articles. Established as a service to Anesthesiology authors, Kudos provides tools for authors to disseminate their research across a broad range of social media platforms and track the reach of that engagement.

Anesthesiology content expansion has not been limited to the online journal. The print journal fills unused white space at the end of articles with rich images that retell the history of our specialty. Through the thoughtful largess of Associate Editor George Bause, M.D., M.P.H., and the Wood Library-Museum, we have doubled the number of these monthly images. Known as Anesthesiology Reflections from the Wood Library-Museum, they remind us of our origins and progress, and quite often with a smile.

Many enhancements have also been made to our research articles to improve the quality and clarity of research reporting and readability. Busy readers may scan the abstracts of articles to select some for more in-depth reading, and they may sometimes read only the abstract. Hence, we are working with authors to ensure that Anesthesiology abstracts can stand alone and convey enough information for a reader to understand the scientific problem, hypothesis, and method for testing the hypothesis, as well as to see presented the key data on which any conclusions are based and to describe clearly those conclusions that are directly supported by the study results. In addition, article abstracts can quickly become overrun with jargon, abbreviations, and acronyms, challenging their comprehension. To improve abstract readability, the use of nonstandard abbreviations and acronyms has been eliminated from article abstracts, titles, and summaries, and we are reducing and discouraging their use in the main body of articles as well. We encourage article titles to be both informative and concise. To improve data reporting and transparency, we now require data variability to be reported more correctly, as either the standard deviation or 95% confidence interval, rather than the standard error of the mean. Similarly, so as not to give readers a sense of false precision, we require that the number of decimal places used to report data must be the same as in the original measurements. If age was measured to the nearest year, it must be reported only to the whole year. Anesthesiology provides guidance to authors on enhancements in article reporting by periodically updating the Instructions for Authors (http://anesthesiology.pubs.asahq.org/public/InstructionsforAuthors.aspx).

Anesthesiology leadership also has changed. Editors Hugh Hemmings, M.D., Ph.D., and David Roth, M.D., Ph.D., retired from their roles, and we are grateful for their leadership and service. Tragically, we unexpectedly lost and miss Jean Mantz, M.D., Ph.D.[2] Anesthesiology was most pleased to welcome as new editors Andrew Davidson, M.B.B.S., M.D., F.A.N.Z.C.A., and Sachin Kheterpal, M.D., M.B.A. They continue the international representation on the Editorial Board, reflecting the international contributorship and readership of the Journal, as well as the ever-increasing breadth of science that we publish. And our Mind-to-Mind founding editor, Carol Cassella, M.D., retired in that role and was replaced by Stephen Harvey, M.D. Our journal has outstanding leadership.

There will be more changes and enhancements to occur in the coming year. We will go where the science takes us.

Competing Interests

Dr. Kharasch is the Editor-in-Chief of Anesthesiology and his institution receives salary support from the American Society of Anesthesiologists for this position.

Correspondence

Address correspondence to Dr. Kharasch: editor-in-chief@asahq.org

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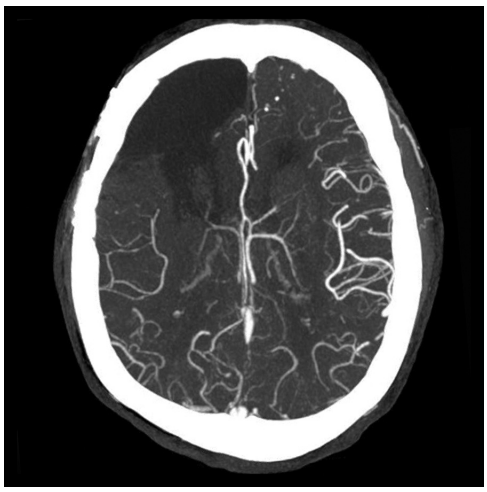
2. Riou B: In memoriam: Jean Mantz, 1960 to 2017. Anesthesiology 2017: 126:991–2

Raising the Alarm on Brain Attacks in Surgical Patients

Are We Doing Enough to Prevent and Treat Postoperative Strokes?

Laurent G. Glance, M.D., Robert G. Holloway, M.D., M.P.H.

THE incidence of postoperative strokes in patients undergoing noncardiac, nonneurologic surgery is between 0.1 and 0.7%.[1,2] Previous studies reported that patients with a history of ischemic stroke are two to three times more likely to experience a postoperative stroke.[1,2] In this issue of Anesthesiology, Christiansen *et al.*[3] report that a history of stroke within 3 months of emergency noncardiac, nonintracranial surgery is associated with a significantly increased risk of a recurrent postoperative stroke using data on 146,694 acute surgeries from the Danish National Patient Registry (DNRP). Specifically, 10% of patients with a history of ischemic stroke within 3 months had a postoperative stroke compared with 2 to 3% of patients with a history of stroke more than 3 months ago and 0.3% in patients with no previous stroke.[3] In a previous study reported in the *Journal of the American Medical Association*, based on 481,183 elective surgeries in the DNRP, this same group reported similar time effects on the risk of postoperative stroke in patients undergoing elective noncardiac surgery: 12.0% in patients with stroke within 3 months, 4.5% in patients with stroke between 3 and 6 months, 1.0 to 2.0% if more than 6 months, and 0.1% in patients with no previous stroke.[4] Unlike the Danish database, previous studies in the United States that reported a much lower risk of recurrent strokes were based on databases (the Nationwide Inpatient Sample and the American College of Surgeons National Surgical Quality Improvement Program database) that grouped all previous strokes together.[1,2] These databases did not include information on how much time had elapsed between previous strokes and surgery.



"The time is right…[to update] existing clinical practice guidelines…[on the] management of patients at high risk for perioperative acute ischemic stroke."

The studies by Christiansen and colleagues[3,4] have important limitations. It is well known that patients with an ischemic stroke are at the highest risk for recurrent stroke soon after the event and that this risk declines over time. The magnitude of postoperative risk found in these studies, however, was considerably greater than that found in a comparable cohort who did not undergo surgery (12.0 *vs.* 3.5%).[5,6] Second, although the International Statistical Classification of Diseases and Related Health Problems, Tenth Revision ischemic stroke diagnoses have been validated in the DNRP,[7] there are no data on whether these diagnoses in the postoperative period accurately reflect recurrent or new infarcts. Preexisting stroke symptoms often worsen in the setting of systemic stress (often called an *anamnestic* response, or bringing out an old memory), and it is possible that symptoms of a recent stroke may transiently worsen in the setting of surgery without acutely infarcted tissue. Third, the impact of increased magnetic resonance imaging use in the detection of small strokes, and even silent stroke, is increasingly recognized.[8] Fourth, the DNRP provides no information on the presumed underlying etiology of the recurrent stroke (*e.g.*, hypoperfusion, atheroembolic, proinflammatory, anesthetic effects, and cerebrovascular dysregulation). Finally, these studies are retrospective and used administrative data from one nation.

These authors[3,4] are to be commended for highlighting an important area that has received little attention in the literature. Perhaps the most important takeaway is the need for additional research to confirm and refine these estimates using different approaches in more diverse populations. In addition, more research is needed to better understand the

Image: Thabele M. Leslie-Mazwi, M.D., Massachusetts General Hospital, Boston, Massachusetts.

Corresponding article on page 9.

Accepted for publication March 27, 2017. From the Departments of Anesthesiology (L.G.G.), Public Health Sciences (L.G.G., R.G.H.), and Neurology (R.G.H.), University of Rochester School of Medicine, Rochester, New York; and RAND Health, RAND, Boston, Massachusetts (L.G.G.).

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pathomechanisms of postoperative strokes, as well as the various antiischemic strategies (*e.g.*, molecular, antiinflammatory, and cell based) to minimize neuronal injury. Until then, however, we recommend the following:

First, the Society for Neuroscience in Anesthesiology and Critical Care recently published a consensus statement, supported but not endorsed by the American Society of Anesthesiologists, on the prevention and management of postoperative strokes.[9] They suggest that surgeons consider delaying elective surgery for 1 month in patients after a stroke. According to this consensus statement, the recommendation to delay elective surgery in patients with recent strokes is supported only by "opinion-based evidence."[9] The data by Christiansen and colleagues[3,4] suggest that the recommended delay for elective surgery might need to be longer, possibly up to at least 3 months after an acute stroke. In addition, in those stroke patients who are recommended for surgery, particular attention should be paid to baseline neurologic deficits so that new postoperative deficits can be identified.

Second, perioperative and surgical teams should have a heightened sense of awareness to new neurologic deficits and clear procedures to engage stroke teams.[10] Although major surgery within the past 14 days is a relative contraindication to fibrinolytic therapy,[11] existing guidelines state that the use of fibrinolytic therapy may be considered in the absence of intracranial or intraspinal surgery (class IIb recommendation).[9,11] However, many surgeons and anesthesiologists are likely to be hesitant to use fibrinolytic therapy in the immediate postoperative period due to the risk of major bleeding at the operative site. For many surgical patients, endovascular thrombectomy may be the only practical approach to restoring perfusion after a stroke caused by proximal artery occlusion. Existing guidelines specify that endovascular therapy is reasonable and can be useful in patients with contraindications to fibrinolytic therapy (class IIa recommendations).[12] Endovascular therapies with mechanical thrombectomy have been described as the second quantum leap in stroke care.[12,13] Not only is endovascular thrombectomy less likely to lead to bleeding complications in surgical patients, it is more likely to lead to better neurologic outcomes. At 90 days, patients with acute ischemic strokes undergoing endovascular therapy are more than 50% more likely to have reduced disability compared with standard lytic therapy.[14] Endovascular thrombectomy may represent a new frontier in the therapy of acute postoperative strokes.

Third, we believe that the care of high-risk patients whose elective surgery cannot be delayed (*e.g.*, cancer surgery) should be regionalized to comprehensive stroke centers with advanced neuroimaging capabilities and neuroendovascular specialists, resources that are not available in most hospitals and primary stroke centers.[15] In 2017 there are 121 comprehensive stroke centers in the United States.[16] For the many patients where this may not be practical (*e.g.*, location is remote from a comprehensive stroke center), however, a clear plan should be in place to manage such patients *via* a telestroke consultation.[17]

Fourth, the American Stroke Association has published clinical practice guidelines on the prevention and early management of patients with acute ischemic strokes.[11,12,18] These clinical practice guidelines do not discuss the management of patients with a recent stroke undergoing surgery or the approach to patients if a new stroke occurs after surgery. In particular, they do not address delaying elective surgery after a recent stroke. The only currently available guideline addressing this issue is from the Society for Neuroscience in Anesthesiology and Critical Care.[12] These guidelines, however, have not been widely disseminated; they have been cited only 20 times since publication in 2014, compared with 868 citations for the American Stroke Association guidelines, also published in 2014. The timing is right to convene a group of neurologists, anesthesiologists, and surgeons to review the literature and write a focused update of existing clinical practice guidelines focusing on the perioperative management of patients at high risk for perioperative acute ischemic stroke.

Many patients fear disabling strokes more than death.[19] We need to do much more than we are doing to better understand, prevent, and treat strokes in high-risk surgical patients.

Competing Interests

The authors are not supported by, nor maintain any financial interest in, any commercial activity that may be associated with the topic of this article.

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Understanding Potential Drug Side Effects

Can We Translate Molecular Mechanisms to Clinical Applications?

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CLINICAL pharmacology is one important basis of modern anesthesiology. In this month's edition of Anesthesiology, Lecker *et al.*[1] report the results of an investigation of high concentrations of tranexamic acid (TXA) on *N*-methyl-D-aspartate (NMDA) and glutamate receptors in cultured murine hippocampal neurons. The investigation was prompted by reports of seizures in patients receiving TXA during cardiac surgery. It is an excellent example of "reverse translation": taking clinical problems to the laboratory to understand their etiology and mechanism.

"[This study] is an excellent example of 'reverse translation': taking clinical problems to the laboratory to understand their etiology and mechanism."

The role of antifibrinolytic therapy for bleeding continues to expand in medical practice. The antifibrinolytic agent most extensively used in 2016 is the lysine analog TXA, a drug developed in the 1950s by Okamoto Utakoin in Japan when searching for a therapy to treat postpartum hemorrhage. From the late 1960s until today TXA has been used in a growing number of surgical and nonsurgical settings to reduce bleeding including menorrhagia, cardiac surgery, orthopedic surgery, and in trauma. The results of the Clinical Randomization of an Antifibrinolytic in Significant Hemorrhage 2 (CRASH-2) trial in trauma patients had a major impact on its growing use in clinical practice.[2] TXA also is used for prophylaxis in patients with hereditary angioedema and may have important antiinflammatory effects as a protease inhibitor.[3] Despite this extensive use, reported side effects are infrequent.[4] In 2009 TXA acid was entered into the World Health Organization list of essential medicines.

In 2010, reports began to emerge suggesting increased nonischemic clinical seizures after cardiac surgery and cardiopulmonary bypass (CPB) with the use of high-dose TXA infusions.[5] Additional analyses from larger retrospective evaluations also reported an increase in convulsive seizures after CPB even when TXA was used at lower doses.[5] Despite these reports, it is important to realize that case reports are anecdotal. Retrospective analyses of clinical databases and even the gold standard of clinical studies, a double-blind, randomized, placebo-controlled prospective trial, can only suggest a potential association or statistical probability between drug administration and adverse effects. After cardiac surgery, seizures have multiple causes that range from emboli to the cerebral circulation, producing cerebral anoxia to other potential drug-induced effects. However, a cause–effect relationship of adverse events is best proven by elucidation of the molecular/pathophysiologic mechanisms of the potential adverse effect.

Multiple mechanisms may be responsible for TXA producing seizures. Current reports suggest TXA increases neuronal excitation by antagonizing inhibitory γ-aminobutyric acid neurotransmission and inhibits neural glycine receptors.[6,7] When one examines the chemical structures of TXA, γ-aminobutyric acid, and glycine and their similarities, additional mechanisms may be involved; however, epsilon aminocaproic acid, another lysine analog antifibrinolytic agent, has not been reported to produce seizures.[5]

Lecker *et al.*[1] report that high concentrations of TXA inhibit NMDA receptors. The NMDA receptor is an ion channel receptor present in neural cells that is activated after glutamic acid or glycine binding to control neurotransmission in what is called synaptic plasticity. Thus, inhibiting these receptors will allow for neuronal excitation and thus

Image: Adobe Stock.

Corresponding article on page 89.

Accepted for publication January 26, 2017. From the Institute of Anesthesiology, Heart and Diabetes Center North Rhine-Westphalia, Bad Oeynhausen, Ruhr-University Bochum, Germany (A.K.); and Department of Anesthesiology, Cardiac Anesthesiology and Critical Care, Duke University School of Medicine, Durham, North Carolina (J.H.L.).

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seizures. However, despite this interesting finding, the TXA concentrations that are effective to inhibit NMDA receptors are much higher than concentrations found in the cerebral spinal fluid and 5- to 500-fold higher than plasma concentrations measured after a high-dose TXA protocol in cardiac surgery.[5,7]As the authors note, such concentrations are currently not systemically achieved *via* intravenous administration but only topically *via* local application.

From a molecular perspective, the chemical structure of TXA is very similar to the amino acid glycine, which in the brain and spinal cord acts as an inhibitory neurotransmitter. In a previous study, Lecker *et al.*[7] reported that TXA, in concentrations found in the cerebrospinal fluid after a standard high-dose protocol in cardiac surgery, inhibits cerebral glycine receptors as a potential mechanism to cause seizures. However, in cardiac surgery the increase in seizures potentially associated with TXA has been observed predominantly in older patients after procedures with CPB.[5] In open-heart surgery, air enters the heart and circulation, and despite efforts to prevent systemic embolism, microbubbles may enter the systemic circulation, produce local microvascular injury, and damage the blood–brain barrier (BBB). Other potential sources of emboli include atheromatous material or calcified plaques from the aorta, thrombotic material from the left side of the heart, particulate matter after valve surgery, and potential right-to-left communication with a patent foramen ovale. Local sites of vascular injury may cause local disturbances of the BBB, increase TXA concentrations at the site of injury of the brain, and potentially promote seizures. This effect may be aggravated in older patients, who already often present with age-related dysfunction of the BBB.[8] Additionally, using newer sensitive laboratory methods, large variations in TXA plasma levels and the interindividual ratio between plasma concentrations and concentrations found in the cerebral spinal fluid have been reported.[7,9] Therefore, additional mechanisms may be responsible for producing seizures in addition to circulating levels of TXA. This is particularly important because the risk of seizures seems insignificant in women receiving approximately 4 g/d TXA for menstrual bleeding, in trauma patients in the large CRASH-2 trial who received 2 g TXA, and in noncardiac surgical patients.[2]

Glycine is also an obligatory co-agonist of the NMDA subtype of ionotropic glutamate receptors that control numerous calcium-dependent processes. These receptors are not only distributed widely in the central nervous system but also in renal, myocardial, and other tissues. The NMDA receptors are currently under intensive investigation as targets for treatment of psychiatric disorders such as depression, schizophrenia, and Alzheimer disease.[10] The NMDA receptors outside the central nervous system also are discussed as potential targets for new pharmacologic targets.[11]

One of the interesting aspects of TXA use is the increasing application of this agent topically. A recent Cochrane review addressed the topical application of TXA in a large variety of clinical settings such as cardiac surgery, knee arthroplasty, and spinal surgery.[12] The authors concluded that topically administered TXA may reduce bleeding and transfusions but expressed concern that safety data, particularly with regard to thromboembolic complications, are missing.[12] High topical concentrations of TXA may lead to lower plasma levels that depend on the dose used, the application site, and local readsorption and may achieve plasma levels that are considerably lower than concentrations measured after a high-dose intravenous protocol in cardiac surgery but are effective at inhibiting systemic hyperfibrinolysis.[1,5]

Important for the elucidation of the causes of adverse drug reactions is the understanding of drug actions on nontarget receptors, proteases, or other pharmacologic processes as well as on-target effects. As in this case, TXA, a hemostatic agent, potentially facilitates seizures. In understanding key functions of NMDA receptors in controlling neurotransmission, their distribution in the different organ systems, and the effects of TXA as noted by Lecker *et al.*,[1,7] molecular mechanisms are important to explain better and potentially earlier-recognized potential side effects. However, there are likely additional mechanisms behind both TXA and seizures after cardiac surgery. Multiple factors may be important in producing seizures that involve multiple receptors, disruption of the BBB, and other potential concomitant events that contribute to the clinical side effects. We look forward to further investigation of the mechanisms of TXA-induced seizures in patients.

Competing Interests

The authors are not supported by, nor maintain any financial interest in, any commercial activity that may be associated with the topic of this article.

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ANESTHESIOLOGY REFLECTIONS FROM THE WOOD LIBRARY-MUSEUM

Stamping Out Pain with Brandy Anesthesia: McDowell's Cystolithotomy of Polk

A 32-cent commemorative U.S. postage stamp was released (*right*) in 1995 on the 200th anniversary of the birth of Tennessee politician James K. Polk (1795 to 1849). As a sickly 12-yr-old, Polk had drunk the brandy prescribed him as his only anesthetic for bladder stone removal. This cystolithotomy likely required that the boy be secured for surgery by leather straps and strong assistants—brandy was an impotent anesthetic. And impotence likely precluded future children for the young patient after his scarring from the rapid perineal dissection of his famous surgeon, Ephraim McDowell (1771 to 1830). A 4-cent commemorative U.S. postage stamp was released (*left*) in 1959 to commemorate McDowell on the 150th anniversary of ovarian surgery, which he pioneered. Ironically, Polk's surgeon, McDowell, hailed as the "Father of Abdominal Surgery," would die from appendicitis and never live to see Polk reject brandy and all alcohol in adulthood as the teetotaling eleventh president of the United States. (Copyright © the American Society of Anesthesiologists' Wood Library-Museum of Anesthesiology.)

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Risks of Cardiovascular Adverse Events and Death in Patients with Previous Stroke Undergoing Emergency Noncardiac, Nonintracranial Surgery

The Importance of Operative Timing

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ABSTRACT

Background: The outcomes of emergent noncardiac, nonintracranial surgery in patients with previous stroke remain unknown.

Methods: All emergency surgeries performed in Denmark (2005 to 2011) were analyzed according to time elapsed between previous ischemic stroke and surgery. The risks of 30-day mortality and major adverse cardiovascular events were estimated as odds ratios (ORs) and 95% CIs using adjusted logistic regression models in *a priori* defined groups (reference was no previous stroke). In patients undergoing surgery immediately (within 1 to 3 days) or early after stroke (within 4 to 14 days), propensity-score matching was performed.

Results: Of 146,694 nonvascular surgeries (composing 98% of all emergency surgeries), 5.3% had previous stroke (mean age, 75 yr [SD = 13]; 53% women, 50% major orthopedic surgery). Antithrombotic treatment and atrial fibrillation were more frequent and general anesthesia less frequent in patients with previous stroke (all $P < 0.001$). Risks of major adverse cardiovascular events and mortality were high for patients with stroke less than 3 months (20.7 and 16.4% events; OR = 4.71 [95% CI, 4.18 to 5.32] and 1.65 [95% CI, 1.45 to 1.88]), and remained increased for stroke within 3 to 9 months (10.3 and 12.3%; OR = 1.93 [95% CI, 1.55 to 2.40] and 1.20 [95% CI, 0.98 to 1.47]) and stroke more than 9 months (8.8 and 11.7%; OR = 1.62 [95% CI, 1.43 to 1.84] and 1.20 [95% CI, 1.08 to 1.34]) compared with no previous stroke (2.3 and 4.8% events). Major adverse cardiovascular events were significantly lower in 323 patients undergoing immediate surgery (21%) compared with 323 successfully propensity-matched early surgery patients (29%; $P = 0.029$).

Conclusions: Adverse cardiovascular outcomes and mortality were greatly increased among patients with recent stroke. However, events were higher 4 to 14 days after stroke compared with 1 to 3 days after stroke. **(ANESTHESIOLOGY 2017; 127:9-19)**

EMERGENCY surgery is one of the most high-risk situations encountered in clinical practice, and risks become especially pronounced in patients with weighty comorbidities, such as cerebrovascular disease.[1] Because time is an important factor in the clinical setting, detailed information on perioperative risks is important to guide decision-making and to inform the patient and relatives about realistic perioperative expectations. More than 800,000 patients experience a stroke in the United States every year, with the majority being ischemic strokes,[2] and the World Health Organization estimates that incidence rates of stroke in Europe will increase by approximately 1.5 million per year from 2000 to 2025.[3] Emergency surgeries in patients with previous stroke are becoming more frequent due to advances in treatment and an increased willingness to treat older and more fragile patients. Stroke has been included in widely used preoperative risk evaluation schemes, such as the revised cardiac risk index by Lee

What We Already Know about This Topic

- There is a steep decline and stabilization of risks within the first 9 months after stroke among patients undergoing elective surgery, but risks after emergency surgery are unknown

What This Article Tells Us That Is New

- After emergency noncardiac, nonintracranial surgery, risks of 30-day major adverse cardiovascular events (acute myocardial infarction, ischemic stroke, or cardiovascular death) were high for patients with stroke less than 3 months before surgery (odds ratio [OR] = 4.7), 3 to 9 months (OR = 1.9) and more than 9 months (OR = 1.6) compared with no previous stroke
- Risks of death (1.6, 1.2, and 1.2) in the same period were also increased
- Risk of major adverse cardiovascular events was significantly lower after immediate (1 to 3 days after stroke) compared with early surgery (4 to 14 days)
- These patterns were similar to that observed in poststroke patients having elective surgery

This article is featured in "This Month in Anesthesiology," page 1A. Corresponding article on page 3. Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site (www.anesthesiology.org). This article has an audio podcast.

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et al.,[4] but the importance of time elapsed between stroke and emergency surgery has not previously been reported and is not considered in current perioperative guidelines.[5,6] A recent study demonstrated a steep decline and stabilization of risks within the first 9 months after stroke among patients undergoing elective surgery.[7] In patients with previous myocardial infarction, a similar decrease in risks followed by stabilization at 6 months has been demonstrated, and perioperative guidelines do address the importance of time between stenting and noncardiac surgery.[8,9] The mechanism for increased risks in patients with recent stroke are thought to be at least partly mediated by deteriorating cerebral autoregulation within the first 5 days after stroke and impaired autoregulation for up to 3 months.[10,11] This condition renders patients vulnerable to secondary neuronal injury during anesthesia, surgical stress, and perioperative care. Indeed, a recent strategy to improve outcomes from emergency surgery includes very early operation, and a recent guideline presented several arguments in favor of surgical repair of hip fractures within 48 h, but does not provide specific guidance on whether this is appropriate in patients with very recent strokes.[12] Herein we hypothesized that very early or more delayed surgery would be associated with better outcomes than surgery conducted at an intermediate time point when autoregulation may be maximally dysregulated.

We investigated the association between time elapsed after ischemic stroke and the risk of adverse events after emergency noncardiac surgery. Although we recognize that postponing emergency surgeries is rarely an option, choosing to operate earlier is sometimes a possibility. Furthermore, our results may provide additional guidance for clinical decision-making and lead to a better understanding of expected perioperative outcomes in this growing and critically ill patient population.

Materials and Methods

Ethics

This study was approved by the Danish Data Protection Agency, Copenhagen, Denmark (reference No. GEH-2014–019 and I-Suite No. 02737). Register-based studies using depersonalized data do not require ethics committee approval or patient consent in Denmark.

Data Sources

In Denmark, all of the citizens have a unique personal identification number, which enables identification and linkage of individual patients across several Danish registers. For this study, we retrieved information on diagnoses at hospital discharge and surgical procedures from the Danish National Patient Register, complete since 1977.[13] All of the information is coded according to international standards including the International Classification of Diseases, Tenth Revision (ICD-10) and the Nordic Medico-Statistical Committee (NOMESCO) Classification of Surgical Procedures.[14] From the Danish Anesthesia Database, complete since 2005, we retrieved information on whether a surgery was elective or emergent, as well as information on body mass index, type of anesthesia, and duration of anesthesia, which was collected as part of the daily clinical work. Information on pharmacotherapy before surgery was retrieved from the Danish Register of Medicinal Product Statistics, which holds information on all claimed prescriptions from Danish pharmacies. The specific drug, coded according to the Anatomical Therapeutic Chemical classification system, and the date of dispense were available. The register is linked to government reimbursement and has proven to be accurate.[15] The Central Population Register provided information on date of birth and sex. The National Causes of Death Register provided information on cause of death and death date.

Study Population

Our study population included all emergency noncardiac and nonintracranial surgeries from 2005 to 2011 for patients more than 20 yr of age. As several procedures were closely linked to a preceding stroke, we excluded cardiac surgeries, surgeries to the carotid arteries, gastrostomies, tracheostomies, and surgical procedures to the arteries of the aortic arch, as done previously.[16] Patients with repeat surgery within the 30-day follow-up period were only included at first surgery. Surgeries beyond a completed 30-day follow-up period were eligible for analyses. See figure 1, Supplemental Digital Content (http://links.lww.com/ALN/B461), for a flow diagram of the inclusion and exclusion criteria for the study population.

We undertook two analytic approaches. Patients with stroke 14 days or less before surgery were stratified as immediate surgery (1 to 3 days between stroke and surgery) and early surgery (4 to 14 days between stroke and surgery) and underwent propensity score–matched analysis and restricted cubic splines analysis to investigate the short-term relationship between time interval to surgery and perioperative outcomes. Longer time intervals were analyzed by logistic regression and restricted cubic spline analyses with patients, also stratified by time between stroke and surgery as patients with no previous stroke, patients with stroke less than 3 months before surgery (stroke less than 3 months), patients with stroke 3 to 9 months before surgery (stroke 3 to 9 months), and patients with stroke more than 9 months before surgery (stroke more than 9 months). Previous ischemic strokes

Submitted for publication June 20, 2016. Accepted for publication March 13, 2017. From The Cardiovascular Research Center, Herlev-Gentofte Hospital, University of Copenhagen, Hellerup, Denmark (M.N.C., C.A., G.H.G., M.E.J.); Department of Cardiology, Glostrup Hospital, University of Copenhagen, Glostrup, Denmark (C.A.); Department of Health Science and Technology, Aalborg University, Aalborg, Denmark (C.T.-P.); Anesthesiology and Critical Care Trials and Interdisciplinary Outcome Network, Department of Anesthesiology, University of Wisconsin, Madison, Wisconsin (R.D.S.); and The Pain Clinic, Department of Anesthesiology, Næstved Hospital, Næstved, Denmark (P.F.J.).

were defined as cerebral infarction (ICD-10: I63) or unspecified stroke (ICD-10: I64) occurring within 5 yr of surgery, because the majority of unspecified strokes in the Danish National Patient Registry have been shown to be of ischemic origin.[17] Transient ischemic attacks and hemorrhagic strokes were not considered because the frequency is low, and the validity and pathophysiology of these diagnoses are different from those of ischemic strokes. Vascular and nonvascular surgeries were analyzed separately in the main analyses, and only nonvascular surgeries were included in sensitivity analyses due to low numbers of vascular surgeries. Vascular surgery was defined as surgery to both arterial and nonarterial vessels according to the NOMESCO classifications (see table 1, Supplemental Digital Content, http://links.lww.com/ALN/B461, which is a list of surgery types according to the NOMESCO classifications).

Study Variables and Outcomes

The uses of pharmacotherapy within 120 days before surgery were grouped according to the Anatomical Therapeutic Chemical classification system. These included lipid modifying agents (C10A), β-blocking agents (C07), agents acting on the renin–angiotensin system (C09), potassium-sparing agents (C03D), thiazides (C03A), calcium channel blockers (C08), digoxin (C01AA05), vitamin K antagonists (B01AA0), glucose-lowering agents (A10), loop diuretics (C03CA01), and antithrombotic therapy as low-dose acetylsalicylic acid (B01AC06), dipyridamole (B01AC07), clopidogrel (B01AC04), or a combination of acetylsalicylic acid and dipyridamole (B01AC30).

Based on ICD-10 coding, we identified previous comorbid conditions as myocardial infarction, chronic obstructive pulmonary disease, anemia, cancer with metastases, renal disease, rheumatic disease, peripheral artery disease, liver disease, diabetes mellitus, chronic heart failure, ischemic heart disease, and atrial fibrillation (coding details are available in table 1, Supplemental Digital Content, http://links.lww.com/ALN/B461). We only considered comorbidities diagnosed or treated within 5 yr before surgery. The diagnoses for comorbidities used in this study have been validated with excellent positive predictive values of 97 to 100% in the Danish National Patient Registry.[17]

Surgeries were divided into 16 categories in accordance with previously published work[14,18] (coding details are available in table 1, Supplemental Digital Content, http://links.lww.com/ALN/B461) and defined as low-, intermediate-, or high-risk surgery in agreement with the European Society of Cardiology perioperative guidelines.[5] Our primary outcomes were 30-day all-cause mortality and a combined endpoint of 30-day major adverse cardiovascular events (MACEs), which included nonfatal ischemic stroke, nonfatal myocardial infarction, and cardiovascular death (any cause of death with ICD-10 code I). Nonfatal ischemic stroke was evaluated separately as a secondary endpoint.

Statistical Analysis

Differences between groups at baseline for continuous and categorical variables were tested using the Student's *t* test and chi-square test. We used three analytical approaches to assess risks of adverse outcomes, including logistic regression models, spline analyses, and propensity-score matching.

Multivariate logistic regression models were used to estimate odds ratios (ORs) with 95% CIs, adjusted for sex, age, body mass index, comorbidities, pharmacotherapy, type of surgery, and surgery risk. Patients with no previous stroke served as the reference. Analyses were performed for the primary and secondary endpoints stratified by vascular and nonvascular surgery. A few patients with missing data on body mass index (approximately 3% were missing for the stroke patients and 2% for no-stroke patients) were included, but considering the very small amount, no attempts were made to impute or otherwise replace the missing data.

Restricted cubic spline regression models adjusted for sex and age were used to analyze time between stroke and surgery as a continuous variable. These analyses included all of the patients with previous stroke undergoing nonvascular surgery. The median time between stroke and surgery for all of the patients (353 days) and patients with stroke 14 days or less before surgery (2 days) served as the reference. Five knots were placed at the 10th, 25th, 50th, 75th, and 90th percentiles of time between stroke and surgery.[19]

Among patients undergoing nonvascular surgery we used propensity-score matching to estimate risks of MACEs in patients having immediate surgery (1 to 3 days after stroke) and early surgery (4 to 14 days after stroke). Propensity-score matching was carried out in two steps. First, a logistic regression model was used to estimate the propensity score for undergoing early surgery, based on all of the variables from table 1 (C statistic for this model was 0.681). Next, we used the gmatch-macro, based on a greedy matching algorithm,[20] to match patients undergoing early surgery in a 1:1 ratio with patients undergoing immediate surgery. Patients were matched on propensity score (maximum deviation = 0.01), sex, and type of surgery in three groups (abdominal, orthopedic, or other surgery). Differences in baseline characteristics between the propensity score–matched groups were assessed using standardized mean differences, with a value of less than 0.10 indicating minimal imbalance between the groups.

We estimated absolute risks of MACEs in clinically relevant patient subgroups undergoing nonvascular surgery, stratified by the presence of stroke, chronic obstructive pulmonary disease, atrial fibrillation, diabetes mellitus, kidney disease, ischemic heart disease, previous stroke, heart failure, sex, and age more than or less than 70 yr, without additional adjustment. In addition, as a sensitivity analysis, we also estimated absolute risks of a MACE for stroke patients, stratified according to the revised cardiac risk index.[4]

Several sensitivity analyses were performed in the group of patients undergoing nonvascular surgery and subsequently

Table 1. Baseline Characteristics: Nonvascular

Characteristic	No Previous Stroke		Stroke < 3 Months		Stroke 3–9 Months		Stroke > 9 Months	
	n	%	n	%	n	%	n	%
Total No.	135,689		2,289		1,090		4,117	
Age, mean (SD), yr	57 (21)		74 (13)		75 (13)		75 (13)	
Men	60,666	44.7	1,137	49.7	509	46.7	1,880	45.7
Body mass index, mean (SD)	25.3 (5.0)		24.2 (4.7)		23.7 (4.6)		24.5 (4.9)	
Missing data, body mass index	2,602	1.9	75	3.3	36	3.3	127	3.1
Medication								
Lipid-lowering agents	16,623	12.3	810	35.4	453	41.6	1,655	40.2
Antithrombotics*	20,884	15.4	1,440	62.9	762	69.9	2,830	68.7
β-blocking agents	14,866	11.0	543	23.7	275	25.2	982	23.9
Renin-angiotensin system inhibitors	22,619	16.7	752	32.9	387	35.5	1,452	35.3
Aldosterone antagonists	3,237	2.4	110	4.8	55	5.0	213	5.2
Glucose-lowering agents	9,730	7.2	328	14.3	148	13.6	633	15.4
Thiazides	11,914	8.8	392	17.1	174	16.0	701	17.0
Calcium channel blocking agents	13,789	10.2	482	21.1	232	21.3	919	22.3
Loop diuretics	13,262	9.8	527	23.0	276	25.3	1,081	26.3
Digoxin	3,672	2.7	186	8.1	90	8.3	335	8.1
Vitamin K antagonist	4,293	3.2	215	9.4	72	6.6	347	8.4
Comorbid diseases								
Myocardial infarction	2,719	2.0	158	6.9	65	6.0	275	6.7
Chronic obstructive pulmonary disease	5,617	4.1	265	11.6	133	12.2	442	10.7
Anemia	6,714	4.9	332	14.5	172	15.8	575	14.0
Metastatic cancer	3,479	2.6	88	3.8	34	3.1	125	3.0
Peptic ulcer	4,716	3.5	203	8.9	102	9.4	406	9.9
Renal disease	3,391	2.5	157	6.9	84	7.7	260	6.3
Rheumatologic disease	1,735	1.3	51	2.2	43	3.9	132	3.2
Peripheral artery disease	3,538	2.6	264	11.5	122	11.2	424	10.3
Liver disease	1,969	1.5	50	2.2	25	2.3	98	2.4
Chronic heart failure	15,210	11.2	661	28.9	323	29.6	1,277	31.0
Ischemic heart disease	7,604	5.6	420	18.3	224	20.6	823	20.0
Atrial fibrillation	6,616	4.9	427	18.7	206	18.9	696	16.9
Venous thromboembolism	2,561	1.9	95	4.2	53	4.9	178	4.3
Diabetes mellitus	10,804	8.0	391	17.1	193	17.7	749	18.2
Surgeries								
Ear/nose/throat	987	0.7	4	0.2	3	0.3	13	0.3
Orthopedic major	40,423	29.8	1,173	51.2	601	55.1	2,239	54.4
Orthopedic minor	22,959	16.9	153	6.7	104	9.5	456	11.1
Abdominal, nonbowel	26,445	19.5	279	12.2	94	8.6	417	10.1
Abdominal, bowel	9,905	7.3	273	11.9	93	8.5	348	8.5
Breast	692	0.5	0		0		11	0.3
Plastic	14,446	10.6	190	8.3	96	8.8	330	8.0
Endocrinology	92	0.1	0		0		0	
Eye	1,879	1.4	7	0.3	13	1.2	24	0.6
Female reproductive	9,213	6.8	23	1.0	16	1.5	38	0.9
Male reproductive	1,377	1.0	11	0.5	6	0.6	43	1.0
Neurologic	2,246	1.7	43	1.9	18	1.7	37	0.9
Thoracic/pulmonary	805	0.6	73	3.2	4	0.4	26	0.6
Urology	4,220	3.1	56	2.4	40	3.7	134	3.3
Surgical risk								
Low	17,869	13.2	206	9.0	113	10.4	373	9.1
Intermediate	117,820	86.8	2,083	91.0	977	89.6	3,744	90.9
High	0		0		0		0	

Results are n (%) if not otherwise mentioned.

*Antithrombotics included any combination of acetylsalicylic acid, dipyridamole, and clopidogrel.

Table 2. Outcomes by Stroke Subgroup

	No Previous Stroke (N = 135,689)		Stroke < 3 months (N = 2,289)		Stroke 3–9 months (N = 1,090)		Stroke > 9 months (N = 4,117)	
Incidence	n	%	n	%	n	%	n	%
30-day all-cause mortality	6,501	4.8	376	16.4	134	12.3	482	11.7
30-day MACE	3,187	2.3	473	20.7	112	10.3	363	8.8
Separately analyzed endpoints*								
Acute myocardial infarction	396	0.3	19	0.8	11	1.0	26	0.6
Ischemic stroke	353	0.3	227	9.9	30	2.8	95	2.3
Cardiovascular death	2,438	1.8	227	9.9	71	6.5	242	5.9

Major adverse cardiovascular events (MACE) included nonfatal myocardial infarction, nonfatal ischemic stroke, and any cardiovascular death.
*Constitute the components of the combined endpoint of MACEs.

repeated in a subgroup of patients undergoing minor or major orthopedic surgery. We estimated stroke-associated risks of 30-day MACEs stratified by anesthesia time (less than 120 min *vs.* 120 min or more), type of anesthesia (general *vs.* other, which included regional anesthesia and/or sedation), and regular *versus* odd operation hours (regular being 7:00 AM to 4:00 PM on weekdays).

Data management and statistical analyses were performed using SAS version 9.4 (SAS Institute Inc, USA). Figures were compiled using R statistical software version 3.2.2 (https://www.r-project.org; accessed February 5, 2017).

Main analyses were decided on *a priori*, including patient selection, variables, outcomes, and statistical methods. Subanalyses and sensitivity analyses were decided on *post hoc* and after inputs from peer review. Throughout the study, efforts were made to comply with the Strengthening the Reporting of Observational Studies in Epidemiology guideline for reporting observational studies.[21]

Results

All Patients: Baseline

We identified 146,694 emergency surgeries with 7,861 patients (5.4%) having previous stroke. Only 3,509 (2.4%) were vascular surgeries, with 365 patients having previous stroke. Eleven percent of the patients had more than one eligible surgery within the study period.

For patients undergoing nonvascular surgery, more than half were women in all of the groups. Previous stroke patients were 7 to 8 yr older, on average, than patients with no previous stroke. All of the comorbidities were more prevalent in patients with previous stroke compared with no-stroke patients (all $P < 0.05$). Major orthopedic surgery accounted for 51 to 55% of surgeries in patients with previous stroke compared with only 30% in patients with no previous stroke (table 2, Supplemental Digital Content, http://links.lww.com/ALN/B461, which contains details on these surgeries and the proportion of surgeries related to fractures). Abdominal nonbowel surgery was more frequent in no-stroke patients (19%) compared with patients with previous stroke (8 to 12%). At baseline, patients with stroke less than 3 months before surgery were largely comparable with patients with stroke 3 to 9 months and more than 9 months before surgery (baseline characteristics are presented in table 1 for nonvascular surgery and in table 3, Supplemental Digital Content, http://links.lww.com/ALN/B461, for vascular surgery).

Long-term Interval between Stroke and Surgery in All Patients: Crude Events and Adjusted Models

The crude number of events for nonvascular surgeries is shown in table 2. Thirty-day MACEs occurred in 20.7% of patients with stroke less than 3 months before surgery and 8.8% of patients with stroke more than 9 months before surgery compared with only 2.3% of patients with no previous stroke. We observed low rates of myocardial infarctions in all of the patient subgroups (1% or less). Ischemic strokes were especially frequent in patients with stroke less than 3 months before surgery (9.9%) compared to patients with more distant stroke (2.3 to 2.8%). Cardiovascular death was the main contributor to the MACE endpoint for no-stroke patients (1.8%), as well as in patients with recent (9.9%) or more distant stroke (5.9%). All-cause mortality was significantly higher after surgery in stroke patients (11.7 to 16.4%) compared with no-stroke patients (4.8%). *P* value for the difference between the stroke groups was less than 0.001 for all of the endpoints.

Results from adjusted logistic regression models stratified by vascular and nonvascular surgery are presented in figure 1. Patients with stroke less than 3 months were at high risk of MACE for both nonvascular surgery (OR = 4.71 [95% CI, 4.18 to 5.32]) and vascular surgery (OR = 3.42 [95% CI, 2.02 to 5.78]) compared with no-stroke patients. Similar findings were seen for all-cause mortality. Patients with stroke more than 9 months before surgery undergoing nonvascular surgery remained at a significantly increased risk of MACE (OR = 1.62 [95% CI, 1.43 to 1.84]) and mortality (OR = 1.20 [95% CI, 1.08 to 1.34]) compared with no-stroke patients. We observed very high risks of repeat ischemic stroke in patients with stroke less than 3 months

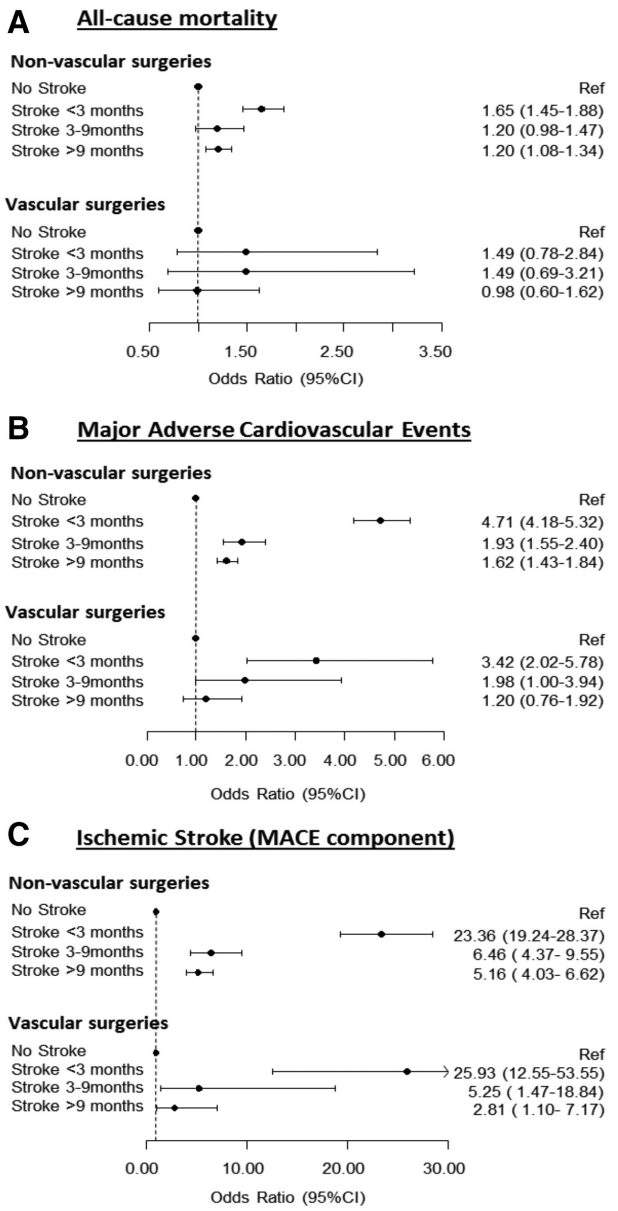

Fig. 1. Odds ratios for major adverse cardiovascular events and all-cause mortality. Data were adjusted for sex, age, body mass index, and all comorbidities, pharmacotherapy, surgery group, and surgery risk, as listed in table 1. MACE = major adverse cardiovascular events; Ref = reference.

before surgery undergoing nonvascular (OR = 23.36 [95% CI, 19.24 to 28.37]) and vascular surgery (OR = 25.93 [95% CI, 12.55 to 53.55]).

Time between stroke and surgery was analyzed as a continuous variable for nonvascular surgery in spline analyses (fig. 2A–C). As time between stroke and surgery increased, risks of MACE, death, and ischemic stroke declined. Patients undergoing surgery approximately 5 months after their index stroke were no longer at a significantly increased risk of MACE (OR = 1.21 [95% CI, 0.98 to 1.49]) or death (OR = 1.20 [95% CI, 0.98 to 1.45]) compared with patients undergoing surgery approximately 12 months after initial stroke (reference; fig. 2A–C).

Long-term Interval between Stroke and Surgery in All Patients: Absolute Risk by Comorbidity, Age, and Sex

The absolute risks of MACE in all patients undergoing nonvascular surgery are presented for a total of 36 clinically relevant patient subgroups, stratified by comorbidities, sex, and age (fig. 3). Risks of MACE in any male stroke patient more than 70 yr of age (16.2%) were lower than those of men more than 70 yr of age with concomitant stroke and chronic obstructive pulmonary disease (23.7%), stroke and myocardial infarction (27.7%), and stroke and kidney disease (22.7%). On the contrary, the impact of comorbidities in addition to previous stroke was less pronounced in women less than 70 yr of age where the absolute risks of MACE included any stroke patient (9.1%), stroke and chronic obstructive pulmonary disease (7.3%), stroke and myocardial infarction (12.1%), and stroke and kidney disease (12.5%). The sensitivity analysis of absolute risks of MACE stratified by the revised cardiac risk index is presented in table 4, Supplemental Digital Content (http://links.lww.com/ALN/B461).

Immediate and Early Surgery: Baseline

A large proportion of patients with previous stroke underwent surgery within 14 days of index stroke (see fig. 2, A and B, Supplemental Digital Content, http://links.lww.com/ALN/B461, which displays time between previous stroke and surgery). Using propensity-score matching, we matched 323 patients undergoing early surgery (4 to 14 days after stroke) with the same number of patients undergoing immediate surgery (1 to 3 days after stroke). Baseline characteristics before and after matching are shown in table 5, Supplemental Digital Content (http://links.lww.com/ALN/B461). After successful matching, standardized mean differences were less than 0.10, indicating minimal imbalance between the groups.

Immediate and Early Surgery: Crude Events and Adjusted Models

In the propensity score–matched population, risk of 30-day MACEs were significantly lower after immediate surgery (69 events) compared with early surgery (93 events; $P = 0.029$). No difference was observed for 30-day all-cause mortality ($P = 0.678$; table 3). In spline analyses, time between stroke and surgery up to 14 days was assessed as a continuous variable (fig. 2A–C). The point estimates indicated that risks of MACE and death after surgery increased within the first 7 and 3 days, respectively, after a stroke; however, 95% CIs were wide and the day-by-day differences not statistically significant.

Sensitivity Analyses: All Patients and Orthopedic Surgery Patients

Risks of MACE associated with each stroke subgroup were estimated in analyses stratified by time of surgery, anesthesia type, and duration of anesthesia, including all patients

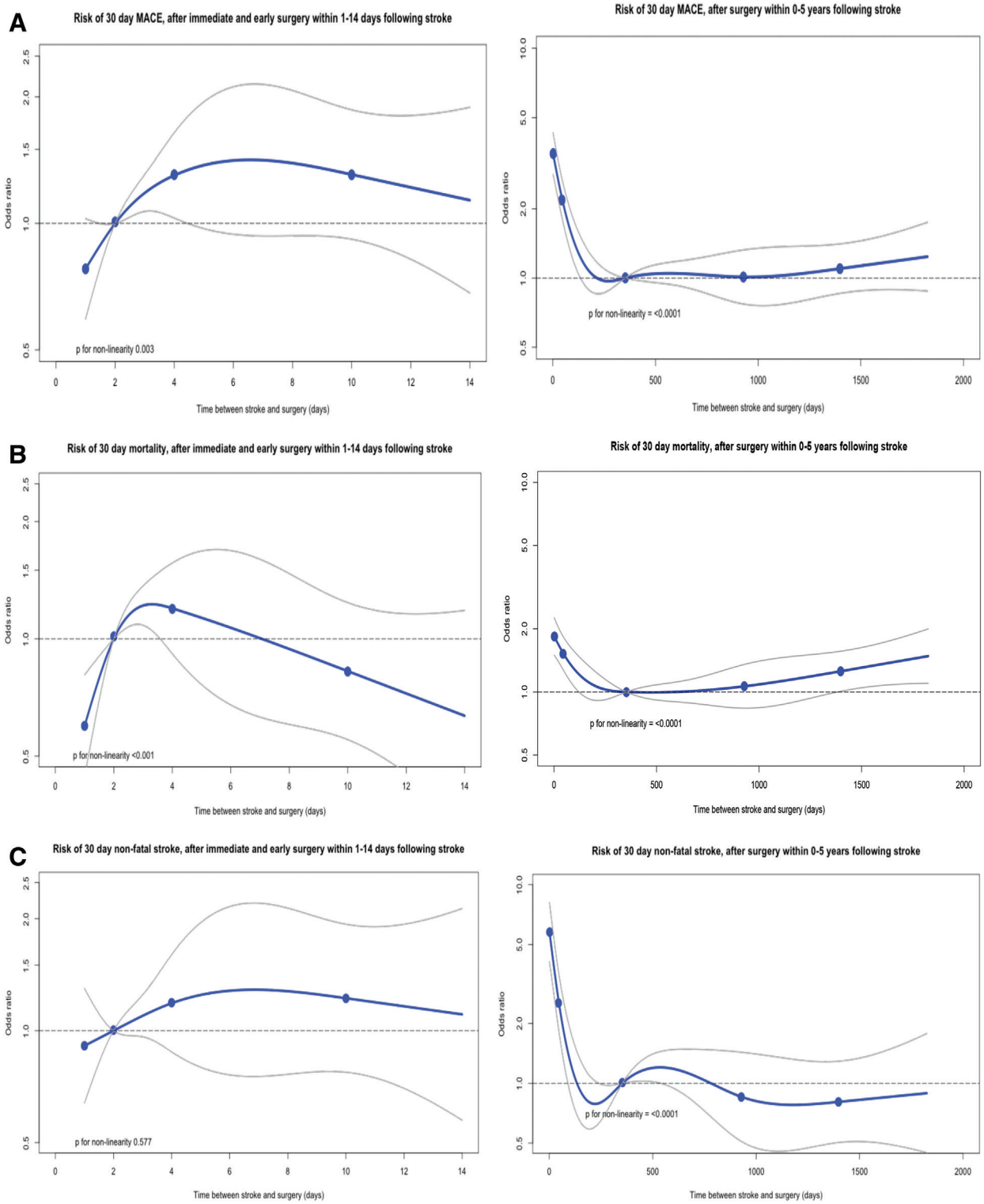

Fig. 2. Spline analyses. Data show the time between stroke and surgery and the risk of adverse outcomes. (*A*) Risk of 30-day major adverse cardiovascular events (MACE), including acute myocardial infarction, ischemic stroke, or cardiovascular death. (*B*) Risk of 30-day all-cause mortality. (*C*) Risk of 30-day nonfatal ischemic stroke. Time between stroke and surgery as a continuous variable were analyzed using a spline regression model adjusted for sex, age, and surgical category. Patients with 2 days between stroke and surgery were used as a reference for the spline analysis of the first 14 days and 353 days for the spline analysis of 0 to 5 yr.

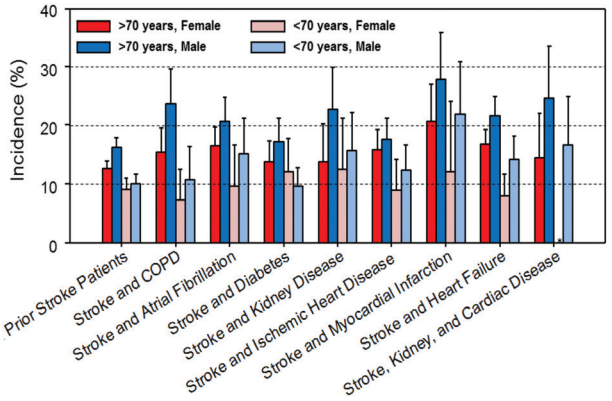

Fig. 3. Incidence of major adverse cardiovascular events (MACE), stratified by patient subgroup (stroke before surgery, specific comorbidities, sex, and age). Cardiac disease was defined as myocardial infarction, ischemic heart disease, or heart failure. *Error bars* represent 95% CIs. *The incidence was not calculated for this particular group due to an insufficient number of events. *P* was significant (less than 0.05) for all groups except for stroke and atrial fibrillation, stroke and kidney disease, stroke and myocardial infarction, and stroke and kidney and cardiac disease ($P > 0.05$). COPD = chronic obstructive pulmonary disease.

undergoing nonvascular surgery (see table 6, Supplemental Digital Content [http://links.lww.com/ALN/B461], which display the sensitivity analysis for the full study cohort). Patients with stroke less than 3 months were at significantly higher risks of MACE with duration of anesthesia less than 120 min (OR = 6.69 [95% CI, 5.44 to 8.23]) compared with duration of anesthesia at 120 min or more (OR = 3.93 [95% CI, 3.39 to 4.56]), with an overall *P* for interaction of less than 0.001. Risks of MACE varied with time of surgery at regular and odd hours, although the estimates for patients with stroke less than 3 months were very similar (surgery performed in regular hours, OR = 5.51 [95% CI, 4.58 to 6.63], and odd hours, OR = 4.25 [95% CI, 3.62 to 4.99]; *P* for interaction = 0.012). Risks of MACE did not differ by type of anesthesia stratified as general anesthesia and other types (*P* for interaction = 0.175). We repeated our main analyses in the subgroup of patients undergoing minor or major orthopedic surgery (46% of the study cohort). Unadjusted absolute risks and ORs for patients with stroke less than 3 months were largely comparable with our main findings, including risks of MACE (OR = 4.25 [95% CI, 3.62 to 5.00]), all-cause mortality (OR = 1.54 [95% CI, 1.29 to 1.84]), and ischemic stroke (OR = 22.28 [95% CI, 17.43 to 28.49]). (See table 7, Supplemental Digital Content [http://links.lww.com/ALN/B461], which shows the main analysis only for the cohort of orthopedic surgery). In the subgroup of orthopedic surgery, we also performed analyses stratified by time of surgery, anesthesia type, and anesthesia duration (see table 8, Supplemental Digital Content [http://links.lww.com/ALN/B461], for variable characteristics and full results). Risks of MACEs did not differ by anesthesia type (*P* for interaction = 0.827) or regular *versus* odd hours of operation (*P* for interaction = 0.101). Risks of MACE were significantly higher in orthopedic surgery patients if duration of anesthesia was less than 120 min *versus* duration of anesthesia more than 120 min (*P* for interaction < 0.001). In the group of patients undergoing orthopedic surgery, 818 patients underwent surgery during the first 14 days after stroke. The majority of these early surgeries (635 surgeries) were on the hip or femoral bone, and, of these, 58% were fracture repairs and only 1% were due to infection of a prosthesis.

Discussion

This nationwide study of patients with and without previous stroke undergoing emergency noncardiac, nonintracranial surgery demonstrated a time-dependent increased risk of 30-day MACE and all-cause mortality associated with previous stroke. Noticeably, among patients with stroke 14 days or less before surgery, we found that undergoing surgery 4 to 14 days after stroke was associated with significantly increased risks of a MACE compared with undergoing surgery within 1 to 3 days of stroke. In a long-term perspective, patients with a stroke less than 3 months before surgery were at elevated risk, which plateaued as time between stroke and surgery exceeded 4 to 5 months. Elderly patients with additional comorbidities, such as kidney disease or previous myocardial infarction in addition to stroke were at an especially high risk of a perioperative MACE. In addition, we found that the risk of a MACE was largely dependent on the duration of anesthesia, whereas the type of anesthesia and surgery at regular *versus* odd hours did not significantly affect the risk of adverse events.

Existing Recommendations

The observed long-term time dependency between stroke and perioperative risks was similar to that observed in a previous study from our group examining previous stroke in patients having elective noncardiac and nonintracranial surgery.[7] The associations in time between stroke and surgery and risks of perioperative adverse events have not previously been investigated in a setting of emergency noncardiac surgery, and only a few studies have previously addressed these issues in an elective surgery setting.[22–25] In addition, this study is the first to look at perioperative risks in patients undergoing surgery 14 days or less after stroke.

The lack of guidance for patients with previous stroke undergoing surgery was addressed in a recent consensus statement from the Society for Neuroscience in Anesthesiology and Critical Care; they suggested that elective surgery should be delayed 1 to 3 months but did not include any guidance for emergency surgery.[26,27] They concluded that the ultimate decision should always be based on the balance between risks of perioperative stroke and the risks of delaying surgery, which must be assessed thoroughly for each patient

Table 3. Outcomes for Propensity-score Matching

Variable	Stroke 1–3 days (N = 323)		Stroke 4–14 days (N = 323)		
	n	%	n	%	*P* Value
30-day all-cause mortality	54	16.7	58	18.0	0.678
30-day MACE	69	21.4	93	28.8	0.029
Separately analyzed endpoints*					
Acute myocardial infarction	4	1.2	3	0.9	0.704
Ischemic stroke	39	12.1	49	15.2	0.251
Cardiovascular death	26	8.0	41	12.7	0.053

Outcomes for propensity score–matching analysis are displayed in n (%) of events and *P* value for difference. Major adverse cardiovascular event (MACE) included nonfatal myocardial infarction, nonfatal ischemic stroke, and any cardiovascular death.

*Data constitute the components of the combined endpoint of MACEs.

in need of emergency surgery. The observed increasing risk of adverse events within the first days after stroke and the long-term increased risk within the first 4 to 5 months may provide guidance for clinicians preparing patients with previous stroke for surgery.

Clinical Importance

Our data suggest that emergency surgery is associated with better outcomes if operations occur within 72 h of the stroke; thereafter, a higher risk period ensues that coincides with dysregulated cerebral autoregulation (see following paragraph). Hence, we hypothesize that anesthesia and surgery may constitute a larger second hit in the poststroke phase when conducted between 4 to 14 days after the initial insult. Nonetheless, the results of this study are important for guiding perioperative decision-making and to inform the patient and relatives about realistic expectations of perioperative outcomes. For example, patients with stroke less than 3 months had a 16% absolute risk of 30-day mortality and a 21% risk of MACE, which is substantial. In addition, estimating absolute risks in clinically relevant subgroups of patients, we found that patient risks were also highly dependent on other comorbidities, sex, and age, as outlined in figure 3. Informing clinicians about expected outcomes in these high-risk patients according to comorbidities and demographics may improve individual preoperative evaluation, rigorous monitoring, and patient care. Medical diseases such as atrial fibrillation and renal disease may be stabilized even in the setting of emergency surgery, and antithrombotic strategies should be carefully considered. Because this study showed a high incidence of repeat ischemic strokes (10% in patients with stroke less than 3 months), increased attention toward perioperative stroke screening seems relevant. The straightforward Face Arm Speech test for identifying neurologic deficits has shown promise, and the routine use of this test in the postoperative setting may be warranted.[28]

Surgery, Anesthesia, and Cerebral Autoregulation

Interestingly, in propensity score–matched analyses, we found that immediate surgery within 1 to 3 days of the index stroke was associated with significantly fewer MACEs than early surgery within 4 to 14 days. Similarly, findings from spline analyses suggested that risks of adverse events and death increased over the first 3 to 7 days, followed by a decline in risks. A systematic review of 23 studies on autoregulation of cerebral blood flow has provided a possible explanation for the increasing risk of adverse events within the first days after stroke and the continuous decrease in risks for the following several months.[29] The review suggests that autoregulation deteriorates during the first 5 days after an ischemic stroke, followed by a recovery period of an estimated three months. These time intervals coincide with our periods of higher risk and hence support biologic plausibility. However, because we did not have information on important perioperative parameters, clinical variables, or the indication for surgery, we urge caution with this interpretation. Future prospective studies with more detailed perioperative data should seek to address these issues in detail.

In our study, subgroup analysis stratified by duration of anesthesia showed an unexpected increased risk of MACE with shorter duration of surgery. One explanation might be that longer anesthesia time is due to more thorough preparation and thus reduced perioperative risks. We estimated preparation time as the difference in anesthesia time and total procedure time, which showed that mean preparation time was in fact longer in patients with previous stroke compared with no-stroke patients (data not shown). We were, however, unable to investigate these associations in depth because surgery- and anesthesia-specific variables, beyond the type of anesthesia used, were not available in our registries.

We hypothesized that general anesthesia might exert more profound effects on the cardiovascular system and thus further aggravate the impaired autoregulation, increasing the risks of adverse outcomes. The analyses stratified by type of anesthesia showed only a nonsignificant association toward increased risks with general anesthesia (70 *vs.* 80% had general anesthesia for patients with and without stroke, respectively), whereas no difference was observed in the subgroup of patients undergoing orthopedic surgery. However, because we lack details of intraoperative care, we cannot

be sure that the patients allocated regional anesthesia had nonanesthetic depth sedation[30] and different hemodynamics compared with the general anesthetic group.

Strengths and Limitations

Our study included a contemporary unselected nationwide cohort of patients undergoing emergency noncardiac surgery. The Danish universal healthcare system ensures equal access to healthcare services irrespective of socioeconomic status. The comprehensive and validated administrative registers and the possibility for linking individual records using a unique personal identifier made possible the adjustment for important confounders, such as demographics, comorbidities, drug use, and surgery-related variables. Despite these efforts, residual confounding cannot be ruled out. The register-based method reduced the risk of report and selection bias, as well as other imprecisions related to data collection. We found that 19% of all previous stroke patients underwent emergency surgery within 14 days of the index stroke. This suggests that the stroke episode and the need for surgery are somehow correlated, but whether the ischemic stroke caused the emergency surgical condition (*i.e.*, the stroke facilitated an injurious fall resulting in a hip fracture) or whether a surgical condition caused a subsequent stroke (*i.e.*, a severe trauma resulting in disseminated intravascular coagulation causing an ischemic stroke) cannot be made out from the data available. In addition, information on in-hospital distributed medications and perioperative care was not available, and changes in medication during hospital admission, including preoperative antithrombotic and anticoagulation therapy, could not be accounted for in our study. No information on periprocedural blood loss or blood transfusions was available. It was not possible to distinguish between thromboembolic and atherothrombotic strokes, which are known to be different in pathophysiology. We have shown previously that stroke patients not undergoing surgery experience a similar reduction in risks of repeat stroke over time but that these risks and reductions are much more pronounced in patients undergoing surgery, which suggests that surgery constitutes a significant risk factor for repeat stroke.[16] Previous literature from our Danish registers suggests an underestimation of myocardial infarction due to the lack of a systematic assessment of the myocardial infarction by troponins after surgery.[31] The use of continuous time variables, such as anesthesia time, may be problematic and dependent on the specific surgery.[32]

Conclusions

In this study, we demonstrated that patients with previous stroke are at high risk of perioperative death and MACE for several months after emergency noncardiac and nonintracranial surgery. Interestingly, for the first 3 to 7 days after stroke, risks seemed to increase as cerebral autoregulation may have deteriorated, suggesting that immediate surgery may be associated with lower risks. We observed that patients were at particularly high risk within the first 3 months of an ischemic stroke, whereas the rapid decrease in risks of adverse events leveled off after approximately 4 to 5 months. Stroke-associated risks were dependent on patient sex, age, and additional comorbidities. Although emergency surgeries might not be feasible to postpone until the risk period subsides, high risk of adverse events and death should be taken into account during the perioperative risk assessment and conveyed when informing the patient and relatives about realistic expectations.

Research Support

Support was provided solely from institutional and/or departmental sources.

Competing Interests

The authors declare no competing interests.

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A Phase 1, Single-center, Double-blind, Placebo-controlled Study in Healthy Subjects to Assess the Safety, Tolerability, Clinical Effects, and Pharmacokinetics–Pharmacodynamics of Intravenous Cyclopropyl-methoxycarbonylmetomidate (ABP-700) after a Single Ascending Bolus Dose

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ABSTRACT

Background: Cyclopropyl-methoxycarbonylmetomidate (ABP-700) is a new "soft" etomidate analog. The primary objectives of this first-in-human study were to describe the safety and efficacy of ABP-700 and to determine its maximum tolerated dose. Secondary objectives were to characterize the pharmacokinetics of ABP-700 and its primary metabolite (cyclopropyl-methoxycarbonyl acid), to assess the clinical effects of ABP-700, and to investigate the dose–response and pharmacokinetic/pharmacodynamic relationships.

Methods: Sixty subjects were divided into 10 cohorts and received an increasing, single bolus of either ABP-700 or placebo. Safety was assessed by clinical laboratory evaluations, infusion-site reactions, continuous monitoring of vital signs, physical examination, adverse event monitoring, and adrenocorticotropic hormone stimulation testing. Clinical effects were assessed with modified observer's assessment of alertness/sedation and Bispectral Index monitoring. Pharmacokinetic parameters were calculated.

Results: Stopping criteria were met at 1.00 mg/kg dose. No serious adverse events were reported. Adverse events were dose-dependent and comprised involuntary muscle movement, tachycardia, and ventilatory effects. Adrenocorticotropic hormone stimulation evoked a physiologic cortisol response in all subjects, no different from placebo. Pharmacokinetics were dose-proportional. A three-compartment pharmacokinetic model described the data well. A rapid onset of anesthesia/sedation after bolus administration and also a rapid recovery were observed. A quantitative concentration–effect relationship was described for the modified observer's assessment of alertness/sedation and Bispectral Index.

Conclusions: This first-in-human study of ABP-700 shows that ABP-700 was safe and well tolerated after single-bolus injections up to 1.00 mg/kg. Bolus doses of 0.25 and 0.35 mg/kg were found to provide the most beneficial clinical effect *versus* side-effect profile. **(Anesthesiology 2017; 127:20-35)**

CYCLOPROPYL-METHOXYCARBONYLMETOMIDATE (CPMM, now ABP-700; The Medicines Company, USA) is a new intravenous anesthetic agent that currently is being developed for the induction and/or maintenance of general anesthesia and sedation in patients undergoing diagnostic or therapeutic procedures. ABP-700 is a second-generation analog of etomidate, a γ-aminobutyric acid (GABA) type A receptor agonist.[1] It contains an ester bond that is subject to rapid hydrolysis by nonspecific tissue esterases, a property that should result in a predictable dose–response relationship. The *in vivo* ester hydrolysis of the methoxycarbonyl moiety in ABP-700 generates a principle active metabolite, cyclopropyl-methoxycarbonyl acid (CPM acid), which is a 1,000-fold less potent activator of the GABA type A receptor compared to ABP-700.[2] This chemical approach to modulation of pharmacology has been used previously in approved drugs such as remifentanil, esmolol, and clevidipine to cause rapid inactivation of

What We Already Know about This Topic

- The clinical use of etomidate is limited by variability in recovery times and inhibition of adrenocortical steroid synthesis
- Cyclopropyl-methoxycarbonylmetomidate (ABP-700) is an etomidate analog that undergoes rapid hydrolysis by nonspecific tissue esterases and does not produce prolonged inhibition of steroid synthesis in animal models

What This Article Tells Us That Is New

- In a first-in-human study, cyclopropyl-methoxy-carbonyl-metomidate (ABP-700) was safe and well tolerated up to a maximum tolerated bolus dose of 1.0 mg/kg
- Onset of hypnosis after bolus administration was rapid as was recovery
- APB-700 did not cause cardiovascular depression, centrally induced respiratory depression, or suppression of the physiologic response of the adrenal axis to adrenocorticotropic hormone stimulation
- Involuntary muscle movements were observed at doses of 0.175 mg/kg and greater

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the compound. This approach also is being applied to sedative-hypnotics to create so-called "soft analogs" that show faster pharmacokinetics and a high therapeutic index.[3,4]

Etomidate was introduced into clinical practice to induce and maintain the hypnotic component of anesthesia while preserving hemodynamic and respiratory stability.[5] However, large population variability in recovery times and significant suppression of adrenocortical steroid synthesis has limited its clinical use.[6] In an attempt to eliminate these side effects while retaining its beneficial cardiovascular and respiratory profile, various new analogs of etomidate have been synthesized and tested in preclinical and animal settings.[2] Of these, ABP-700 showed promising pharmacology in rats.[7] When tested in beagle dogs, it was observed that recovery after ABP-700 was rapid both after single-bolus and continuous infusion.[1] Also, adrenocortical recovery occurred within approximately 90 min, which is not significantly different from propofol.[1]

In this article, results from a first-in-human, Phase 1, single-center, double-blind, placebo-controlled study of ABP-700 after a single ascending bolus dose are reported. The primary objectives were to describe the safety and efficacy of ABP-700 and to determine its maximum tolerated dose (MTD). Secondary objectives were to characterize the pharmacokinetics of ABP-700 and its primary metabolite (CPM acid), to assess the clinical effects of ABP-700, and to investigate the pharmacokinetic–pharmacodynamic relationships. We also tested the influence of a single-dose fentanyl pretreatment on the clinical and side effect profile with two of the most promising ABP-700 dosages.

Materials and Methods

Study Management and Registration

This trial was conducted at the QPS early Phase 1 unit, Groningen, The Netherlands, in cooperation with the Department of Anesthesiology at the University Medical Center Groningen, University of Groningen, The Netherlands, in accordance with the Declaration of Helsinki, in compliance with good clinical practice and applicable regulatory requirements. Ethics committee approval was obtained (Medische Ethische Toetsings Commissie Stichting Bebo, Assen, The Netherlands, NL48312.056.14, being the responsible ethics committee for QPS), and the study was registered at a public registry (Dutch Trial Register, NTR4545) before the start of the study. All volunteers provided written informed consent before participation.

Subjects

Healthy nonsmoking men and women aged between 18 and 45 yr with a body mass index (BMI) between 17.5 and 30 kg/m^2, American Society of Anesthesiologists physical status of I or II, and without risk of a difficult airway (modified Mallampati score I or II) were eligible for this study. Women were included if they were of nonchildbearing potential, *i.e.,* had to have undergone one of the following sterilization procedures at least 6 months before the first dose: hysteroscopic sterilization, bilateral tubal ligation or bilateral salpingectomy, hysterectomy, bilateral oophorectomy, or be postmenopausal with amenorrhea for at least 1 yr before the first dose and have follicle-stimulating hormone serum levels consistent with postmenopausal status (follicle-stimulating hormone levels of less than 30 IU/l). Subjects were excluded in case of a history or presence of significant disease or disease risk. In addition, volunteers must have refrained from, or not anticipate, the use of any medication, alcohol, or (illicit) drug abuse. Participants should not have had surgery within 90 days before drug dosing, a history of febrile illness within 5 days before dosing, not have participated in another clinical trial within 90 days before dosing, and not be pregnant or lactating. Subjects with a history or presence of adrenal insufficiency as defined by serum cortisol level less than 6 μg/dl at screening also were excluded from participation.

Study Execution

This study was set up as a Phase 1, single-center, double-blind, placebo-controlled, single ascending-dose study of ABP-700. In total, 60 volunteers were divided into 10 cohorts. In each cohort, six subjects received a single IV bolus dose of ABP-700 or placebo in a 5:1 ratio. The actual dosages of ABP-700 were 0.03, 0.10, 0.25, 0.35, 0.50, 0.75, and 1 mg/kg. The starting dose of 0.03 mg/kg was chosen as a conservative starting point based on preclinical efficacy and toxicology data and following guidelines from the European Medicines Agency (London, United Kingdom). Two cohorts of six volunteers received a single IV bolus dose of 0.25 or 0.35 mg/kg ABP-700 or placebo preceded by 1 μg/kg fentanyl in a 5:1 ratio. An additional cohort (cohort 10) of six volunteers was added, receiving a single IV bolus dose of ABP-700, 0.175 mg/kg or placebo in a 5:1 ratio to fine-tune the dose–response relationship.

Subjects were admitted to the clinical pharmacology unit 1 day before study drug administration. Subjects were required to fast for a minimum of 8 h overnight before study drug administration and continued to fast for at least 4 h thereafter. Water was not permitted from 2 h before until 1 h after dosing. Consumption of foods and beverages containing caffeine, alcohol, or grapefruit was prohibited 24 h, 48 h, and 10 days before

This article is featured in "This Month in Anesthesiology," page 1A. Timothy Brennan, Ph.D., M.D., served as Handling Editor for this article. M.M.R.F.S. and B.I.V. contributed equally to this article and should both be considered as first authors. Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site (www.anesthesiology.org).

Submitted for publication March 1, 2016. Accepted for publication March 27, 2017. From the Department of Anesthesiology, University of Groningen, University Medical Center Groningen, The Netherlands (M.M.R.F.S., B.I.V., D.J.E., A.R.A., P.M., S.M., K.v.A.); Department of Anesthesia and Perioperative Medicine, Ghent University, Gent, Belgium (M.M.R.F.S.); QPS Netherlands, BV, Groningen, The Netherlands (I.d.D.); QPS LLC, Newark, Delaware (T.C.); and The Medicines Company, Parsippany, New Jersey (J.A.C., S.P.S.).

dosing, respectively, and throughout subjects' admission to the study facility. Before dosing, all subjects were transported to a dedicated treatment room equipped similarly to an operating room used in the University Medical Center Groningen that included monitoring equipment as well as respiratory support, including a tracheal intubation kit, anesthesia machine (Primus®; Dräger, Germany), and a fully equipped emergency "crash" cart immediately available. Subjects breathed room air before, during, and postdosing of ABP-700. In the event that an oxygen saturation (Spo_2) level less than 90% was not resolved by stimulation or jaw thrust, additional oxygen (5 l/min) was delivered *via* nasal prongs (Microstream®; Medtronic, Ireland). If required, brief manual ventilatory support was allowed with the use of a tight-fitting face mask. Before drug administration, two intravenous cannulas were inserted. The intravenous cannula for study drug administration was placed downstream of the arterial sampling and monitoring cannula, which was inserted into the radial artery after local anesthesia. The second intravenous cannula was placed in the opposite arm to draw the venous blood samples. All subjects received minimal crystalloid intravenous fluids during the drug administration period *via* the cannula for drug administration.

Because ABP-700 is derived from etomidate, a drug associated with involuntary muscle movements (IMMs), and because of the preclinical findings that ABP-700 also is associated with IMM, including myoclonic jerking,[1] midazolam was made available as safety medication at an initial 1-mg bolus dose for amelioration of severe myoclonus if observed. Additional midazolam doses of 1 mg could be administered at the discretion of the anesthesiologist–investigator. During the conduct of the study, a board-certified anesthesiologist was present throughout the dosing period until full recovery of the subject.

Subjects remained supine until recovery and were supine or semirecumbent in bed until the removal of the arterial line. When supine or semirecumbent, subjects were allowed to rise for brief periods under supervision. Subjects did not engage in strenuous activity at any time during the confinement period. Subjects were asked to avoid exercise 72 h before clinical laboratory tests at screening, check-in (1 day before dosing), and follow-up. Subjects remained in the clinic through completion of all scheduled postdose procedures on day 2 and returned for a follow-up visit 4 to 6 days after dosing.

After each cohort, the principal investigator (PI), sponsor, and ethics committee evaluated all available data relevant to the safety, tolerability, pharmacokinetics, and clinical effects of ABP-700 to proceed to the next dose level. Once an MTD was identified, no further dose escalation occurred. Stopping criteria for dose escalation were a grade 3 or higher dose-limiting toxicity event as defined in the September 2007 Food and Drug Administration Guidance*; Spo_2 less than 90% not resolved by simple stimulation, jaw thrust, or supplemental oxygen administered *via* nasal prongs; any serious adverse events (AEs) that were considered by the PI to be related to study drug; and any clinically significant AEs that the sponsor and PI considered a safety concern. Because methanol is a byproduct of the ABP-700 metabolism and was detected during animal studies (data on file), it was indicated to search for significant changes in clinical or laboratory parameters indicative of methanol exposure with the potential for toxicity including, but not limited to, evidence of metabolic acidosis.

*Toxicity Grading Scale for Healthy Adult and Adolescent Volunteers Enrolled in Preventive Vaccine Clinical Trials with the following exceptions: local injection-site reactions that are grade 2 or higher, bradycardia must be considered clinically significant, and QTcF change from baseline greater than 100 ms or total QTcF greater than 500 ms.

Primary Study Endpoints

Safety and tolerability of ABP-700 were assessed by evaluation of AEs, physical examination, safety laboratory tests (serum chemistry, hematology, arterial blood gas, urinalysis, and coagulation), serum methanol concentration, intermittent 12-lead electrocardiograms, temperature, and infusion-site reaction monitoring. Electrocardiograms were evaluated for PR interval, QRS interval, and QTcF interval. AEs were defined as the incidences of treatment-emergent adverse experiences per system organ class according to MedDRA (version 16.1; MedDRA MSSO, USA) from the period of arrival at the clinic up to the follow-up visit.

All volunteers were monitored continuously with a Philips MP50 monitor (Philips, The Netherlands) measuring continuous three-lead electrocardiogram and for heart rate, continuous pulse oximetry, noninvasive blood pressure every minute (at lower limb level), continuous invasive blood pressure *via* the radial artery cannula, respiration rate, respiration pattern, and end-tidal carbon dioxide (Microstream; Medtronic). High-frequency electronic data were captured from 1 min predose until 15 min after full recovery.

An adrenocorticotropic hormone (ACTH) stimulation test was performed to evaluate the effect of ABP-700 on adrenal function. Screening and baseline cortisol levels were attained before 9:00 AM after 1-h rest in the supine position and after at least an 8-h fast. Adrenocortical stimulation commenced with an IV bolus administration of 250 μg synthetic ACTH 60 min after ABP-700 administration. Cortisol levels were measured at 1 and 2 h after ACTH administration. The MTD was reached when two or more volunteers met stopping criteria in any particular dosing cohort.

Secondary Study Endpoints

The clinical hypnotic–anesthetic drug effect of ABP-700 was evaluated by means of the Modified Observer's Assessment of Alertness/Sedation (MOAA/S) score, as shown in table 1. Clinical effect was defined as a MOAA/S less than 5. Onset of deep sedation/anesthesia was defined as the first postdose transition to a MOAA/S value less than 3 and offset of deep sedation/anesthesia as the transition to a MOAA/S value greater than 2. Duration of sedation/anesthesia was defined as the time between onset and offset. MOAA/S scoring was performed 1 min before dosing and at approximately 15 s, 30 s, 1 min postdose, and every minute thereafter for a

Table 1. Modified Observer's Assessment of Alertness/Sedation (MOAA/S) score

Responsiveness	Score
Responds readily to name spoken in normal tone	5 (alert)
Lethargic response to name spoken in normal tone	4
Responds only after name is called loudly and/or repeatedly	3
Responds only after mild prodding or shaking	2
Responds only after painful trapezius squeeze	1
Does not respond to painful trapezius squeeze	0

minimum of 15 min after full recovery. Subjects were considered recovered when three consecutive MOAA/S scores of 5 were obtained. In addition, a processed electroencephalographic measure, the Bispectral Index (BIS), was applied as a continuous measure of cerebral drug effect. BIS (software revision 1.13; Medtronic) was derived from two-channel frontal electroencephalogram using an Fpz-F7 and Fpz-F8 referential montage and calculated with the BIS-VISTA monitor (Medtronic) using the bilateral electrodes BIS® Sensor electrodes (Medtronic). An average BIS value was calculated by use of data from the left and right BIS values. Electrode impedance was less than 5 kOhm. The smoothing interval of the BIS® monitor (Medtronic) was set at 15 s. BIS values range from 100 to 0, with lower values denoting more drug effect.

Pharmacokinetic–Pharmacodynamic and Clinical Effect Evaluation

The pharmacokinetics of ABP-700 and its metabolite (CPM acid) were studied. Arterial and venous blood samples were collected. Arterial samples were drawn before ABP-700 injection and at 0.5, 1, 2, 3, 4, 8, 12, 20, 30, 45, 60, 90, 120, and 180 min after ABP-700 injection. Venous samples were drawn predose, 1.5, 3.5, 7, 13, 21, 35, 75, 125, 185, 240 min and 6, 8, and 12 h postdosing. Blood samples were collected in prechilled Vacutainers (Becton Dickinson, USA) containing NaF/Na_2EDTA to inhibit nonspecific esterase activity (BD cat. No. 367587; Becton Dickinson). Samples were stored on wet ice for no longer than 30 min until centrifuged at 5°C for 7 min at 1,800*g* to separate the plasma. Plasma was transferred by the use of disposable pipettes into cryovials and placed immediately on dry ice until transferred to a –80°C freezer within a total allotted time of 60 min.

Plasma concentrations of ABP-700 and metabolite CPM acid were measured by high-performance liquid chromatography with tandem mass spectrometric detection using the SCIEX API 4000 LC/MS/MS System with a TurboIonSpray® interface (AB SCIEX, Canada) in positive mode at QPS.

Deuterium-labeled D5-ABP-700 and D5-CPM-acid were used as internal standards. To 0.05 ml plasma, the internal standard solution and acetonitrile were added. After mixing and centrifuging, part of the supernatant was transferred onto the Ostro Protein Precipitation and Phospholipid Removal Plate, 25 mg (Waters Chromatography B.V., The Netherlands). By the use of positive pressure, the supernatant was eluted and collected in a 96-well plate. Before analysis, a dilution step with Milli-Q ultrapure water (Merck Milliport, The Netherlands) and acetonitrile was applied. Liquid chromatography was performed on a C18 column (50 × 3.0 mm, 5 µm; Advanced Chromatography Technologies Ltd, UK) mounted in line with a 4 × 3.0-mm C18 guard column (Advanced Chromatography Technologies Ltd) on an Agilent 1100/1200 LC system (Agilent Technologies, USA). The column temperature was maintained at 50°C. The mobile phase A was 0.1% formic acid in water, and the mobile phase B was 0.1% formic acid in 50% acetonitrile. For sample elution, a gradient of 30 to 95% B was applied over a period of 1 min at a flow rate of 1 ml/min. The approximate elution times for ABP-700 and CPM acid were 2.1 and 1.7 min, respectively. The nominal mass transitions monitored were 315.2 to 211.1 and 301.3 to 197.0 *m/z* for ABP-700 and CPM acid, respectively. The method was validated over a concentration range of 5.00 to 1,250 ng/ml for ABP700 and 25.0 to 6,250 ng/ml for CPM acid. Precision and accuracy were demonstrated for the validation samples within a single run of six aliquots (within-run for repeatability) and between different runs (between-run for reproducibility) distributed over at least 2 days. Validation samples were prepared in blank human NaF/Na2EDTA plasma by spiking known concentrations of ABP-700 and CPM acid. For precision, acceptance criteria of coefficient of variation (CV%) not to exceed 20.0% at lower limit of quantification (LLOQ) or 15% at all other levels was met for all samples. For accuracy, acceptance criteria of percentage of relative error not to exceed 20.0% at LLOQ or 15% at all other levels was met for all samples. LLOQ for ABP-700 and CPM acid was determined at 5 and 25 ng/ml, respectively.

Noncompartmental pharmacokinetic analyses of concentration–time data of both arterial and venous plasma ABP-700 and its primary metabolite (CPM acid) were conducted with Phoenix® WinNonlin®, version 6.3 (Pharsight Corporation, USA). Only plasma pharmacokinetic profiles that contained more than five consecutive data points with a quantifiable concentration value were considered evaluable. Actual elapsed times from dosing were used to estimate all individual plasma pharmacokinetic parameters for evaluable subjects. Observed predose concentrations were set as missing to generate C_0 values, which were calculated as the extrapolated concentration at time 0 (computed for parent only). Systematic exposure was calculated with the area under the drug concentration–time curve. More detailed information on the applied methods to analyze the noncompartmental pharmacokinetics of the concentration–time data of plasma ABP-700 (both arterial and venous) and its primary metabolite can be found in Supplemental Digital Content 1 (http://links.lww.com/ALN/B437).

In addition, compartmental pharmacokinetic and pharmacodynamic models were developed with NONMEM (Icon Development Solutions, USA). The time course of ABP-700

was modeled with a three-compartmental pharmacokinetic model with volumes *V1*, *V2*, *V3*; elimination clearance *CL*; and intercompartmental clearances *Q2* and *Q3*. Arterial observations were related to the central compartment. Venous and metabolite concentrations were not used. All parameters were scaled linearly with total body weight. Mixing delay for the pharmacokinetic was set to 15 s. Residual error in pharmacokinetic observations was assumed to be proportional to the predicted concentration. Pharmacokinetic observations reported as lower than the LLOQ were ignored.

We modeled BIS using a sigmoidal E_{max} model driven by an effect compartment concentration (*Ce*) connected to the plasma compartment by a first-order rate constant ($ke_{0,BIS}$). The equation of the pharmacodynamic model was as follows:

$$\frac{dCe}{dt} = ke_{0,BIS} \cdot (C - Ce)$$

$$Effect = E_0 + (E_{max} - E_0) \cdot \frac{Ce^{\gamma}}{Ce_{50}^{\gamma} + Ce^{\gamma}} + \varepsilon$$

Where *C* and *Ce* are the concentrations in the central (*V1*) and effect compartments, E_0 is the baseline pharmacodynamic measure when no drug is present, E_{max} is the maximum possible drug effect, Ce_{50} is the *Ce* associated with 50% of the maximum effect, γ is the steepness of the concentration *versus* response relation, and ε represents additive residual error to the pharmacodynamic observations. Signal delay in the BIS due to epoch generation and smoothing also was set to 15 s.

MOAA/S observations were treated as ordered categorical responses and modeled with a proportional-odds method. The model estimates the cumulative probabilities of MOAA/S scores. Let *S* denote an observed score, the logits *lx*, of the probabilities that $S = 0$, $S \leq 1$, $S \leq 2$, $S \leq 3$, $S \leq 4$, are:

$$l_{S=0} = b_0 + DEFF \cdot Ce$$
$$l_{S\leq 1} = b_0 + b_1 + DEFF \cdot Ce$$
$$l_{S\leq 2} = b_0 + b_1 + b_2 + DEFF \cdot Ce$$
$$l_{S\leq 3} = b_0 + b_1 + b_2 + b_3 + DEFF \cdot Ce$$
$$l_{S\leq 4} = b_0 + b_1 + b_2 + b_3 + b_4 + DEFF \cdot Ce$$

Where the b_0 is a fixed-effect parameter representing the logit of the probability for score 0 and b_1 though b_4 represent the difference in logits between the scores. *DEFF* is also a fixed-effect parameter for the model, and *Ce* represents the effect-site concentration of ABP-700, calculated with a first-order rate constant ($ke_{0,MOAA/S}$) in a similar manner as done for BIS. The corresponding probabilities are given by:

$$PC_x = \frac{e^{l_x}}{1 + e^{l_x}}$$

The actual probabilities, p_x, of observing a particular score are: $P_{S=0} = PC_{S=0}$, $P_{S=1} = PC_{S\leq 1} - PC_{S=0}$, $P_{S=2} = PC_{S\leq 2} - PC_{S\leq 1}$, $P_{S=3} = PC_{S\leq 3} - PC_{S\leq 2}$, $P_{S=4} = PC_{S\leq 4} - PC_{S\leq 3}$, $P_{S=5} = 1 - PC_{S\leq 4}$.

For BIS and MOAA/S PD model estimation, the individual predicted plasma concentrations were used as the driving force for the effect compartment. This is known as the sequential method,[8,9] also known as the Individual Pharmacokinetic Parameters (IPP) method. Covariate search was not performed due to the limited variability in age, weight, sex, and BMI in the studied individuals.

Model parameters were assumed to be log-normally distributed or constant across the population. For estimates of logarithmic interindividual variability, we report the estimated variance and the coefficient of variation.

Uncertainty in estimated model parameters was evaluated by estimating the upper and lower 95% confidence limits by spline-interpolation of the likelihood profiles. We determined what increase/decrease in each parameter is required to increase NONMEM objective function by 3.84.

To quantify the pharmacokinetic predictive performance for an observation, we calculated the performance error[10] (PE_{PK}) and absolute performance error (APE_{PK}) as follows:

$$PE_{PK} = \frac{C_{observed} - C_{predicted}}{C_{predicted}} \times 100\%$$
$$APE_{PK} = |PE_{PK}|$$

For these measures, the median values are reported. The median PE_{PK} ($MdPE_{PK}$) indicated bias, and median APE_{PK} ($MdAPE_{PK}$) indicates precision.

Clinical Observations

This study was intended to define a MTD for ABP-700. However, it was reasoned that well-tolerated doses would need to be assessed for potential further clinical testing. Therefore, to evaluate the potential clinical utility of every specific dose, we analyzed various relevant clinical observations described by the attending anesthesiologist during the dosing together with a visual inspection of the individual vital signs trends.

IMMs were anticipated with clinical testing of ABP-700 based both on preclinical observations[1] and also by virtue of the parent compound etomidate's known effects on muscle movement in humans.[11,12] No standardized nomenclature or scoring system was implemented to characterize IMM, as it has not been done for etomidate in the past. Instead, we described the extensiveness of the observed IMMs, rather than their nature. As such, "extensive movements" are movements defined as IMMs that involve the whole body or a considerable part of it. "Few movements" are movements that occur in few body parts, such as both arms or the face.

Breathing was monitored by means of capnography (end-tidal carbon dioxide) and capnography-derived respiratory rate monitoring. Apnea was defined as an absence of breathing for 20 s or more. Tachypnea was defined as a breathing frequency of 20 breaths/min or more. Sinus tachycardia was defined as a heart rate of 100 beats/min or more and if this meant a significant change from baseline (due to possible volunteer stress). Increased blood pressure was defined as a mean arterial pressure greater than 110 mmHg[13] if this meant a significant change from baseline. Desaturation was defined as a decrease of Spo_2 less than 95%.

Data Recording and Statistics

All vital signs data, MOAA/S, BIS, respiratory function, comments, and a subset of pharmacokinetic sampling time were stored electronically with a dedicated and validated electronic data capturing device (Rugloop II; DEMED, Belgium). Visual and computerized methods of data validation were applied to ensure accurate, consistent, and reliable data for the subsequent statistical analysis.

The sample size was based on previous investigations adequately studying safety, efficacy, clinical effect, and pharmacokinetic–pharmacodynamic behavior of new compounds while minimizing the exposure of volunteers to the compound. Descriptive statistics are displayed as mean ± SD or geometrical mean ± 95% CI unless indicated otherwise.

Results

Subjects

In total, 154 subjects were screened by the contract research organization (QPS, The Netherlands). Of these, 60 subjects were admitted to the study, of whom 50 received ABP-700 and 10 received a placebo. All subjects who were enrolled completed the study; no subject withdrew from the study. Mean height, weight, BMI, and age were similar among the groups, as shown in table 2.

Safety and Tolerability

Treatment-emergent adverse experiences were reported in 41 subjects, with 94 AEs reported in total (table 3). Of these, three subjects (30%) in the placebo group reported at least one AE. The majority of AEs were of mild intensity (93.7%). The number and severity of reported AEs increased with ascending doses of ABP-700. No volunteers withdrew from the study due to AEs. Of the 41 subjects experiencing at least one AE, all had at least one AE of mild intensity. Three subjects also experienced at least one AE of moderate intensity. No AEs of severe intensity were reported. AEs that were "possibly" related to the study treatment were reported for 37 subjects. Four subjects had AEs that were unlikely related, and 15 subjects experienced AEs that were unrelated to the study medication.

Table 2. Demographics

Characteristics	Placebo (N = 10)	ABP-700 (N = 50)
Sex, n (%)		
Male	10 (100)	49 (98)
Female	0 (0)	1 (2)
Age, yr, mean (SD)	23 (3.7)	25 (3.0)
Ethnicity, n (%)		
Not Hispanic or Latino	10 (100)	49 (98.0)
Hispanic	0 (0)	1 (2.0)
Race, n (%)		
Asian	1 (10)	2 (4)
Black or African American	0 (0)	1 (2)
White	9 (90)	47 (94)
Smoking status, n (%)		
Never	10 (100)	58 (96.7)
Stopped	0 (0)	2 (3.3)
If yes, subject quit smoking >6 months ago? Yes	0 (0)	2 (100)
Height, cm, mean (SD)	182 (6)	182 (7)
Weight, kg, mean (SD)	74 (7)	76 (7)
Body mass index, kg/m^2, mean (SD)	22 (1.8)	23 (1.8)
Alcohol consumption, units/week, median (range)	4.5 (0–12)	7 (0–14)

ABP-700 = cyclopropyl-methoxycarbonylmetomidate.

Table 3. Adverse Events

Event	ABP-700 (N = 50)	Placebo (N = 10)
Muscle twitching	15	0
Apnea	13	0
Hyperventilation*	10	0
Sinus tachycardia	9	0
Catheter site–related reaction	6	1
Eye disorder†	5	1
Blood pressure increased	4	0
Restlessness	4	0
Nausea/vomiting	2	0
Abnormal respiration‡	3	0
Decreased oxygen saturation	3	0
Neurologic anesthetic complication§	3	0
Hiccups	3	0
Injection-site pain	2	0
Myoclonus	2	0
Headache	2	0
Presyncope‖	2	0
Fatigue	2	0
Euphoric mood	2	0

Listing of adverse events occurring in two or more ABP-700–treated subjects: *verbatim term: breathing disorder and paradoxical chest wall movements; †verbatim terms: rhythmic movement of eyes, eye lid fluttering, and eye lid twitching; ‡verbatim term: sigh; §verbatim term: emergence delirium; and ‖verbatim term: vasovagal reaction (during placement of arterial line).

ABP-700 = cyclopropyl-methoxycarbonylmetomidate.

For each cohort, figure 1 presents the hemodynamic and respiratory data. More information on vital signs data can be found in Supplemental Digital Content 2 (http://links.lww.com/ALN/B438), which are figures plotting the individual data on heart rate, Spo_2, mean arterial blood pressure measured using a noninvasive blood pressure cuff, mean arterial blood pressure using an invasive blood pressure method, respiratory rate, frontal muscles electromyographic activity and BIS measured by the Vista Monitor (Medtronic, Ireland), and end-tidal carbon dioxide for each volunteer receiving ABP-700 or placebo.

Table 4 lists the various tolerability parameters. The occurrence and severity of IMM was reported as dose-dependent. IMM was characterized primarily as muscle twitching or myoclonic activity, but movements including clonic, dystonic, and other variants of IMM also were included and reported as IMM. Figure 1 and table 4 show stable hemodynamics without any occurrence of bradycardia

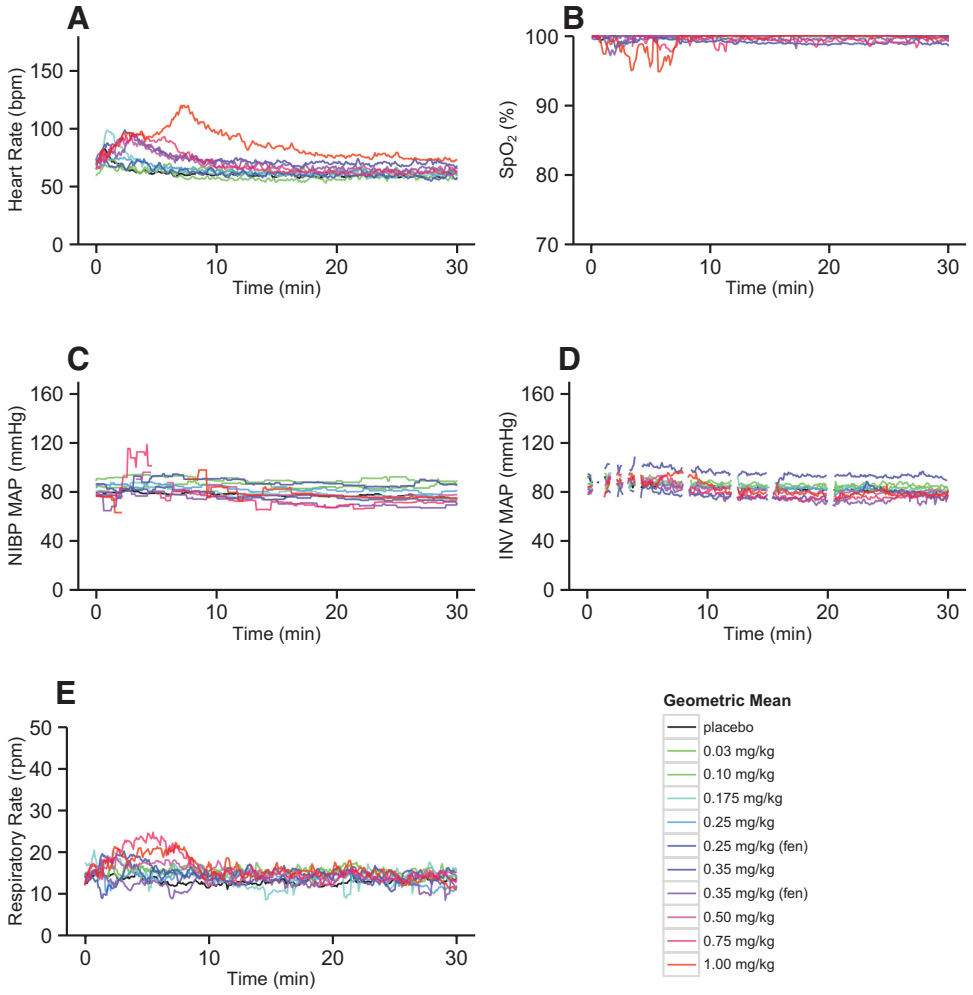

Fig. 1. Vital signs. (*A*) Heart rate. (*B*) Oxygen saturation (Spo_2). (*C*) Mean arterial pressure as measured by no-invasive blood pressure monitoring (NIBP MAP). (*D*) Mean arterial blood pressure as measured by invasive arterial blood pressure monitoring (INV MAP). (*E*) Respiration rate. Data are presented as geometrical means per cohort. Fen = fentanyl.

or hypotension after bolus injection. In the higher dosing cohorts, an increase in heart rate and mean arterial blood pressure was observed after similar time course as the cerebral drug effect of ABP-700.

Decreased Spo_2 was reported by seven subjects in the highest dose cohorts and was considered mild and self-limiting (fig. 1). Depression in respiratory rate as measured by capnography, mostly related to IMM resulting in short-lasting and self-limiting upper airway obstruction, was found in 13 volunteers and was dose-related (table 4). Short-lasting chin lift was required in four of these higher dose volunteers to maintain a patent airway. Except in the two highest dosages, where tachypnea was seen, overall respiration rate did not change during the study period, as shown in figure 1.

ABP-700 was not tolerated by three subjects because of the occurrence of severe IMM accompanied by hemodynamic disturbances. One of these subjects had received 0.75 mg/kg ABP-700, and the other two subjects had received 1.00 mg/kg ABP-700. Per protocol, midazolam was given in these three subjects. Total dosages of midazolam ranged from 2 to 5 mg. As such, in the group of 1.00 mg/kg ABP-700 the stopping criteria of the study were met, and it was concluded that 1.00 mg/kg of ABP-700 was the MTD.

Serum chemistry, hematology, urinalysis, coagulation, serum methanol concentration, and temperature did not change during the study period (data not shown). In particular, serum methanol levels were not detectable at all doses tested. Arterial blood gas parameters (pH, $Paco_2$, Pao_2, HCO_3, Na^+, Cl^-, anion gap) did not change significantly between baseline and 15 min postdose (see Supplemental Digital Content 3, http://links.lww.com/ALN/B439, a table with the arterial blood gas sampling values at baseline and 15 min postdose for each ABP-700 dosing cohort and placebo).

On the 12-lead electrocardiogram, a small decrease in the PR interval and QRS interval was observed after bolus injection of ABP-700 of all dosing groups. The changes are not dose-dependent and not clinically significant. A minor increase in the QTcF interval can be seen after bolus injection of ABP-700 (all dosing groups) without dose

Table 4. Evaluation of Tolerability of ABP-700 Given to Healthy Volunteers

	Placebo	0.03 mg/kg	0.10 mg/kg	0.175 mg/kg	0.25 mg/kg	0.25 mg/kg + fen	0.35 mg/kg	0.35 mg/kg + fen	0.50 mg/kg	0.75 mg/kg	1.00 mg/kg	Total
Involuntary muscle movements												
No movements	10	5	5	1	0	1	0	1	0	0	1	24
Few movements	0	0	0	3	5	3	2	4	0	0	0	17
Extensive movement	0	0	0	1	0	1	3	0	5	5	4	19
Ventilation												
Respiratory depression	0	0	0	1	1	1	0	3	1	2	4	13
Tachypnea	0	0	0	1	0	1	0	1	2	5	3	13
Desaturation	0	0	0	0	1	0	0	2	0	1	3	7
Vital signs												
Hypertension	0	0	0	2	0	0	2	0	0	1	3	8
Hypotension	0	0	0	0	0	0	0	0	0	0	0	0
Tachycardia	0	0	0	2	0	0	2	1	1	3	4	13
Bradycardia	0	0	0	0	0	0	0	0	0	0	0	0
Catheter site–related reactions	1	1	0	1	1	0	0	1	0	1	1	7
Nausea/vomiting	0	0	0	0	0	0	0	1	0	1	1	3

Values are number of volunteers. ABP-700 = cyclopropyl-methoxycarbonylmetomidate; fen = fentanyl.

dependency or clinical significance. ACTH stimulation evoked an adrenal cortisol response in all subjects treated with ABP-700. Plasma cortisol concentrations increased by at least 200 nM/l at 60 or 120 min post-ACTH stimulation, indicating no adrenal suppression (fig. 2). There was no difference observed between placebo and the ABP-700 dose levels, other than differences that would be considered with the normal variability of the test.

ACTH testing was not performed for the cohorts that received fentanyl as a pretreatment, because these cohorts were repeats of previous dose levels. Cortisol levels of cohort 10, which received 0.175 mg/kg ABP-700, were not analyzed because an ACTH-stimulation test in previous cohorts that received a higher dose of ABP-700 (0.25, 0.35, 0.50, 0.75, and 1.00 mg/kg) did not reveal any adrenocortical suppression.

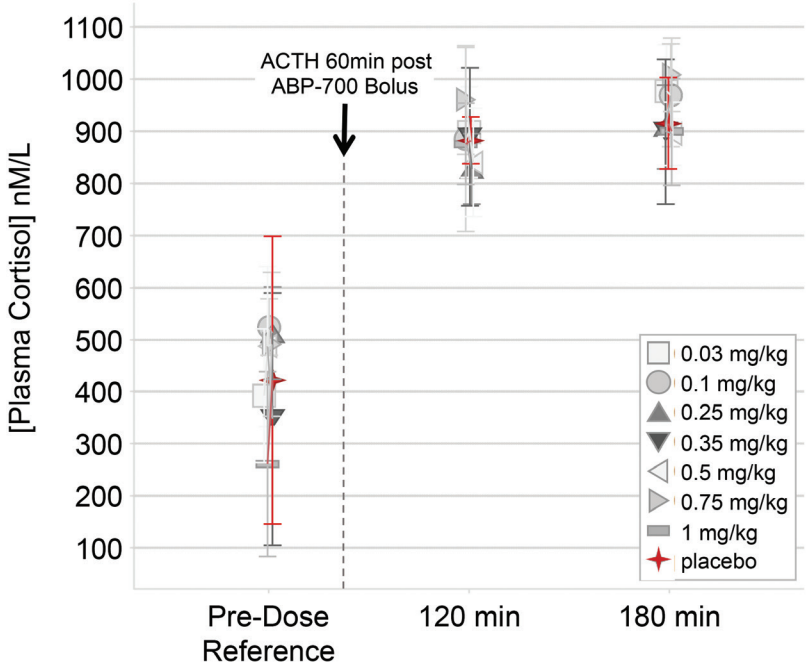

Fig. 2. Human adrenocortical response to adrenocorticotropic hormone (ACTH) stimulation after bolus administration of cyclopropyl-methoxycarbonylmetomidate (ABP-700) (0.03 to 1.0 mg/kg) or placebo. Adrenocortical response before and after administration of 250 μg synthetic ACTH 1 h after bolus ABP-700. Predose cortisol reference levels were obtained before 9 AM on the day of ABP-700 administration. Values are mean cortisol levels (± SD) for ABP-700 and placebo groups. n = 5 by ABP-700 dose and n = 7 for placebo.

Noncompartmental Pharmacokinetics

For each cohort, the time courses of the arterial and venous concentrations of ABP-700 and of the arterial of CPM acid are displayed in figure 3. The most important noncompartmental pharmacokinetic parameter values for ABP-700 (both arterial and venous) and its metabolite CPM acid are shown as tables in Supplemental Digital Content 1 (http://links.lww.com/ALN/B437). Overall, pharmacokinetics were dose-proportional. Clear differences between arterial and venous concentrations and pharmacokinetic parameters were observed.

Compartmental Pharmacokinetics

No significant covariate relationships with age, weight, height, or sex were found. The weight linear scaled model achieved an Akaike information criterion of –2,700.61 with a median absolute percentage error of 1.98 (24.5)%. The summarized equations of the final pharmacokinetic model are as follows:

$$SIZE = WGT / 70kg$$
$$V1(L) = V1_{ref} \cdot SIZE \cdot \exp(\eta 1)$$
$$V2(L) = V2_{ref} \cdot SIZE \cdot \exp(\eta 2)$$
$$V3(L) = V3_{ref} \cdot SIZE \cdot \exp(\eta 3)$$
$$CL(L \cdot \min^{-1}) = CL_{ref} \cdot SIZE \cdot \exp(\eta 4)$$
$$Q2(L \cdot \min^{-1}) = Q2_{ref} \cdot SIZE \cdot \exp(\eta 5)$$
$$Q3(L \cdot \min^{-1}) = Q3_{ref} \cdot SIZE \cdot \exp(\eta 6)$$

Symbols $V1_{ref}$, $V2_{ref}$, $V3_{ref}$, CL_{ref}, $Q2_{ref}$, and $Q3_{ref}$ are the estimated compartmental volumes and clearances for a 70-kg individual, and symbols $\eta 1$ to $\eta 6$ represent random variances. Estimated parameters are shown in table 5. The population and individual predictions *versus* time and observed

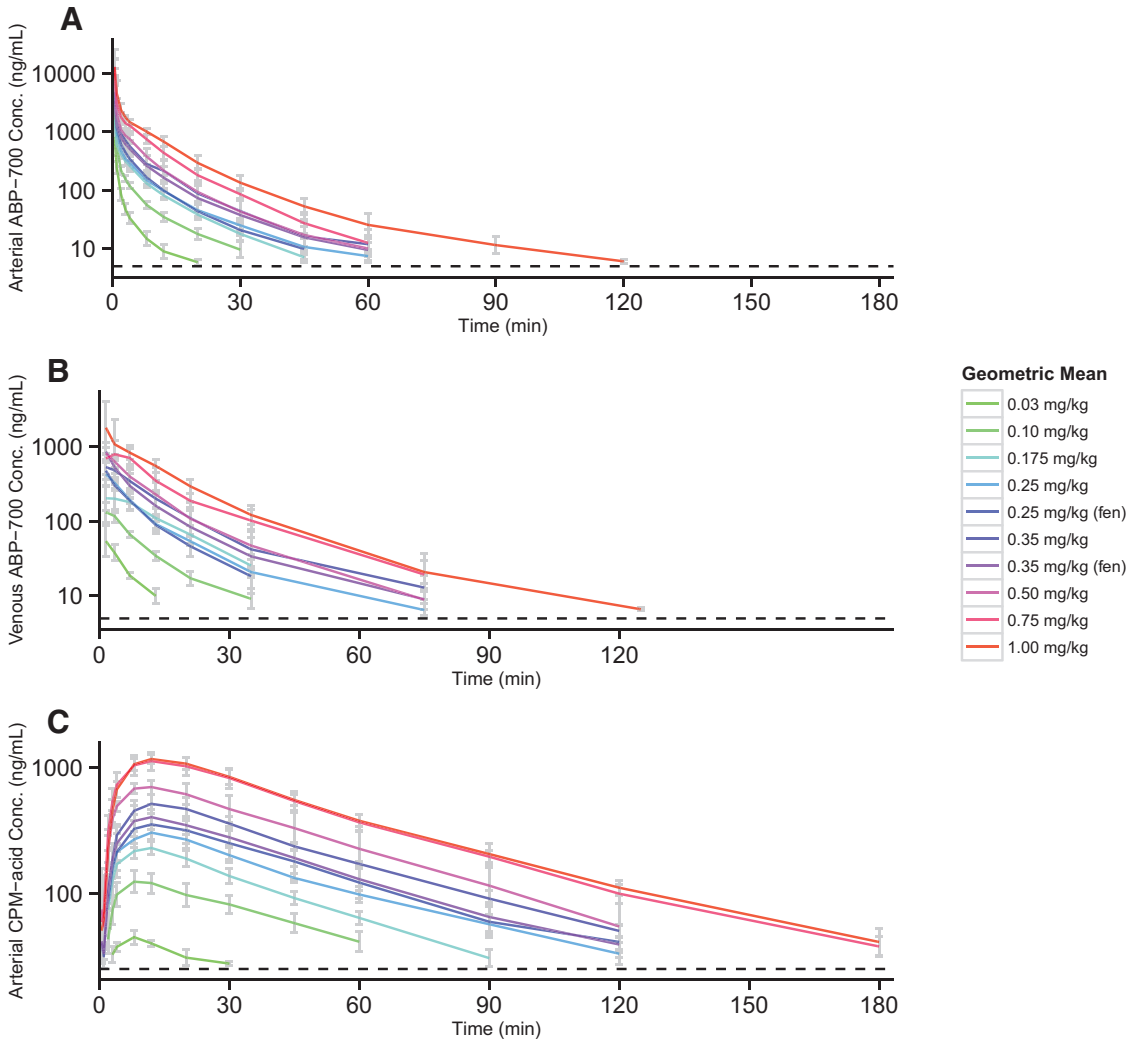

Fig. 3. Pharmacokinetics of cyclopropyl-methoxycarbonylmetomidate (ABP-700) and its primary metabolite cyclopropyl-methoxycarbonyl acid (CPM acid). (*A*) Arterial plasma concentrations of ABP-700. (*B*) Venous plasma concentrations of ABP-700. (*C*) Arterial plasma concentrations of the ABP-700 metabolite, CPM acid. Data are presented as geometrical means ± 95% CI per cohort. The bolus of ABP-700 was administered at time 0 min.

Table 5. Estimated Model Parameters and Population Variances in the Final Pharmacokinetic Model

Parameters	Estimated Value	95% Confidence Limits	
		Lower	Upper
$V1_{ref}$, l	0.527	0.427	0.649
$V2_{ref}$, l	3.02	2.47	3.71
$V3_{ref}$, l	5.18	4.59	5.85
CL_{ref}, l/min	1.66	1.57	1.77
$Q2_{ref}$, l/min	1.14	0.951	1.41
$Q3_{ref}$, l/min	0.336	0.297	0.382
	Variance, ω^2	CV, %	
$\eta 1$	0.323	61.8	
$\eta 2$	0.352	64.9	
$\eta 3$	0.106	33.5	
$\eta 4$	0.0178	13.4	
$\eta 5$	0.249	53.2	
$\eta 6$	0.0804	28.9	
	Residual error (SD)		
Arterial observations	0.103		

$V1_{ref}$, $V2_{ref}$, $V3_{ref}$, CL_{ref}, $Q2_{ref}$, and $Q3_{ref}$ are the estimated compartmental volumes and clearances for a 70-kg individual, and symbols $\eta 1$ to $\eta 6$ represent random variances.

CV = coefficient of variation.

ABP-700 arterial concentrations and the likelihood profiles are documented as figures in Supplemental Digital Content 4 (http://links.lww.com/ALN/B440) and 5 (http://links.lww.com/ALN/B441), respectively.

Clinical Effects and Pharmacodynamics

For each cohort, the time course of the BIS and frontal electromyographic activity are plotted in figure 4. At the lowest dose of ABP-700 administered (0.03 mg/kg), there was no observed effect on BIS. Subjects receiving dose levels of 0.1 mg/kg ABP-700 or greater showed a dose-dependent response to the treatment, as indicated by the rapidly decreasing BIS values (within 1 to 2 min). Duration of cerebral drug effect was longer in higher dosing cohorts. Due to increased electromyographic activity,[14] as shown in figure 4, the decrease in BIS in the 0.75 mg/kg cohort paradoxically was delayed. For BIS model development, Ce_{50} and $ke_{0,BIS}$ were assumed and log-normally distributed across the population. Estimating baseline BIS resulted in numerical instability, and its value was fixed to 90. The summarized equations of the final models are as follows:

$$Ce_{50} = Ce_{50,TYP} \cdot \exp(\eta 1)$$
$$ke_{0,BIS} = ke_{0,BIS,TYP} \cdot \exp(\eta 2)$$

Symbols Ce_{50} and $ke_{0,\mathrm{BIS}}$ represent estimated model parameters in the individual; $Ce_{50,\ TYP}$ and $ke_{0,BIS,TYP}$ represent the typical population model parameters; and $\eta 1$ and $\eta 2$ represent population variances. Estimated parameters are shown in table 6. The population and individual predictions *versus* time and BIS and the likelihood profiles for the model parameters are shown as figures in Supplemental Digital Content 6 (http://links.lww.com/ALN/B442) and 7 (http://links.lww.com/ALN/B443).

Figure 5 shows the individual MOAA/S scores. In the placebo and 0.03-mg/kg dose groups, no signs of clinical effect were observed in any subjects. Onset of clinical effect was observed starting with the 0.10 mg/kg dose group. In the 0.175-mg/kg dose group, four subjects (80%) reached clinical effect, and two subjects (40%) reached deep sedation/anesthesia. In the cohorts with a dose of 0.25 mg/kg and higher, both clinical effect and deep sedation/anesthesia was reached in 100% of the subjects. The duration of both the clinical effect and sedation increased with escalating dose. Neither the time to onset of clinical effect nor the time to onset of deep sedation/anesthesia was dose-related.

For MOAA/S PD model development, we assumed log-normally distributed population variability in drug effect.

$$DEFF = DEFF_{TYP} \cdot \exp(\eta 1)$$
$$ke_{0,MOAA/S} = ke_{0,MOAA/S,TYP}$$

Symbols $DEFF$ and $ke_{0,MOAA/S}$ represent estimated model parameters in the individual, $DEFF_{TYP}$ and $ke_{0,MOAA/S,TYP}$ represent the typical population model parameters, and $\eta 1$ represents population variance. For the final model, the estimated parameters are shown in table 7. The population and individual predictions *versus* time and MOAA/S and the likelihood profiles for the model parameters are shown as figures in Supplemental Digital Content 8 (http://links.lww.com/ALN/B444) and 9 (http://links.lww.com/ALN/B445).

Influence of Fentanyl

After completion of the preplanned cohorts and evaluation for tolerability and potential clinical utility, the 0.25- and 0.35-mg/kg doses of ABP-700 were considered as the two most promising doses and were repeated in the presence of fentanyl pretreatment (1 μg/kg). The administration of fentanyl resulted in decrease in the incidence and extent of IMM and also in less tachycardia when compared with the ABP-700 dose without fentanyl (table 4). Fentanyl pretreatment did not alter the time to onset of clinical effect or onset of sedation, nor did it alter the duration of sedation or clinical effect (data not shown). As shown in figure 4, a more pronounced clinical effect of ABP-700 as measured by BIS was observed with fentanyl pretreatment than without, although this did not result in a significant covariate in the pharmacodynamic model parameters. Fentanyl did result in somewhat higher frequency of respiratory depression and desaturation events. The administration of fentanyl did not result in a substantial alteration of the pharmacokinetics of either ABP-700 or its metabolite CPM acid, as shown by both noncompartmental and compartmental modeling (analysis not shown).

Discussion

Cyclopropyl-methoxycarbonylmetomidate, or ABP-700, is a short-acting, soft analog of etomidate and was developed to avoid the adrenocortical suppression while preserving

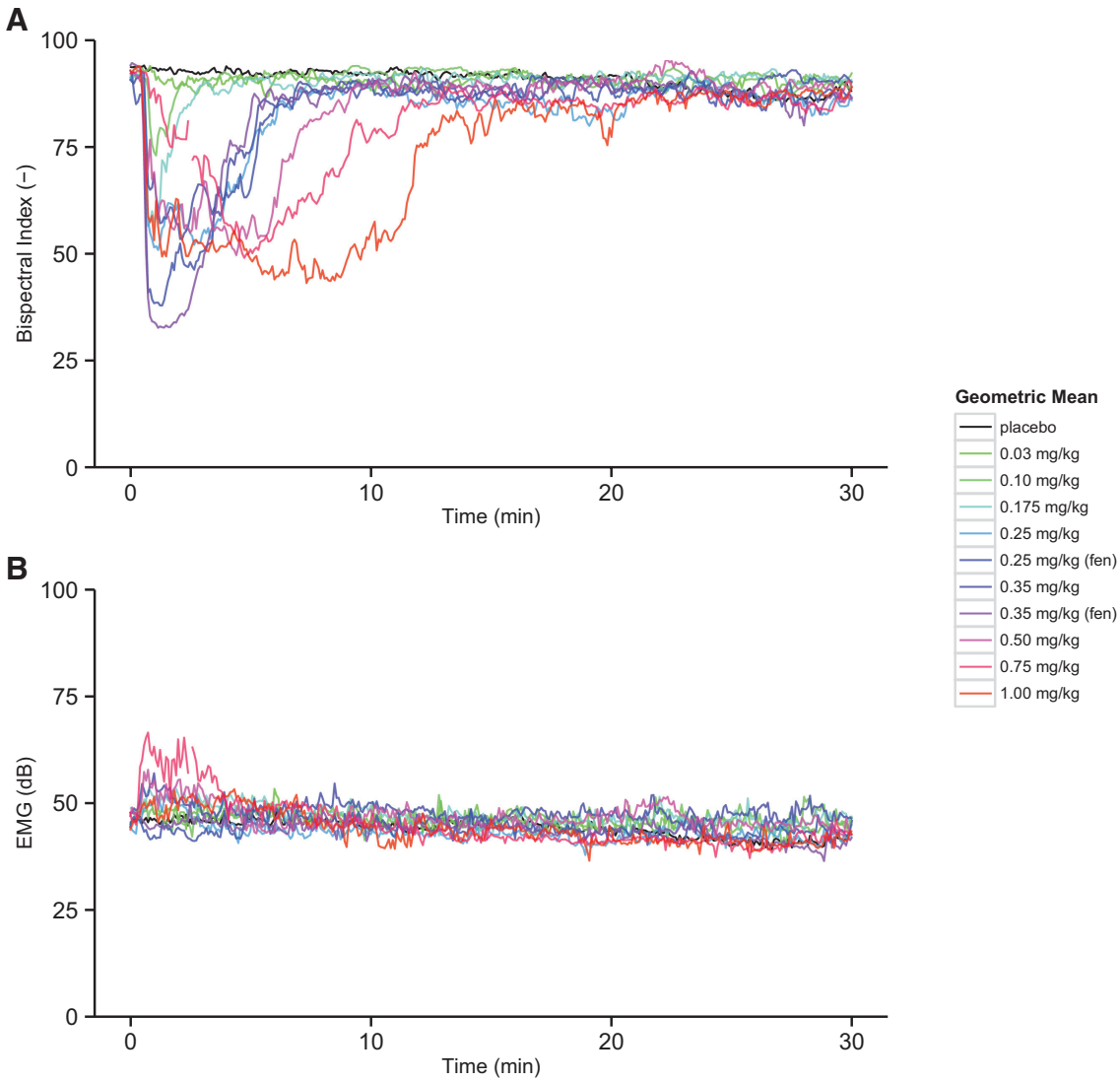

Fig. 4. Hypnotic effect of cyclopropyl-methoxycarbonylmetomidate. (*A*) Depth of sedation and anesthesia, as measured by the Bispectral Index and (*B*) frontal electromyographic activity (EMG). Fen = fentanyl.

the beneficial dose–response profile of etomidate.[5] In this first-in-human study, we found that ABP-700 was safe and well-tolerated after single-bolus injections of up to a maximum of 1.0 mg/kg. The two most promising dosages for potential clinical utility as a bolus induction agent were thought to be 0.25 and 0.35 mg/kg.

As expected, based on the preclinical studies,[2,7,15] ABP-700 demonstrated hypnotic–anesthetic properties. Clinical effect was first seen after a bolus dose of 0.175 mg/kg of ABP-700 (in four of five volunteers). At doses of 0.25 mg/kg and greater, a decrease in MOAA/S score to 0 was observed in all subjects. The time to onset of deep sedation/anesthesia was found to be dose-independent and extremely short, around 30 s after bolus injection, which is somewhat shorter than etomidate and thiopental[16] and significantly shorter than propofol.[17] Duration of deep sedation/anesthesia was dose-dependent. Offset of deep sedation/anesthesia was characterized by an abrupt and rapid return to a MOAA/S score of 5 in most of the volunteers. There were very few recorded instances of MOAA/S 2 or 3 during either induction or emergence from deep sedation/anesthesia. BIS profiles showed a similar time course of drug effect to MOAA/S scores, characterized by a rapid, dose-independent decline, with lowest value between 45 and 55, except for the cohort that received a bolus of 0.75 mg/kg ABP-700, due to possible electromyographic artifacts (fig. 4).[14]

Pharmacodynamic modeling for both MOAA/S and BIS illustrate the steep relation between the ABP-700 effect-site concentration and the cerebral drug effect. Although possibly influenced by the intermittent nature of MOAA/S scores in this study, figure 6 clearly shows the small range in effect-site concentrations between the probabilities for different MOAA/S scores. It is remarkable that the difference in ABP-700 effect-site concentration between 50% probability of MOAA/S score 5 and 0 is only around 500 ng/ml. The steepness also is evident in the large γ found in the BIS sigmoid pharmacodynamic model. The effect-site

Table 6. Estimated Model Parameters and Population Variances in the Final BIS Pharmacodynamic Model

Parameters	Estimated Value	95% Confidence Limits	
		Lower	Upper
E_0	90	—	—
E_{max}	0	—	—
$Ce_{50,\ TYP}$, ng/ml	1,200	1,060	1,350
γ	7.24	6.83	7.66
$ke_{0,\ BIS,TYP}$, l/min	0.156	0.132	0.179
	Variance, ω^2	CV, %	
$\eta 1$	0.139	38.6	
$\eta 2$	0.742	105	
	Residual error (SD)		
BIS observations	6.35		

E_0 is the baseline pharmacodynamic measure when no drug is present; E_{max} is the maximum possible drug effect; $Ce_{50,\ TYP}$ is the typical population model parameter for the effect-site concentration of ABP-700 associated with 50% of the maximum effect; γ is the steepness of the concentration versus response relation; $ke_{0,\ BIS,TYP}$ is the typical population model parameter for the first-order rate constant between plasma and effect-site compartment; and $\eta 1$ and $\eta 2$ represent population variances.

BIS = Bispectral Index; CV = coefficient of variation; *TYP* = population typical value.

concentration reaching 50% of drug effect as measured by BIS is found around 1,200 ng/ml and validates the effect-site concentration range found when using MOAA/S score to quantify cerebral drug effect. A small hysteresis effect between plasma and effect site is observed and is characterized by a fast $ke_{0,\ MOAA/S}$ of 0.488/min. A smaller $ke_{0,\ BIS}$ was found; however, this value might be biased because of the delay in the BIS monitor during very fast changes in cerebral drug effect.[18]

Clinical observations related to IMM, ventilation, and vital signs were dose-dependent and consistent with the mechanism of action of ABP-700 and its structurally related analog, etomidate.[5,16] From a dosage of 0.175 mg/kg of ABP-700 upwards, IMM were observed in most of the volunteers. These IMMs varied from few muscle movements of a more twitching character to more extensive muscle movements that appeared to be of an intermittent myoclonic nature. In various volunteers, these muscle twitches and myoclonic movements were considered mild and not always considered as an AE by the attending anesthesiologist. In three cases of severe IMM in the highest two dose cohorts, midazolam was administered, which successfully attenuated these movements, consistent with what was seen during preclinical studies.[1] The occurrence of IMM is a common observation among anesthetic agents, such as etomidate,[19] propofol,[20] and the novel anesthetic agent AZD-3043.[21] The etiology of these IMMs is not entirely clear. They are not thought to be associated with electroencephalographically measured epileptiform discharges[16,22] and have been hypothesized to be a subcortically mediated or triggered process.[11]

ABP-700 did not cause cardiovascular depression, as seen in figure 1 and table 4. However, increases in heart rate and blood pressure were observed in the higher dosing groups and followed a similar time course as the clinical effect. Increases in heart rate with stable blood pressure have been reported previously for etomidate[23] and also with the inhaled agent desflurane.[24] Some short-lasting episodes of increased blood pressures (mean arterial blood pressure greater than 110 mmHg) were found, but without any requirement for treatment.

ABP-700 has a remarkably stable respiratory profile. No centrally induced respiratory depression was recorded. Decreases in amplitude and frequency of respiration rate were induced mostly by the IMM, resulting in a brief and self-limiting upper airway obstruction. Per protocol, brief chin lift was applied in four volunteers after 20 s of airway obstruction to maintain a patent airway. Two of these volunteers had received 0.75 mg/kg ABP-700, and two had received 1.00 mg/kg. Tachypnea was observed in parallel with the hypnotic-anesthetic effect in the two highest dosing groups, receiving 0.75 and 1.00 mg/kg AB-700 boluses. Self-limiting changes in Spo_2 were only recorded occasionally. The notion that deep sedation/anesthesia is possible without any appreciable respiratory depression is obviously of potential clinical importance. Etomidate is associated with less respiratory depression than propofol but at deep sedation/anesthesia doses, both decrease medullary respiratory drive and right-shift the hypercarbic ventilator response.[25,26] Although the stable respiratory rate and arterial carbon dioxide levels seen with ABP-700 are suggestive of preservation of respiratory drive, this MTD dose study was not intended to thoroughly evaluate ABP-700 effects on respiratory function.

Consistent with preclinical findings,[1,15] ABP-700 did not suppress the physiologic response of the adrenal axis to ACTH stimulation in human volunteers. A significant increase of at least 200 nM at 60 or 120 min poststimulation occurred and was not different from subjects who received a placebo. Although not directly compared in this study, adrenal responsiveness after single-bolus exposure to ABP-700 is superior to the substantial adrenal suppression caused by bolus dosing of etomidate.[6]

Noncompartmental pharmacokinetics were studied for both ABP-700 (arterial and venous) and its metabolite CPM acid (arterial). The results showed rapid elimination of ABP-700 across all dose regimens. The mean clearance values observed in this study were relatively high compared with hepatic blood flow. Clearance and volume of distribution estimated for the venous blood samples were all higher than those estimated for the arterial blood samples, reflecting the rapid metabolism of ABP-700 by esterases during the arterial to venous transport. ABP-700 plasma exposure increased dose proportionally as the dose increased from 0.03 to 1.00 mg/kg for both arterial and venous blood samples. Dose-proportional arterial plasma exposures of CPM acid also were observed.

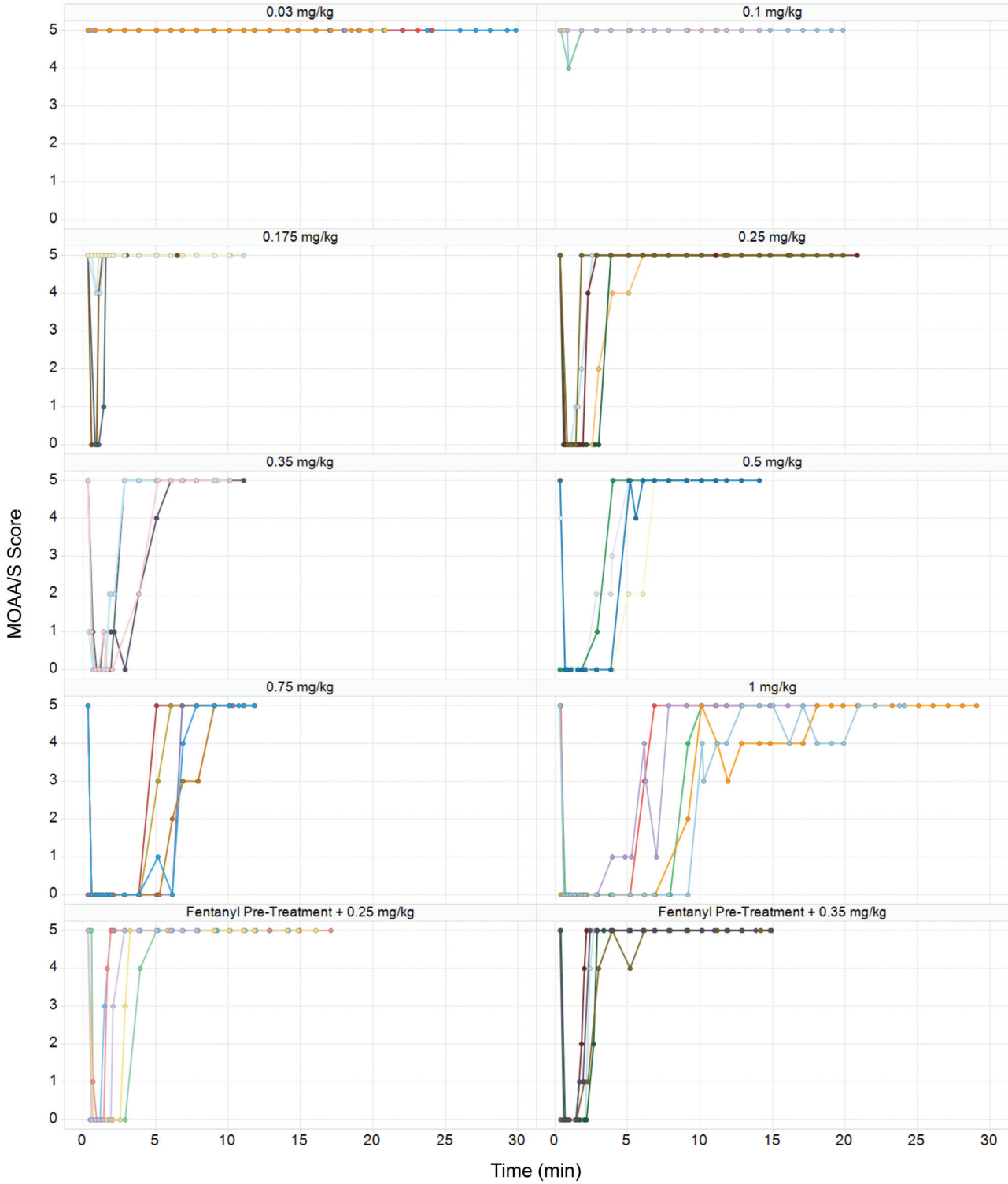

Fig. 5. Modified Observers Assessment of Alertness and Sedation (MOAA/S) scores by subject. The data are displayed by individual subject who received cyclopropyl-methoxycarbonylmetomidate (n = 50) and per cohort (10 cohorts in total). Data for placebo were omitted because no subject was scored less than 5.

A three-compartment pharmacokinetic model was developed to describe the time course of arterial drug concentration. The final model accurately predicts the arterial concentrations of ABP-700 after a single-bolus injection in the central compartment. Venous samples were not used for model estimation. For soft drugs, it is well known that venous samples are potentially very misleading in describing the clinical behavior of a drug through modeling.[27] The

Table 7. Estimated Model Parameters and Population Variances in the Final MOAA/S Pharmacodynamic Model

Parameters	Estimated Value	95% Confidence Limits	
		Lower	Upper
$DEFF_{TYP}$	5.25	4.41	6.23
$ke_{0\ MOAA/S,TYP}$	0.488	0.443	0.537
b_0	−10.1	−11.7	−8.77
b_1	0.713	0.459	1.02
b_2	0.518	0.307	0.817
b_3	0.407	0.222	0.670
b_4	1.02	0.735	1.36
	Variance, ω^2	CV, %	
η_1	0.0472	22.0	

b_0 is a fixed-effect parameter representing the logit of the probability for score 0; b_1 through b_4 represent the difference in logits between the scores; DEFF is also a fixed-effect parameter for the model; $ke_{0,\ MOAA/S,\ TYP}$ is the typical population model parameter for the first-order rate constant between plasma and effect-site compartment; and $\eta 1$ represents the population variance.

CV = coefficient of variation; *MOAA/S* = Modified Observers Assessment of Alertness and Sedation; $DEFF_{TYP}$ = typical population parameter for a fixed-effect parameter for the model.

values for volumes of distribution and clearance echoes the noncompartmental kinetics, although one should be aware that a compartmental model based on single-bolus data only is very biased and often characterized by a misspecified central compartmental volume.[28] A more realistic model has to be developed, adding data from continuous-infusion investigations, hereby also exploring other than linear models, more accurate size descriptors than weight, and more covariates.

The use of fentanyl as a premedication to dosages of 0.25 mg/kg ABP-700 and 0.35 mg/kg ABP-700 did not alter the onset, duration, and recovery profile of ABP-700 as measured by MOAA/S. Nevertheless, a more pronounced decrease in BIS was seen. Less extensive IMMs were observed with the addition of fentanyl. It is well known IMM due to etomidate can be attenuated by pretreatment with low doses

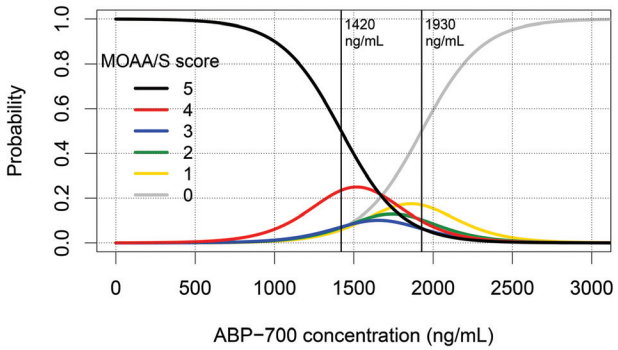

Fig. 6. Population predictions of Modified Observers Assessment of Alertness and Sedation (MOAA/S) scores *versus* cyclopropyl-methoxycarbonylmetomidate (ABP-700) effect-site concentrations. The *vertical lines* to the *x-axis* denote the effect-site concentration at a 50% probability for having an MOAA/S score of 5 and 0, respectively.

of commonly used drugs during procedural care, such as opioids and benzodiazepines.[11,12] Although speculative, given the chemical derivation of ABP-700 as an etomidate analog, the origin of IMM seen with ABP-700 may be mechanistically similar to that seen with etomidate.

Consistent with known effects, some respiratory and hemodynamic changes were associated with fentanyl administration before ABP-700. This study was not designed to assess these changes, so it is not possible to comment on their clinical significance. Because ABP-700 is not expected to have any analgesic properties, its use in procedural sedation or anesthesia will require concomitant dosing of opioids.

In general, there were no substantial differences in both noncompartmental and compartmental pharmacokinetic parameters for ABP-700 and CPM acid after a bolus dose of 0.25 and 0.35 mg/kg of ABP-700 without and with 1 μg/kg fentanyl pretreatment, respectively. However, these comparisons on pharmacokinetics of ABP-700 or CPM acid between the subjects that have been administered ABP-700 alone or coadministered with fentanyl might be biased and were performed for exploratory purposes. The influence of fentanyl on the pharmacokinetics of ABP-700 and CPM acid should be studied in greater detail within a properly designed drug–drug interaction study.

As always, studies of this type have limitations. One is that the clinical hypnotic–anesthetic drug effect of ABP-700 was assessed by the MOAA/S scale. A problem with using the MOAA/S scale is that the volunteer is stimulated, which might result in an arousal poststimulus and biasing the hypnotic–anesthetic continuum. Furthermore, clinical observations are made by clinicians and are therefore prone to subjectivity. As there were three attending anesthesiologists involved in this study, some variability in MOAA/S assessments is possible.

Another limitation of this study is that BIS monitoring is not validated for monitoring the depth of anesthesia achieved with ABP-700. However, BIS is an established form of monitoring for other GABA-acting drugs,[29] and the BIS pattern follows the MOAA/S pattern closely. Therefore, it can be assumed that the BIS data are valid for ABP-700. In addition, as this is a safety and tolerability Phase 1 study, findings should not be extrapolated to any clinical practice guidelines yet.

We can conclude that ABP-700 was safe and well tolerated after single-bolus injections of up to a maximum tolerated bolus dose of 1.0 mg/kg. A bolus dose of 0.25 and 0.35 mg/kg was found to have a sufficiently favorable clinical effect *versus* side-effect profile to be explored in future studies. These dosages of ABP-700 showed dose responsive hypnotic–anesthetic characteristics and a safety and tolerability profile that warrants further investigation and development.

Acknowledgments

The authors acknowledge the support of Rob Spanjersberg, C.N., C.R.C., Clinical Research Coordinator, and Anne-Sophia

Koene, M.D., Research Fellow (Department of Anesthesiology, University Medical Center Groningen, The Netherlands); Khalid Abd-Elaziz, M.D., Research Physician, Wouter Dijkstra, Pharm.D., Clinical Trial Pharmacist, Gonda Renkema, B.Sc., Clinical Study Coordinator, and Mariska Beukers, M.Sc., Project Manager (QPS Netherlands, BV, Groningen, The Netherlands); David Grayzel, M.D. (CEO, Annovation Biopharma, Cambridge, now Partner, Atlas Venture, Cambridge, Massachusetts); Kevin Pojasek, Ph.D. (COO, Annovation Biopharma, Cambridge, Massachusetts, now CEO, Quartet Medicine, Cambridge, Massachusetts); Scott Chappel, Ph.D. (CSO, Annovation Biopharma, Cambridge, Massachusetts, now CSO, Surface Oncology, Cambridge, Massachusetts); Amy DiRico, B.S. (Clinical Project Director, Phase One, LLC, Norwich, Connecticut); and Chau Hwei Fu, Ph.D. (Global PK Head, QPS LLC, Newark, Delaware) during the trial and the preparation of the manuscript.

Research Support

This study was a sponsor-initiated study by The Medicines Company, Parsippany, New Jersey. The sponsor covered the costs related to this study.

Competing Interests

Dr. Struys: his research group/department received grants and funding from The Medicines Company (Parsippany, New Jersey), Masimo (Irvine, California), Fresenius (Bad Homburg, Germany), Acacia Design (Maastricht, The Netherlands), Medtronic (Dublin, Ireland), and honoraria from The Medicines Company, Masimo, Fresenius, Baxter (Deerfield, Illinois), Medtronic, and Demed Medical (Temse, Belgium). Dr. Absalom: his research group/department received grants and funding from The Medicines Company, Masimo, Fresenius, Acacia Design, Medtronic, and he has received honoraria from The Medicines Company and Janssen Pharmaceutica NV (Beerse, Belgium). Dr. Meyer attended one advisory board from The Medicines Company, for which his department received an honorarium. Dr. Meier received honoraria from Abbott Vascular (Hoofddorp, The Netherlands). He also attended one advisory board from The Medicines Company, for which his department received an honorarium. Dr. Daas is an employee of QPS Netherlands, BV, Groningen, The Netherlands. Dr. Chou is an employee of QPS LLC, Newark, Delaware. Dr. Campagna is an employee of The Medicines Company. Mr. Sweeney is an employee of The Medicines Company and former employee of Annovation Biopharma (Cambridge, Massachusetts). The other authors declare no competing interests.

Reproducible Science

Full protocol available at: m.m.r.f.struys@umcg.nl. Raw data available at: m.m.r.f.struys@umcg.nl.

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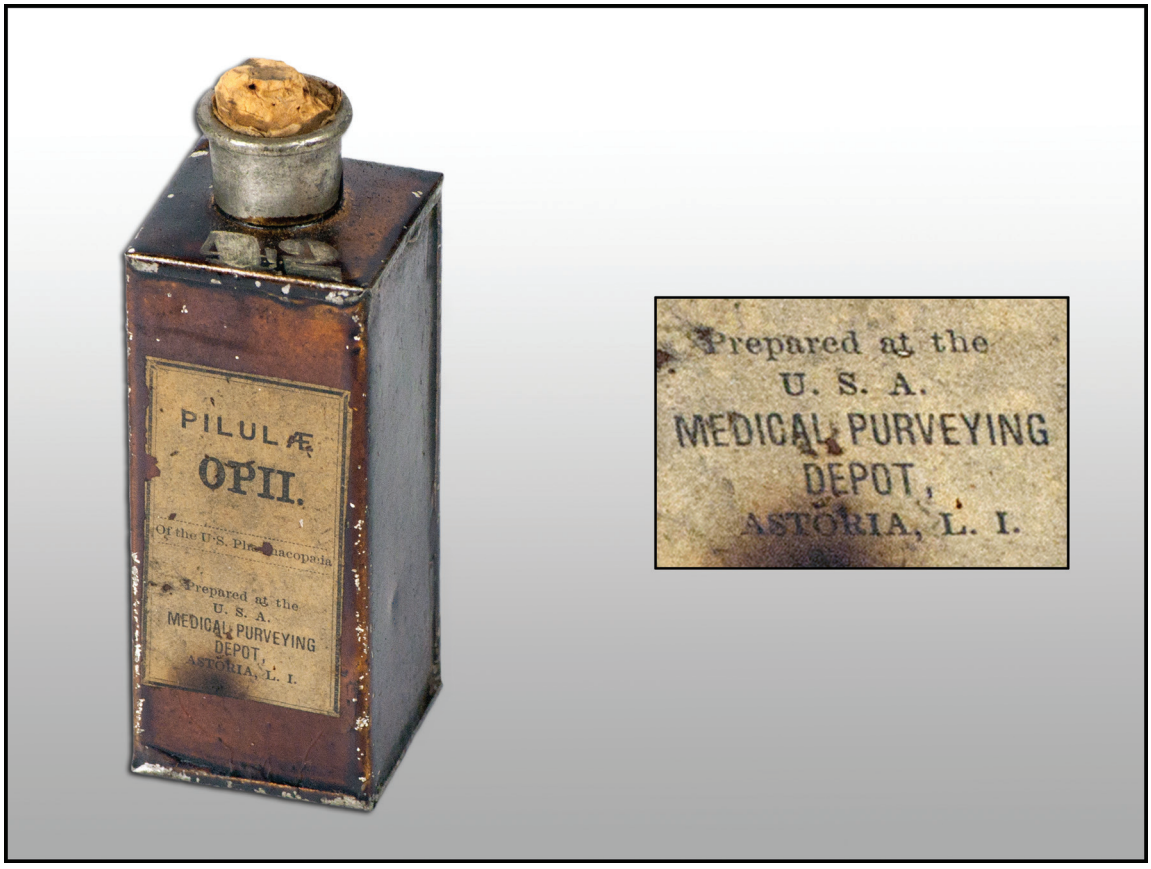
ANESTHESIOLOGY REFLECTIONS FROM THE WOOD LIBRARY-MUSEUM

Clean Analgesia? A Civil War Tin for Pills of Opium…and Soap



For small-scale production of opium pills during America's Civil War, a drachm (3.89 g) of the powdered drug was mixed with 12 grains (0.72 g) of hard dry soap and molded with a dash of water into a cylindrical mass for division into 60 pills. However, the Union Army required a much more industrial scale of pill rolling. At its Medical Purveying Depot in Astoria, Long Island, New York, the Union Army employed 12 women to roll out an average totaling 60,000 opium pills daily. Men working the nearby printing press generated paper labels (*right*) for the japanned tins (*left*) that were corked after being filled with *Pilulae Opii* (Latin: little balls or pills of opium). The soap was considered an "excipient" or inert filler for the analgesic opium. Remarkably, soap was so routinely compounded with opium that, to conceal from patients that they were receiving opium, a physician could simply prescribe opium as *Pilulae Saponis Compositae* or "Compound Pills of Soap." (Copyright © the American Society of Anesthesiologists' Wood Library-Museum of Anesthesiology.)

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Goal-directed Fluid Therapy Does Not Reduce Primary Postoperative Ileus after Elective Laparoscopic Colorectal Surgery

A Randomized Controlled Trial

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ABSTRACT

Background: Inadequate perioperative fluid therapy impairs gastrointestinal function. Studies primarily evaluating the impact of goal-directed fluid therapy on primary postoperative ileus are missing. The objective of this study was to determine whether goal-directed fluid therapy reduces the incidence of primary postoperative ileus after laparoscopic colorectal surgery within an Enhanced Recovery After Surgery program.

Methods: Randomized patient and assessor-blind controlled trial conducted in adult patients undergoing laparoscopic colorectal surgery within an Enhanced Recovery After Surgery program. Patients were assigned randomly to receive intraoperative goal-directed fluid therapy (goal-directed fluid therapy group) or fluid therapy based on traditional principles (control group). Primary postoperative ileus was the primary outcome.

Results: One hundred twenty-eight patients were included and analyzed (goal-directed fluid therapy group: n = 64; control group: n = 64). The incidence of primary postoperative ileus was 22% in the goal-directed fluid therapy and 22% in the control group (relative risk, 1; 95% CI, 0.5 to 1.9; P = 1.00). Intraoperatively, patients in the goal-directed fluid therapy group received less intravenous fluids (mainly less crystalloids) but a greater volume of colloids. The increase of stroke volume and cardiac output was more pronounced and sustained in the goal-directed fluid therapy group. Length of hospital stay, 30-day postoperative morbidity, and mortality were not different.

Conclusions: Intraoperative goal-directed fluid therapy compared with fluid therapy based on traditional principles does not reduce primary postoperative ileus in patients undergoing laparoscopic colorectal surgery in the context of an Enhanced Recovery After Surgery program. Its previously demonstrated benefits might have been offset by advancements in perioperative care. **(Anesthesiology 2017; 127:36-49)**

POSTOPERATIVE gastrointestinal dysfunction that occurs in absence of surgical complications, frequently defined as primary postoperative ileus, is one of the major determinants of in-hospital recovery after colorectal surgery.[1,2] Despite advancements in surgical and perioperative care, primary postoperative ileus remains an unpleasant complication that not only delays early enteral feeding and increases caregivers' workload but also increases morbidity,[3] prolongs hospitalization,[4] and increases medical costs.[5,6]

Experimental and clinical trials have shown that both fluid excess[7–15] and hypovolemia[16] can significantly affect the recovery of bowel function and impair anastomotic healing.[11,17,18] Early studies have shown that individualization of fluid therapy based on more objective measures of

What We Already Know about This Topic

- Individualization of intraoperative fluid administration (goal-directed fluid therapy) has been shown to be of benefit in many studies
- The majority of these studies were uncontrolled, and confounding factors might have affected the results

What This Article Tells Us That Is New

- This randomized blinded trial assessed effects of goal-directed fluid therapy on primary postoperative ileus after laparoscopic colorectal surgery, within a well-established Enhanced Recovery After Surgery program
- The incidence of primary postoperative ileus was identical (22%) in the goal-directed fluid therapy control groups
- Previous benefits of goal-directed fluid therapy may have been offset by subsequent improvements in perioperative and surgical care

This article is featured in "This Month in Anesthesiology," page 1A. Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site (www.anesthesiology.org). This article has an audio podcast.

Submitted for publication July 23, 2016. Accepted for publication April 3, 2017. From the Department of Anesthesia (J.C.G.-I., A.T., D.M., F.C., G.B.), Department of Surgery (B.L.S., A.S.L., P.C., N.P., L.S.F.), and Steinberg–Bernstein Centre for Minimally Invasive Surgery and Innovation, Department of Surgery (N.P., L.S.F.), McGill University Health Centre, Montreal, Quebec, Canada.

 Anesthesiology 2017; 127:36-49

hypovolemia (goal-directed fluid therapy) accelerates the recovery of bowel function[19,20] and reduces hospitalization[21,22] and overall complications,[22] especially in high-risk patients.[23,24] However, the majority of these studies were conducted in an uncontrolled clinical setting, where several perioperative confounding factors might have affected postoperative outcomes. In fact, more recent evidence[25–29] has not confirmed these results in patients treated with Enhanced Recovery After Surgery programs, especially when the laparoscopic approach is used. In these patients, the implementation of several integrated evidence-based perioperative interventions, each shown to improve clinical outcomes after colorectal surgery, may provide similar benefits as those observed with goal-directed fluid therapy.[25–29] It also must be considered that the number and type of interventions included in the Enhanced Recovery After Surgery programs vary between different centers, making it difficult to determine and generalize the impact of a single intervention on postoperative outcomes.

In light of this controversial evidence, the impact of goal-directed fluid therapy on specific postoperative complications and in a context of an Enhanced Recovery After Surgery program remains unknown. Specifically, there is a lack of high-quality studies primarily investigating the effect of goal-directed fluid therapy on the recovery of bowel function[28] in a controlled clinical setting in which perioperative interventions influencing bowel function are standardized.

The aim of this study was to determine the impact of goal-directed fluid therapy on the incidence of primary postoperative ileus in patients undergoing laparoscopic colorectal surgery and treated with a well-established, center-specific Enhanced Recovery After Surgery program. It was hypothesized that patients treated with goal-directed fluid therapy would experience less primary postoperative ileus than patients receiving fluid therapy based on traditional principles.

Materials and Methods

Trial Design and Study Subjects

This randomized (1:1) parallel-group patient and assessor-blinded trial was approved by the Research Ethics Board of the McGill University Health Centre, Montreal, Quebec, Canada (study No. 12-177-SDR), and the study procedures were carried out in accordance with ethical standards (ClinicalTrials.gov registration: NCT01818375). Patients were recruited between January 2013 and August 2015 at the Montreal General Hospital, a university-affiliated tertiary center. Consecutive patients scheduled for elective laparoscopic colorectal resection were approached by the research investigators (A.T., D.M., J.C.G.-I.) at the preoperative clinic, and written consent was obtained from eligible patients. Patients were excluded if they were younger than 18 yr old, required emergency surgery, had undergone previous esophageal or gastric surgery, had esophageal varices or cancer, coarctation of the aorta, chronic atrial fibrillation, severe aortic stenosis, preoperative bowel obstruction, coagulopathies, contraindications to epidural analgesia, if they were chronically treated with opioids, and if they did not read or communicate in French or English.

The morning of surgery, eligible patients were assigned randomly by a stratified computer-based block randomization to receive goal-directed fluid therapy based on near-maximal stroke volume optimization (goal-directed fluid therapy group)[30] or fluid therapy based on traditional principles[31] (control group). These include the replacement of preoperative fasting deficit (4/2/1 rule), volume expansion after the induction of anesthesia, and the replacement of insensible blood loss and third-space loss (Supplemental Digital Content 1, http://links.lww.com/ALN/B446, which includes a table that describes the fluid management in the two groups). Randomization was stratified by the surgical indication of creating a stoma. Group allocation was concealed using sequentially numbered sealed brown envelopes, opened the morning of surgery by the research investigators (A.T. and D.M.).

Perioperative Care

Patients were treated according to a well-established Enhanced Recovery After Surgery program specific for patients undergoing elective colorectal surgery initially implemented at our institution in 2008[32] and subsequently modified (Supplemental Digital Content 2, http://links.lww.com/ALN/B447, which includes a table that describes the Montreal General Hospital Enhanced Recovery After Surgery program for colorectal surgery).

Anesthesia and Analgesia Management. On the day of surgery, patients were transferred to the preoperative anesthesia area, where preoperative weight was measured and an intravenous catheter was inserted. After the recording of baseline hemodynamic variables, lactated Ringer's 27 ml/kg[33] was infused before induction of anesthesia in patients of the control group who received mechanical bowel preparation (4 l polyethylene glycol electrolyte lavage; GoLytely®, Braintree Laboratories, USA). A thoracic epidural catheter was inserted between T8 and T12 and a test dose of 3 ml lidocaine 2% with epinephrine (5 μg/ml) was used to confirm the correct placement. Presence of sensory block was assessed before surgery with an ice test, and in presence of primary failure epidural catheters were replaced before induction of anesthesia. No subsequent epidural local anesthetics were administered intraoperatively to minimize the hemodynamic effects of epidural blockade. General anesthesia was induced with propofol (2 mg/kg) and remifentanil (1 μg/kg) and maintained with desflurane or sevoflurane in a mixture of 40% oxygen and 60% air. Intraoperatively, analgesia was provided with remifentanil infusion (0.05 to 0.25 $\mu g \cdot kg^{-1} \cdot min^{-1}$) titrated to keep heart rate and blood pressure within ±20% of the baseline values. Rocuronium was used to facilitate orotracheal intubation and maintain

adequate neuromuscular blockade during surgery (train-of-four count less than 2). Lungs were ventilated with a tidal volume of 8 ml/kg and with a positive end-expiratory pressure of 5 cm H_2O. End-tidal carbon dioxide was maintained between 35 and 40 mmHg by adjusting the respiratory rate. Postoperative nausea and vomiting prophylaxis was achieved with dexamethasone (8 mg) and droperidol (0.625 mg). At the end of surgery, remifentanil was discontinued, 10 ml lidocaine 2% was bolused in the epidural catheter, and ketorolac (30 mg) was administered if not contraindicated. Then, an epidural mixture of bupivacaine (0.1 mg/ml) and fentanyl (3 μg/ml) was started and infused for 48 h. Celebrex and acetaminophen also were prescribed for the entire hospitalization, unless contraindicated. Systemic opioids were administered after the epidural was discontinued or before if clinically required.

Intraoperative Hemodynamic Monitoring and Management. Electrocardiogram activity, invasive blood pressure, and oxygen saturation were measured in every patient. After induction of anesthesia, a disposable esophageal Doppler probe (DP12 Probe; Deltex Medical Ltd., United Kingdom) was inserted into the distal third of the esophagus in every patient. Optimal blood flow signal was identified from the descending aorta in the supine position and displayed on the esophageal Doppler monitor (CardioQ-ODM; Deltex Medical Ltd.) by the treating anesthesiologist in the goal-directed fluid therapy group and by two research investigators (A.T. and D.M.) in the control group. The machine was calibrated to provide data averaged more than 10 cycles.[34]

In the goal-directed fluid therapy group, the patient was positioned in steep Trendelenburg, and after 30 s from the change in position esophageal Doppler–derived hemodynamic variables and standard cardiovascular parameters were recorded. If stroke volume increased by more than 10%, the patient was repositioned flat, 200 ml of 6% hydroxyethyl starch 130/0.4 in 0.9% sodium chloride (Voluven®, Fresenius Kabi, Ltd., United Kingdom) was administered in 5 min, and a new stroke volume measurement obtained. This process was repeated until changing in position did not result in an increase of more than 10% in stroke volume. At this point, it was assumed that stroke volume had reached the plateau of the Frank-Starling curve (near-maximal stroke volume), and the patient was considered volume optimized. The final head-down cardiovascular measurement that did not result in an increase in stroke volume by more than 10% was recorded (Trendelenburg), the patient was repositioned flat, and surgery commenced. This method was described previously to minimize the cardiovascular effects of the pneumoperitoneum and of the changes in position during surgery.[35] After having established the pneumoperitoneum and positioned the patient in Trendelenburg, near-maximal stroke volume was maintained during surgery[30] (Supplemental Digital Content 3, http://links.lww.com/ALN/B448, which includes the goal-directed fluid therapy algorithm). A background maintenance infusion of lactated Ringer's 1.5 ml · kg^{-1} · h^{-1} was administered until the end of surgery.[36]

In the control group, the esophageal Doppler monitor was turned away from the anesthesia care provider, and the screen was covered with a surgical towel soon after the induction of anesthesia. The cardiovascular response obtained 30 s after positioning the patient in steep Trendelenburg and before starting the pneumoperitoneum was measured and recorded (Trendelenburg). Anesthesiologists were blinded to the measurements obtained with the esophageal Doppler for the entire duration of the study. Additional fluids were administered if clinically deemed based on the judgment of the anesthesiologist in charge.

In both groups, blood products were administered when clinically indicated and based on previously reported laboratory cutoffs.[19] Vasopressors and inotropes also were administered based on the clinical judgment of the treating anesthesiologist.

Surgical Technique. Surgery was performed by three experienced fellowship-trained colorectal surgeons (S.L., P.C., and B.L.S.) as previously described[37]

Postoperative Care. At the end of surgery, patients were transferred into the postanesthesia care unit, and an intravenous infusion of lactated Ringer's 1.5 ml · kg^{-1} · h^{-1} was started. After meeting the postanesthesia care unit discharge criteria, patients were discharged to the surgical unit and lactated Ringer's infusion was reduced to 15 ml/h (to keep the vein open) until 8:00 AM the following morning, when intravenous fluids were discontinued. Additional intravenous fluids were administered by the anesthesiologist in charge in the postanesthesia care unit or by the surgical team on the surgical unit as per usual care. The day of surgery patients were encouraged to drink clear fluids (1.5 l/day), and a solid diet as tolerated was started the morning after surgery. The acute pain service visited patients daily to optimize pain control. The surgical team and the acute pain service were blinded to patients' randomization. Patients were discharged if they were afebrile, they tolerated an oral diet, their pain was well controlled (Numeric Rating Score less than 4), and they ambulated independently.

Study Outcomes, Measurements, and Data Collection

The primary outcome was the incidence of primary postoperative ileus during the hospital stay. There is a lack of a standard and validated definition of primary postoperative ileus. Traditional criteria used to define primary postoperative ileus commonly include time-based endpoints such as the time required to pass gas and/or bowel movements or time to tolerate oral diet. These criteria poorly identify patients with significant postoperative gastrointestinal dysfunction in the context of an Enhanced Recovery After Surgery program, as after colorectal surgery patients are fed as tolerated in the immediate postoperative period, independently of the presence of such criteria. Based on these considerations and after having performed a literature review, in 2012 an interdisciplinary consensus was

achieved among anesthetists and colorectal surgeons working at the Montreal General Hospital on how to diagnose and manage postoperative ileus in the context of an Enhanced Recovery After Surgery program. It was found that primary postoperative ileus in the absence of postoperative complications was associated with a median increase of 2 days in length of hospital stay. Beginning on postoperative day 1, patients with primary postoperative ileus were identified by the presence of two or more clinical indicators of gastrointestinal dysfunction, at least one for each of the two following criteria: (1) presence of vomiting OR abdominal distension and (2) absence of flatus/stool OR not tolerating oral diet, in the absence of any precipitating complications. Secondary outcomes included Quality of Recovery score,[38] 30-day complications, readiness to be discharged, length of hospital stay, and readmission rates. Postoperative complications were defined *a priori* (Supplemental Digital Content 4, http://links.lww.com/ALN/B449, which includes the definitions of postoperative complications) and their severity graded by using the Clavien–Dindo classification[39] and the Comprehensive Complication Index.[40]

Hemodynamic variables were measured by the treating anesthesiologist in the goal-directed fluid therapy group and by the two research investigators (A.T. and D.M.) in the control group, all well trained on how to obtain and interpret esophageal Doppler–derived hemodynamic measurements. The esophageal Doppler probe was refocused if necessary, and esophageal Doppler–derived hemodynamic variables were measured 5 min after induction of anesthesia (baseline), in steep Trendelenburg (Trendelenburg), and every 15 min until the end of surgery (end of surgery), before the epidural was bolused. Postoperatively, patients were instructed to drink from a specific 250-ml cup to measure daily oral fluid intake. Patients also were weighed every morning before breakfast. Postoperative gastrointestinal function was assessed by the research investigator (J.C.G.-I.) after dinner was served to the patient. The amount of systemic opioid consumption was measured daily and converted to intravenous morphine equivalents.[41]

Preoperative and intraoperative data were collected by two study investigators (A.T. and D.M.), whereas postoperative data were collected by a third study investigator (J.C.G.-I.), who was blinded to patients' randomization and to the entire intraoperative management. The study investigators were not involved in clinical decision-making and did not have access to the data collected by the other investigators. Data were recorded initially on specific data-collection sheets and then transferred into two separate databases, one containing preoperative and intraoperative data and the other postoperative data. The two databases were merged only when the study was terminated.

Sample Size Calculation and Statistical Analysis

Based on 40% incidence of primary postoperative ileus observed in 114 patients who underwent elective colorectal surgery in the context of an Enhanced Recovery After Surgery program at the Montreal General Hospital, by using the same criteria previously described, a power analysis indicated that a sample size of 64 patients in each group was required to show a 50% primary postoperative ileus reduction in patients treated with goal-directed fluid therapy (one-sided Student's *t* test), with a power of 0.8 and a type 1 error (α) = 0.05. The hypothesis that goal-directed fluid therapy would reduce to 20% the incidence of primary postoperative ileus was based on the observation that in 2012, the incidence of primary postoperative ileus at our institution was higher (40%) than that reported in other centers,[42] despite a well-established Enhanced Recovery After Surgery program that included several perioperative interventions shown to accelerate the bowel recovery (*e.g.,* selective use of mechanical bowel preparation, carbohydrate-rich beverage, early feeding, laparoscopic surgery, epidural, chewing gum, opioid-sparing strategies, and others). At that time, perioperative fluid management was the only element that was not standardized. We therefore hypothesized that goal-directed fluid therapy, by administering intravenous fluids based on more objective measures of hypovolemia, would significantly reduce the incidence of primary postoperative ileus.

Analysis was performed on an intention-to-treat basis and as per protocol. The primary outcome was evaluated with the chi-square test or Fisher exact test if appropriate. A preplanned subgroup analysis of the primary outcome was conducted in patients not receiving a stoma, in patients undergoing colonic surgery, and in patients undergoing rectal surgery. As the proportion of patients who received mechanical bowel preparation was significantly different between the two groups ($P = 0.021$), a nonplanned adjusted analysis was conducted to calculate the relative risk (RR) of primary postoperative ileus, by adjusting for the use of mechanical bowel preparation. Secondary outcomes were evaluated with the Student's *t* test for normally distributed data, the Wilcoxon–Mann–Whitney U test for not normally distributed, and the chi-square test or Fisher exact test when appropriate. Repeated-measures linear mixed model analysis was used to assess and compare intraoperative hemodynamic variables, postoperative pain intensity, opioid consumption, and time spent out of bed over time and between groups. The Tukey *post hoc* test was used for *post hoc* analysis.

Continuous variables are reported as mean ± SD or median (interquartile range), and categorical and ordinal variables as absolute number (percentage). RR with 95% CI also is reported for categorical variables.

Statistical analysis was performed with SPSS, version 23 (IBM Corp., USA) or STATA, version 14 (StataCorp, USA). All statistical tests were two-sided, and $P < 0.05$ was considered to indicate statistical significance.

Results

Patients' Characteristics, Operative Data, and Anesthesia Care

A total of 196 patients were assessed for eligibility, of whom 135 were randomized, 68 to the goal-directed fluid

therapy group and 67 to the control group. One patient in each group did not receive the allocated intervention (goal-directed fluid therapy group: one patient withdrew the consent before starting surgery; control-group: in one patient planned laparoscopic surgery was changed to laparotomy). Two patients in each group dropped out as surgery was aborted because of intraperitoneal carcinomatosis. The intervention was discontinued in one patient in the goal-directed fluid therapy group because the intravenous catheter through which intravenous fluids were administered was disconnected accidentally during the intervention, and this was recognized only at the end surgery. A total of 128 patients were analyzed on an intention-to-treat basis (64 in the goal-directed fluid therapy and 64 in the control group) and 115 patients were analyzed per protocol (56 in the goal-directed fluid therapy and 59 in the control group), as in eight patients in the goal-directed fluid therapy group and in five patients in the control group laparoscopic surgery was converted to laparotomy (fig. 1). Baseline patients' characteristic, operative data, and anesthesia care were similar between the two groups, except for the use of mechanical bowel preparation that was more frequent in the goal-directed fluid therapy group (P = 0.021; table 1 and Supplemental Digital Content 5, http://links.lww.com/ALN/B450, which includes a table reporting patients' comorbidities in the two groups).

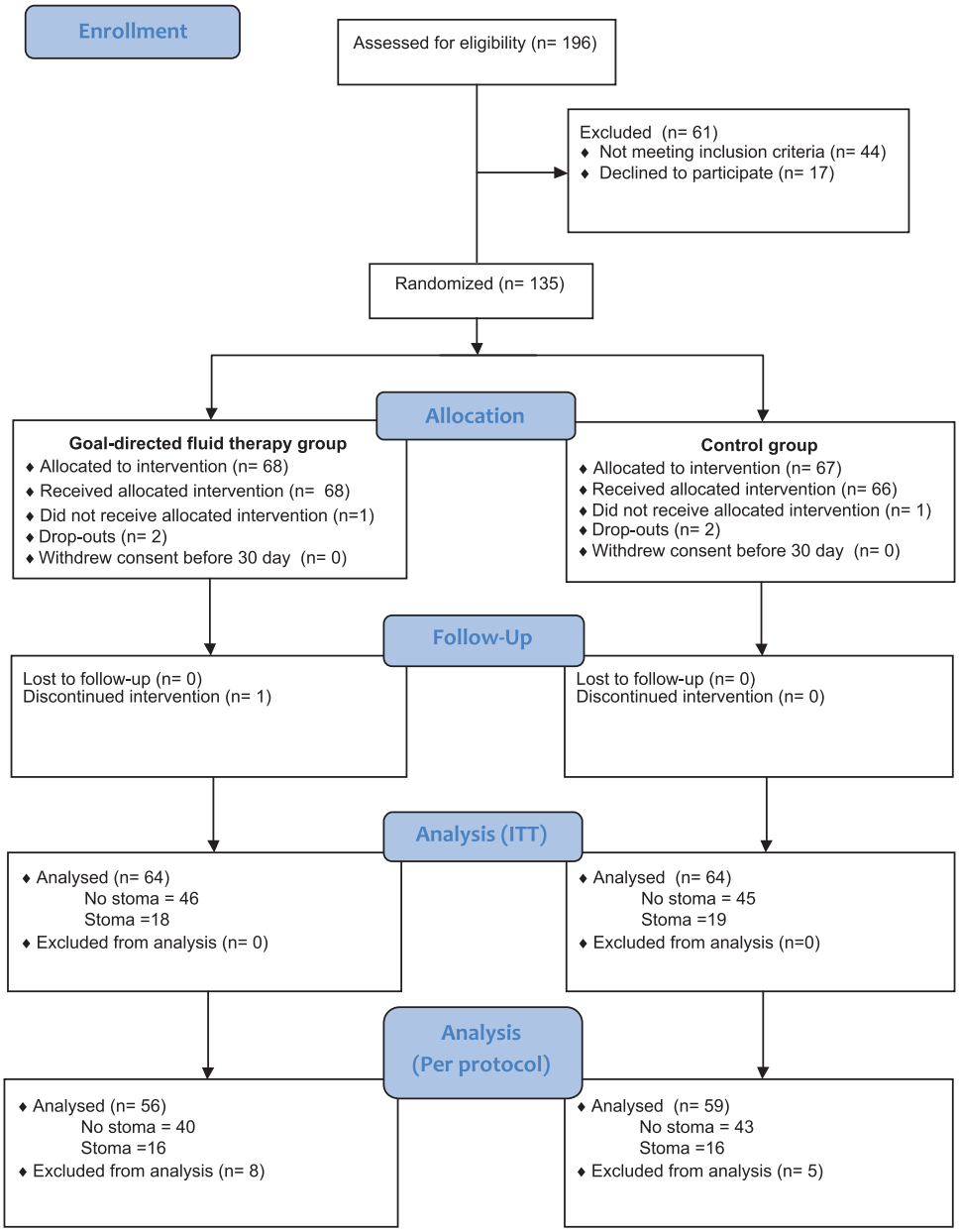

Fig. 1. Consolidated Standards of Reporting Trials (CONSORT) diagram. ITT = intention to treat.

Table 1. Baseline Patients' Characteristics, Operative Data, and Anesthesia Care

	Goal-directed Fluid Therapy (n = 64)	Control (n = 64)	*P* Value
Age, yr	63 ± 15	61 ± 15	
Sex M/F, n	31/33	40/24	
Weight, kg	71.1 (62.2–85.1)	76.5 (67.6–84.7)	
BMI	24.9 (22.4–28.6)	26.1 (23.4–29.1)	
BSA, m^2	1.8 ± 0.2	1.9 ± 0.2	
ASA physical status (I/II/III/IV), n	6/42/14/2	8/38/18/0	
CR-POSSUM score			
Physiology	8 (7–10)	8 (7–9)	
Operative	7 (7–7)	7 (7–7)	
Predictive mortality (%)	1.8 (0.9–9.3)	1.8 (0.9–2.6)	
Charlson comorbidity index	2 (2–3)	2 (1–3)	
Preoperative hemoglobin, g/dl	12.8	13.4	
Indication for surgery, n (%)			
Colorectal cancer	48 (75)	43 (67)	
Inflammatory bowel disease	6 (9.4)	9 (14)	
Diverticulitis	4 (6.3)	7 (10)	
Others*	6 (9.4)	5 (7.8)	
Type of surgery, n (%)			
Colonic	39 (61)	39 (60)	
Ileocecal resection	1 (1.6)	7 (10.9)	
Right hemicolectomy	20 (31)	19 (30)	
Left hemicolectomy	5 (7.8)	4 (6.3)	
Subtotal colectomy	0 (0)	3 (4.7)	
Sigmoidectomy	11 (17)	6 (9.4)	
Total colectomy	2 (3.1)	0 (0)	
Rectal	25 (39)	25 (39)	
Rectal anterior resection	10 (16)	8 (12)	
Rectal low anterior resection	8 (12)	9 (14)	
Proctocolectomy	6 (9.4)	5 (7.8)	
Abdominal perineal resection	1 (1.6)	3 (4.7)	
Stoma, n (%)	18 (28)	19 (30)	
Bowel preparation, n (%)			
4 l GoLYTELY®	36 (56)	23 (36)	
2 Fleet enemas	12 (19)	17 (27)	
Preoperative carbohydrate drinks,† n (%)			
Yes‡	47 (73)	45 (71)	
Yes, according to the quantity indicated§	23 (36)	26 (42)	
Preoperative fasting time, h			
Solid‖	36 (19–40)	34 (17–38)	
Fluid#	4 (3–6)	4 (3–6)	
Duration of surgery, min	189 (144–269)	183.5 (133–254)	0.564
Laparoscopic time, min	108 (68–146)	101 (71–143)	0.506
Conversion to open, n (%)	8 (12)	5 (7.8)	0.380
Final temperature, °C	36.1 ± 0.8	35.9 ± 0.6	0.269
Et desflurane, %	4.4 ± 0.6	4.6 ± 0.7	0.103
Et sevoflurane, %**	1.4 ± 0.3	1.3 ± 0.3	0.617
Remifentanil, $\mu g \cdot kg^{-1} \cdot min^{-1}$	0.1 (0.1–0.2)	0.1 (0.1–0.2)	0.083
Intraoperative ketorolac (30 mg), n (%)	49 (77)	50 (78)	0.754

Data are presented as mean ± SD, median (interquartile range), or absolute numbers (percentage).

*Benign adenoma (six patients in the goal-directed fluid therapy group and three patients in the control group), fecal incontinence (one patient in the control group) terminal ileum stricture (one patient in the control group). †Morning dose. ‡Data from one patient in the control group is missing. §Data from two patients in the control group are missing. ‖Data from two patients in the control group and one patient in the goal-directed fluid therapy is missing. #Data from one patient in the control group is missing. **Eighteen patients received sevoflurane (nine patients in the goal-directed fluid therapy and nine patients in the control group).

ASA = American Society of Anesthesiologists; BMI = body mass index; BSA = body surface area; CR-POSSUM = Colorectal-Physiological and Operative Severity Score for the enUmeration of Mortality and morbidity; Et = end-tidal; M/F = male/female.

Intraoperative Fluid Administration, Vasopressors, and Hemodynamic Data

Patients in the goal-directed fluid therapy group received less intravenous fluids ($P < 0.001$; mainly less crystalloids, $P < 0.001$) but a greater volume of colloids ($P < 0.001$). Estimated blood loss was not different, phenylephrine was used more frequently in the control group ($P = 0.020$), and none of the patients required inotropes (table 2). At baseline, stroke volume and cardiac output were higher in the control group (differences of least squares means, −9.6 ml; 95% CI, −16.6 to −2.6; $P = 0.008$, and differences of least squares means, −0.6 l/min; 95% CI, −1.2 to −0.1; $P = 0.024$, respectively). Overall, stroke volume and cardiac output changes over time significantly differed between the two groups ($P < 0.001$). This difference was driven mainly by a more pronounced increase of stroke volume and cardiac output from baseline to Trendelenburg position in the goal-directed fluid therapy group (stroke volume goal-directed fluid therapy group: differences of least squares means, 24.3 ml; 95% CI, 18 to 30.5; stroke volume control group: differences of least squares means, 12.1 ml; 95% CI, 6 to 18.3; cardiac output goal-directed fluid therapy group: differences of least squares means, 1.1 l/min; 95% CI, 0.7 to 1.6; cardiac output control group: differences of least squares means, 0.4 l/min; 95% CI, −0.1 to 0.86). Only in the goal-directed fluid therapy group did stroke volume and cardiac output remain significantly higher compared with baseline throughout surgery ($P < 0.001$). Intraoperative stroke volume and cardiac output values were higher in the goal-directed fluid therapy group; however, the differences between the two groups did not reach statistical significance at any of the other time intervals (Supplemental Digital Content 6, http://links.lww.com/ALN/B451, which includes two figures reporting stroke volume and cardiac output between groups during surgery). Mean arterial pressure changes over time were similar between the two groups.

Table 2. Preoperative and Intraoperative Intravenous Fluids, Vasopressors, Blood Loss, and Transfusions

	Goal-directed Fluid Therapy (n = 64)	Control (n = 64)	*P* Value
Preoperative period			
Replacement of preoperative intravascular deficit due to MBP,* ml	—	2,094 ± 395	—
Intraoperative period			
Total volume of intravenous fluid, ml	1,535 (1,000–2,272)	2,370 (1,779–3,071)	**< 0.001**
Lactated Ringer's			
ml	500 (323–687)	2,102 (1,600–2,528)	**< 0.001**
ml · kg^{-1} · h^{-1}	2 (2–2)	8.6 (7–11)	**< 0.001**
Colloids, ml	900 (400–1,400)	0 (0–500)	**< 0.001**
Prepneumoperitoneum boluses	400 (200–400)†	—‡	—
NaCl 0.9%,§ ml	194 (150–268)	179 (146–234)	0.132
Total volume of intravenous fluids, ml			
Colonic surgery	1,375 ± 667	2,243 ± 874	**< 0.001**
Rectal surgery	2,342 ± 981	2,958 ± 978	**0.031**
EBL, ml	175 (100–400)	150.0 (100–400)	0.708
Colonic surgery	100 (100–200)	100 (100–200)	0.519
Rectal surgery	400 (125–850)	400 (200–800)	0.914
Erythrocytes			
Patients receiving erythrocytes, n (%)	5 (7.8)	1 (1.6)	0.094
Number of units (2/4/8)	3/1/1	1/0/0	0.100
Volume, ml	0 (0–0)	0 (0–0)	na‖
Vasopressor, n (%)			
Phenylephrine	53 (83)	58 (91)	0.193
n (%)	39 (61)	51 (80)	**0.020**
µg	80 (0–300)	180 (80–440)	**0.016**
Ephedrine			
n (%)	40 (62)	43 (67)	0.496
mg	10 (0–25)	10 (0–20)	0.947
Phenylephrine continuous infusion, n (%)	0 (0)	0 (0)	na
Urine output, ml · kg^{-1} · h^{-1}	1.2 (0.8–1.8)	1.4 (0.8–2.6)	0.148

Data are presented as mean ± SD, median (interquartile range), or absolute numbers (percentage). *P* values in bold represent statistically significant results ($P < 0.05$).

*Preoperative intravenous fluids (4 l GoLYTELY®) were administered only in patients of the control group who received mechanical bowel preparation (35.9%). In two patients, the preoperative deficit due to the use of mechanical bowel preparation could not be completed before surgery because the operating room schedule was changed at the last minute. †In the goal-directed fluid therapy group, 55 patients (86%) needed stroke volume optimization before pneumoperitoneum. ‡In the control group, 40 patients (62.5%) increased stroke volume at baseline by more than 10% when positioned in steep Trendelenburg before pneumoperitoneum. §Amount of normal saline solution used to dilute antibiotics, potassium chloride when needed, and remifentanil. ‖No statistics were computed because there were not enough valid cases to perform the Mann–Whitney U test.

EBL = estimated blood loss; MBP = mechanical bowel preparation; na = not applicable; NaCl 0.9% = normal saline.

Postoperative Data

In the postanesthesia care unit, the two groups were comparable with regard to the amount of intravenous fluids, systemic opioids, and vasopressors received. Postoperative nausea and vomiting, the number of hypotension episodes, urine output, and length of stay in the postanesthesia care unit also were similar.

On the surgical unit, patients received a similar amount of intravenous fluids, and oral intake was not different. Postoperative weight gain and urine output were higher in the control group on day 1 (P = 0.002 and P = 0.004, respectively; table 3). Postoperative pain intensity at rest and while ambulating, systemic opioid consumption, and time spent out of bed were similar between the two groups. Postoperative pain scores were significantly lower in the goal-directed fluid therapy group on day 0 (P = 0.018), but this difference was not clinically significant (table 4).

Outcomes

Primary Outcome and Gastrointestinal Function. Overall, the incidence of primary postoperative ileus was similar between the two groups, on intention-to-treat analysis (22% in the goal-directed fluid therapy group and 22% in the control group, RR, 1; 95% CI, 0.5 to 1.9; P = 1.000) and per protocol (P = 1.000). By adjusting for the use of mechanical bowel preparation, the risk of developing primary postoperative ileus did not change ($RR_{adjusted}$, 1; 95% CI, 0.5 to 1.9; P = 0.094). Recovery measures of gastrointestinal function and gastrointestinal symptoms also were similar (table 5).

Secondary Outcomes. Quality of Recovery score, readiness to be discharged, length of hospital stay, overall 30-day medical and surgical complications, emergency department visits, and readmission rates were not different. More patients in the goal-directed fluid therapy developed intra- or retroperitoneal abscesses (P = 0.048; table 6 and Supplemental Digital Content 7, http://links.lww.com/ALN/B452, which includes a table that describes medical morbidity in the two groups).

Discussion

The results of this study indicate that intraoperative goal-directed fluid therapy aiming to achieve near-maximal stroke volume optimization compared with fluid therapy based on traditional principles does not reduce the incidence of primary postoperative ileus in patients undergoing laparoscopic colorectal surgery in the context of a well-established Enhanced Recovery After Surgery program.

Several trials conducted in patients undergoing abdominal surgery but treated with conventional care have shown that inadequate fluid therapy delays the recovery of gastrointestinal function.[7–11] Experimental and clinical studies have demonstrated that intestinal edema as a result of excessive fluid administration inhibits gastrointestinal transit and impairs anastomotic healing.[7–10,13,14,17,18,43] In contrast, fluid restriction has been shown to accelerate the recovery of bowel function and to facilitate the early intake of oral diet.[11,13,14] However, due to the heterogeneity of the study designs, the

Table 3. Postoperative Fluid Balance, Weight Balance, and Postoperative Hypotension

	Goal-directed Fluid Therapy (n = 64)	Control (n = 64)	*P* Value
Patients receiving intravenous infusion after day 0, n (%)	31 (48)	28 (44)	0.723
Input, ml			
Total intravenous crystalloids,* ml	319 (247–2,967)	607 (291–3,234)	0.269
Total oral fluid intake,† ml	7,681 (5,625–10,350)	6,525 (3,968–10,050)	0.571
Output			
Urine output, day 0‡	700 (450–1,440)	1,450 (700–2,000)	**0.004**
Total gastrointestinal losses,§ ml	75 (0–1,475)	50 (0–1,912)	0.984
Weight balance,‖ kg			
Day 1 – day 0	1.1 ± 1.9	2.1 ± 1.7	**0.002**
Day 2 – day 1	0.6 ± 1.6	0 ± 1.7	0.054
Day 3 – day 2	–0.8 ± 1.4	–0.5 ± 1.4	0.321
Hypotension,# n (%)	15 (23)	16 (25)	1.000
Orthostatic hypotension,** n (%)	2 (3.2)	8 (12)	0.096

Data are presented as mean ± SD, median (interquartile range), or as absolute numbers (percentage). *P* values in bold represent statistically significant results (P < 0.05).

*Total amount of intravenous crystalloids received from surgical unit admission until hospital discharge. †Total oral fluid intake measured from surgical unit admission until hospital discharge. ‡Urine output on day 0 measured from surgical unit admission until 8:00 AM of day 1 (Foley catheters were removed on the morning of day 1 as per Enhanced Recovery After Surgery protocol). Urine output on day 0 could not be measured in seven patients of the goal-directed fluid therapy group and in 10 patients of the control group as Foley catheters were removed in postanesthesia care unit because of patients' discomfort. §Total gastrointestinal losses measured from surgical unit admission until hospital discharge. ‖Postoperative weight could not be measured 11 times in the goal-directed fluid therapy group and six times in the control group because of patients' refusal or because patients were discharged early on day 2 or day 3 (day 1: two patients in goal-directed fluid therapy group and 1 patient in control group; day 2: one patient in the goal-directed fluid therapy group and one patient in the control group; day 3: eight patients in goal-directed fluid therapy group and four patients in the control group). #Systolic blood pressure less than 90 mmHg or less than 20% of the baseline value. **A decrease of at least 20 mmHg in systolic blood pressure on assuming an upright posture from a supine position.

Table 4. Postoperative Pain Intensity and Management and Time Spent Out of Bed

	Goal-directed Fluid Therapy (n = 64)	Control (n = 64)	*P* Value
Pain, static			0.189
NRS day 0	1 ± 2	2 ± 2	
NRS day 1	2 ± 2	2 ± 2	
NRS day 2	2 ± 2	2 ± 2	
NRS day 3	2 ± 2	2 ± 2	
Pain, coughing			**0.018**
NRS day 0	2 ± 2	3 ± 3	**0.018**
NRS day 1	4 ± 2	4 ± 2	0.491
NRS day 2	4 ± 3	4 ± 2	0.435
NRS day 3	3 ± 3	3 ± 2	0.575
Pain, ambulating			0.189
NRS day 0	1 ± 2	2 ± 2	
NRS day 1	2 ± 2	2 ± 2	
NRS day 2	2 ± 2	2 ± 2	
NRS day 3	2 ± 2	2 ± 2	
Days with thoracic epidural analgesia, days	3 (2–3)	3 (2–3)	0.840
Systemic opioids,* mg			1.000
Total, in the first 3 days	10 (3–21)	12.4 (7–25)	*0.344*
Day 0	0 ± 0	0 ± 0	
Day 1	3 ± 7	1 ± 3	
Day 2	6 ± 7	9 ± 8	
Day 3	7 ± 9	7 ± 9	
Celebrex† with thoracic epidural analgesia, n (%)	9 (14)	10 (16)	1.000
Celebrex† after thoracic epidural analgesia, n (%)	48 (75)	51 (80)	0.673
No. patients receiving milk of magnesia, n (%)	52 (81)	45 (72)	0.297
Time spent out of bed, min			0.935
Day 0	26 ± 68	23 ± 54	
Day 1	167 ± 189	232 ± 220	
Day 2	185 ± 177	255 ± 387	
Day 3	163 ± 151	198 ± 136	

Data are presented as mean ± SD or as absolute numbers (percentage). Linear mixed model analysis: *P* values refer to the group main effect, and to the pairwise comparison; *P* values in italic: Wilcoxon–Mann–Whitney U test. *P* values in bold represent statistically significant results ($P < 0.05$).

Postoperative pain (NRS 0 to 10) could not be assessed 15 times because patients' refusal or because patients were discharged early on day 2 or day 3 (day 0: one patient in the control group; day 1: one patient in the control group; day 2: one patient in the goal-directed fluid therapy group; day 3: eight patients in the goal-directed fluid therapy group and four patients in the control group). Systemic opioids consumption could not be measured 14 times because missing data or patients were already discharged (day 1: one patient in the goal-directed fluid therapy group; day 2: one patient in the goal-directed fluid therapy group; day 3: eight patients in the goal-directed fluid therapy group and four patients in the control group). Time spent out of bed could not be assessed 12 times because patients were already discharged (eight patients in the goal-directed fluid therapy group and four patients in the control group).

*Intravenous morphine equivalents. †Celebrex 200 mg *per os* every 12 h.

NRS = Numeric Rating Scale.

lack of a universal definition characterizing a restrictive fluid management, and the absence of a standardized perioperative care,[44] it remains difficult to establish the real impact of fluid restriction on postoperative morbidity.[45–47]

Individualization of fluid therapy based on more objective measures of hypovolemia, commonly called goal-directed fluid therapy, has shown not only to accelerate the recovery of gastrointestinal function[19,22] but also reduce postoperative complications[22] and hospitalization,[21,22] especially in high-risk patients,[23,24] and mainly when compared with liberal fluid administration.[21] Because of these benefits, it has been recommended in patients undergoing major abdominal surgery.[36,48–50]

The results of the present study do not support the use of goal-directed fluid therapy to reduce the incidence of primary postoperative ileus in this specific patient population and perioperative clinical context, despite a larger intraoperative volume of intravenous fluids in the control group and a more pronounced and sustained increase of stroke volume and cardiac output in the goal-directed fluid therapy group during surgery.

Several reasons can explain these findings. First, patients were treated with several perioperative Enhanced Recovery After Surgery interventions that have been shown to facilitate the recovery of gastrointestinal function after abdominal surgery, such as the use of preoperative carbohydrate drinks, laparoscopic surgery, thoracic epidural analgesia, opioid-sparing analgesia, and early feeding, which might have contributed to minimizing the occurrence of primary postoperative ileus in both groups.[2] This also has been demonstrated by two recent meta-analyses that have shown that when patients are treated with a more rational fluid management, and in the context of an Enhanced Recovery After Surgery program, the

Table 5. Incidence of PPOI and Recovery Gastrointestinal Function

	Goal-directed Fluid Therapy (n = 64)	Control (n = 64)	RR (95% CI)	*P* Values	$RR_{adjusted}$ (95% CI)	*P* Values
Primary postoperative ileus,* n (%)						
ITT analysis	14 (22)	14 (22)	1 (0.5–1.9)	1.000	1 (0.5–1.9)	0.094
Per protocol	12 (21)	12 (20)	1 (0.5–2.1)	*1.000*	1.1 (0.5–2.1)	0.225
No stoma, n (%)						
ITT analysis	9 (17)	7 (16)	1.2 (0.5–3.1)	0.615	1.3 (0.5–3.1)	0.316
Per protocol	7 (17)	7 (16)	1.1 (0.4–2.8)	0.882	1.1 (0.4–2.7)	0.272
Stoma, n (%)						
ITT analysis	5 (28)	(37)	0.7 (0.3–1.9)	0.556	0.8 (0.3–2)	0.226
Per protocol	5 (31)	5 (31)	1 (0.3–2.8)	1.000	1.1 (0.4–2.9)	0.477
Colonic surgery n (%)						
ITT analysis	9 (23)	6 (15)	1.5 (0.6–3.8)	0.389	1.4 (0.6–3.5)	0.370
Per protocol	7 (21)	6 (16)	1.3 (0.5–3.5)	0.597	1.2 (0.5–3.3)	0.385
Rectal surgery n (%)						
ITT analysis	5 (20)	8 (32)	0.6 (0.2–1.6)	0.333	0.7 (0.3–1.7)	0.147
Per protocol	5 (23)	6 (29)	0.8 (0.3–2.2)	0.661	0.9 (0.3–2.3)	0.298
Primary postoperative ileus						
Diagnosis, day 1/2/3/≥4, n						
ITT analysis	3/5/4/2	4/7/1/2	na	0.517	na	—
Per protocol	3/3/4/2	4/5/1/2	na	0.486	na	—
Duration, days						
ITT analysis	2 (1–3)	3 (2–3)	na	0.318	na	—
Per protocol	2 (1–4)	3 (2–4)	na	0.389	na	—
Time to first flatus, h						
ITT analysis	20 (12–26)	20 (12–24)	na	0.843	na	—
Per protocol	20 (13–26)	19 (13–24)	na	0.796	na	—
Time to first bowel movement, h						
ITT analysis	21 (16–36)	22 (16–28)	na	0.884	na	—
Per protocol	22 (16–36)	22 (15–28)	na	0.784	na	—
Nausea, n (%)						
ITT analysis	41 (64)	37 (58)	1.1 (0.8–1.5)	0.469	1.2 (0.9–1.6)	0.697
Per protocol	37 (66)	33 (56)	1.2 (0.9–1.6)	0.265	1.3 (0.9–1.7)	0.660
Vomiting,† n (%)						
ITT analysis	25 (39)	25 (39)	1 (0.6–1.5)	1.000	1.1 (0.7–1.6)	0.492
Per protocol	23 (41)	21 (36)	1.1 (0.7–1.8)	0.547	1.3 (0.8–2.1)	0.601
Abdominal distension, n (%)						
ITT analysis	49 (77)	55 (86)	0.9 (0.7–1)	*0.257*	0.9 (0.8–1.1)	0.938
Per protocol	43 (77)	51 (86)	0.9 (0.7–1.1)	*0.230*	0.9 (0.8–1.1)	0.856
Diet intolerance,‡ n (%)						
ITT analysis	15 (23)	17 (27)	0.9 (0.5–1.6)	*0.839*	0.9 (0.5–1.6)	0.582
Per protocol	14 (25)	14 (24)	1 (0.5–2)	*1.000*	1.1 (0.6–2.1)	0.967
Nasogastric tube insertion, n (%)						
ITT analysis	9 (14)	11 (17)	0.8 (0.4–1.8)	*0.808*	0.8 (0.4–1.8)	0.293
Per protocol	8 (14)	8 (14)	1 (0.4–2.6)	*1.000*	1.2 (0.5–2.9)	0.770
Number of skipped meals, n						
ITT analysis	1 (0–2)	1 (0–5)	na	0.551	na	-
Per protocol	1 (0–2)	1 (0–4)	na	0.770	na	-

Data are presented as median (interquartile range), as absolute numbers (percentage), or as RR (95% CI); *P* values in italic: Fisher exact test. The analysis was adjusted for the use of mechanical bowel preparation.

*Absence of gas but presence of stool was not considered a clinical indicator of gastrointestinal dysfunction. †Including patients who did not meet the criteria for primary postoperative ileus. ‡Diet intolerance: at the end of the day patients were asked to judge whether they tolerated the meals they ate during the day. Patients who did not eat any meal during the day were considered not tolerating diet.

ITT = intention to treat; na = not applicable; PPOI = primary postoperative ileus; RR = relative risk.

benefits of goal-directed fluid therapy are offset by advancements in perioperative care.[28,29] Second, patients in the control group were able to eliminate fluid excess, as indicated by a higher urine output the day of surgery and by a marginal weight gain (less than 2.5 kg) on day 1. This suggests that the volume of intravenous fluids received in the control group might have not been high enough to cause sufficient interstitial edema to determine a high incidence of primary postoperative ileus or postoperative complications. Finally, approximately two thirds of patients in both groups were at

Table 6. QoR Score, LOS, 30-day Postoperative Complications, 30-day ED Visits, and 30-day Readmissions

	Goal-directed Fluid Therapy (n = 64)	Control (n = 64)	RR (95% CI)	*P* Value
QoR on day 2	14 (13–16)	14 (13–16)	na	0.648
Readiness to be discharged, days	3 (2–4)	3 (3–5)	na	0.561
LOS, days	4 (3–5)	4 (3–5.7)	na	0.922
30-day mortality, n (%)	0 (0)	0 (0)	na	na
Patients with at least one 30-day complication, n (%)	28 (44)	25 (39)	1.1 (0.7–1.7)	0.590
In-hospital	22 (34)	20 (31)	1.1 (0.7–1.8)	0.707
Postdischarge	9 (14)	8 (12)	1.1 (0.5–2.7)	0.795
Patients with at least one 30-day medical complication, n (%)	18 (28)	14 (22)	1.3 (0.7–1.3)	0.414
Cardiovascular	3 (4.7)	2 (3.1)	1.2 (0.3–8.7)	*1.000*
Respiratory	3 (4.7)	0 (0)	na	*0.244*
Infectious	12 (19)	8 (12)	1.5 (0.7–3.4)	0.330
Other	9 (14)	9 (14)	1 (0.4–2.3)	1.000
Patients with at least one 30-day surgical complication, n (%)	20 (31)	18 (28)	1.1 (0.6–1.9)	0.699
Primary postoperative ileus	14 (22)	14 (22)	1 (0.5–1.9)	1.000
Anastomotic leakage	3 (4.7)	0 (0)	na	*0.244*
Bleeding	3 (4.7)	3 (4.7)	1 (0.2–4.8)	*1.000*
Bowel perforation	1 (1.6)	0 (0)	na	*1.000*
Mechanical bowel obstruction	0 (0)	1 (1.6)	na	*1.000*
Wound dehiscence	0 (0)	1 (1.6)	na	*1.000*
Other	0 (0)	2 (3.1)	na	*0.496*
Patients admitted to ICU,* n (%)	2 (3.1)	1 (1.6)	2 (0.2–21.5)	*1.000*
Patients reoperated within 30 days, n (%)	1 (1.6)	3 (4.7)	0.3 (0–3.1)	*0.619*
30-day Clavien–Dindo classification, n (%)				
I	10 (16)	10 (16)	1 (0.4–2.3)	1.000
II	11 (17)	10 (16)	1.1 (0.5–2.4)	0.811
IIIa	5 (7.8)	2 (3.1)	2.5 (0.5–12.4)	*0.440*
IIIb-IVb	2 (3.1)	3 (4.7)	0.7 (0.1–3.9)	0.648
30-day CCI	0 (0–20.9)	0 (0–11.3)	na	0.483
Patients visiting ED within 30 days, n (%)	13 (20)	9 (14)	1.4 (0.7–3.1)	0.349
Patients readmitted within 30 days, n (%)	8 (12)	6 (9.4)	1.3 (0.5–3.6)	0.571

Data are presented as median (interquartile range), as absolute numbers (percentages), or as RR (95% CI); *P* value in italic: Fisher exact test.

*ICU admission during primary LOS.

CCI = Comprehensive Complication Index; ED = emergency department; ICU = intensive care unit; LOS = length of hospital stay; na = not applicable; QoR = Quality of Recovery; RR = relative risk.

low risk for postoperative complications, and the benefits of goal-directed fluid therapy have been demonstrated mainly in high-risk patients.[23,24]

The main strength of this study is that it specifically evaluates the impact of goal-directed fluid therapy on the recovery of bowel function in the context of standardized and evidence-based perioperative care, limiting the risk of bias due to several perioperative confounding factors. However, it must be acknowledged that Enhanced Recovery After Surgery programs include variable interventions, different among institutions, potentially limiting the generalizability of these results in centers with different perioperative care. For example, the impact of goal-directed fluid therapy on the recovery of bowel function might have produced favorable results in patients treated with systemic opioids and not with epidural analgesia, as it is well established that thoracic epidural analgesia facilitates the recovery of bowel function. Our institutional protocol is to use epidural analgesia for patients undergoing laparoscopic rectal surgery but not colonic surgery, based on results of a previous study showing better pain control with epidural analgesia in the first 48 h after laparoscopic rectal surgery compared with systemic opioids plus intravenous lidocaine.[37] In the current study, it was decided to standardize the analgesia technique to minimize the risk of bias, providing epidural analgesia to all patients. Although the use of epidural analgesia in laparoscopic colorectal surgery remains controversial, it is still used in established Enhanced Recovery After Surgery programs.[49]

Several limitations must be acknowledged. First, patients in the control group received a large volume of intravenous fluids, greater than what currently is recommended,[36] but similar to what is still infused in clinical practice.[51–54] Although the fluid regimen used is based on outdated perioperative fluid therapy principles,[45,48] it is consistent with what is recommended in

widely used anesthesia textbooks.[31] The two groups also differed in the type of fluids, and this might have affected the primary outcome more than the infusion regimen. However, to the best of our knowledge, colloid use alone has not been associated with postoperative gastrointestinal dysfunction, as also demonstrated by the results of our secondary analyses (data unsubmitted with the current manuscript). Similarly, the fluid regimen (volume and timing of fluid administration) was also significantly different between the two groups, further confounding the interpretation of these results.

Second, a more rational goal-directed fluid therapy protocol based on stroke volume optimization when clinically deemed, rather than on preemptive near-maximal stroke volume optimization, might have led to better results, as many patients in the control group were able to maintain adequate systemic perfusion (cardiac output), despite a submaximal stroke volume. Third, despite randomization, a higher proportion of patients in the goal-directed fluid therapy group received mechanical bowel preparation. However, after adjustment for the use of mechanical bowel preparation, the risk of developing primary postoperative ileus was unchanged ($RR_{adjusted}$, 1; 95% CI, 0.5 to 1.9.; $P = 0.094$), although the study was not powered to detect a significant difference is this subgroup of patients. Fourth, although we did not exclude obese patients, the average body mass index in the study population may be lower than in other populations, and these results may not be generalizable. Fifth, in the absence of a universal and validated definition of ileus, we used a definition based on an interdisciplinary consensus achieved among anesthesiologists and surgeons, based on literature review and focusing on clinically relevant symptoms. However, the incidence of primary postoperative ileus was similar to what has been reported previously in the context of an Enhanced Recovery After Surgery program.[15] Moreover, a secondary analysis including all patients of the study has shown that patients with primary postoperative ileus, but without any other complications, had a median increase in length of hospital stay of 4 days, and that primary postoperative ileus was an independent predictor of delayed readiness for discharge and prolonged hospital stay ($P < 0.001$ and $P = 0.001$, respectively; data unsubmitted with the current manuscript). Although the sample size is limited and these results need further validation, these findings suggest that this definition of primary postoperative ileus might accurately identify patients with a clinically meaningful gastrointestinal dysfunction in the context of an Enhanced Recovery After Surgery program. Finally, this study might have insufficient statistical power to determine whether goal-directed fluid therapy can reduce primary postoperative ileus, as its incidence was lower than expected. The expected incidence of primary postoperative ileus in the control group might have been overestimated, probably because some patients in the historical group used to calculate the sample size received intravenous morphine patient-controlled analgesia or had open surgery, factors known to delay the recovery of bowel function after colorectal surgery.[2] It also is possible that the volume of fluids received in the historical group might have been significantly higher or lower than what was infused in the control group, contributing to a higher incidence of primary postoperative ileus. Unfortunately, we could not accurately retrieve this information, as the volume and type of fluids infused during surgery was poorly reported in the anesthetic charts. In addition, these results might be in part explained by the participation effect, as patients in clinical trials tend to have better outcomes regardless of the treatment they receive.[55]

In conclusion, within its limitations, this study shows that intraoperative goal-directed fluid therapy compared with fluid therapy based on traditional fluid management does not reduce the incidence of primary postoperative ileus in patients undergoing laparoscopic colorectal surgery in the context of an Enhanced Recovery After Surgery program. Its previously demonstrated benefits might have been offset by advancements in perioperative and surgical care. Nonetheless, fluid therapy always should be based on physiologic and scientific principles, to minimize the risk of complications associated with fluid overload and hypovolemia, especially in high-risk surgical patients.

Acknowledgments

The authors acknowledge Deltex Medical Ltd. (Chichester, United Kingdom) for loaning the esophageal Doppler monitor.

Research Support

Supported by the Gerard R. Douville Award, granted to Dr. Baldini, McGill University Health Center Research Institute, Montreal, Quebec, Canada.

Competing Interests

Dr. Baldini's academic research funding was used to purchase the esophageal Doppler probes. The other authors declare no competing interests.

Reproducible Science

Full protocol available at: gabriele.baldini@mcgill.ca. Raw data available at: gabriele.baldini@mcgill.ca.

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Epidural Neostigmine *versus* Fentanyl to Decrease Bupivacaine Use in Patient-controlled Epidural Analgesia during Labor

A Randomized, Double-blind, Controlled Study

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ABSTRACT

Background: The addition of opioids to epidural local anesthetic reduces local anesthetic consumption by 20% but at the expense of side effects and time spent for regulatory compliance paperwork. Epidural neostigmine also reduces local anesthetic use. The authors hypothesized that epidural bupivacaine with neostigmine would decrease total hourly bupivacaine use compared with epidural bupivacaine with fentanyl for patient-controlled epidural analgesia.

Methods: A total of 215 American Society of Anesthesiologists physical status II, laboring parturients requesting labor epidural analgesia consented to the study and were randomized to receive 0.125% bupivacaine with the addition of either fentanyl (2 μg/ml) or neostigmine (2, 4, or 8 μg/ml). The primary outcome was total hourly local anesthetic consumption, defined as total patient-controlled epidural analgesia use and top-ups (expressed as milliliters of 0.125% bupivacaine) divided by the infusion duration. *A priori* analysis determined a group size of 35 was needed to have 80% power at $\alpha = 0.05$ to detect a 20% difference in the primary outcome.

Results: Of 215 subjects consented, 151 patients were evaluable. Demographics, maternal and fetal outcomes, and labor characteristics were similar among groups. Total hourly local anesthetic consumption did not differ among groups ($P = 0.55$). The total median hourly bupivacaine consumption in the fentanyl group was 16.0 ml/h compared with 15.3, 14.6, and 16.2 ml/h in the 2, 4, and 8 μg/ml neostigmine groups, respectively ($P = 0.55$).

Conclusions: The data do not support any difference in bupivacaine requirements for labor patient-controlled epidural analgesia whether patients receive epidural bupivacaine with 2 to 8 μg/ml neostigmine or epidural bupivacaine with 2 μg/ml fentanyl. **(Anesthesiology 2017; 127:50-57)**

TRADITIONALLY epidural infusions for labor analgesia have consisted of a combination of local anesthetic plus an adjuvant opioid. The addition of an opioid to epidural local anesthetic reduces the dose of local anesthetic needed for analgesia, thereby minimizing side effects from local anesthetic blockade, especially maternal motor block and, potentially, hypotension. However, these epidurally administered opioids can produce side effects themselves, including pruritus and decreased fetal heart rate variability.[1] For these reasons, there has been interest in nonopioid adjuvants to reduce epidural local anesthetic dose. The cholinesterase inhibitor, neostigmine, produces analgesia when given intrathecally or epidurally, *via* increased acetylcholine stimulation of spinal muscarinic and possibly nicotinic receptors.[2]

Studies of intrathecal neostigmine in the mid to late 1990s demonstrated analgesic efficacy and lack of neurologic injury but also dose-dependent, severe nausea and vomiting, and further clinical development was abandoned.[3,4] In contrast, epidural administration of neostigmine has been shown in both adults and children to reduce local anesthetic requirements in the postoperative setting without nausea and vomiting.[5–9] Epidural neostigmine also has been shown in small, single-dose studies to reduce epidural local

What We Already Know about This Topic

- Single- and intermittent-dose epidural neostigmine reduces local anesthetic requirement for labor analgesia
- Effects of adding neostigmine to epidural local anesthetic infusion on local anesthetic consumption for labor analgesia are unknown

What This Article Tells Us That Is New

- Adding neostigmine (2, 4, or 8 μg/ml) to bupivacaine for patient-controlled epidural analgesia during labor did not reduce bupivacaine requirement compared with bupivacaine plus fentanyl

This article is featured in "This Month in Anesthesiology," page 1A.

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 Anesthesiology 2017; 127:50-57

anesthetic requirement for labor analgesia to a degree similar to that of opioids, including a study in which epidural analgesia was titrated *via* patient-controlled epidural analgesia (PCEA).[10–12] In contrast to opioids, there are no large randomized controlled studies evaluating the effects of epidural neostigmine as an adjunct to local anesthetics in the obstetric population for continuous PCEA during labor. The purpose of the current study was to compare the effects of epidural neostigmine, 2, 4, or 8 μg/ml, with that of a commonly used concentration of fentanyl (2 μg/ml) when added to 0.125% bupivacaine *via* PCEA during labor. We hypothesized that epidural bupivacaine with neostigmine would reduce total hourly bupivacaine use compared with epidural bupivacaine with fentanyl for labor analgesia. A secondary analysis was intended to evaluate the clinical dose response of 2 to 8 μg/ml of epidural neostigmine on local anesthetic consumption for labor analgesia compared with 2 μg/ml of fentanyl if significant clinical differences were detected for all doses of neostigmine studied.

Materials and Methods

The study was registered before recruitment of the first subject (http://www.clinicaltrials.gov; NCT00779467), was performed under Investigational New Drug (No. 42281) oversight by the U.S. Food and Drug Administration, and was reviewed on an ongoing basis by a data safety monitoring board. After approval from the institutional review board (Wake Forest School of Medicine, Winston-Salem, South Carolina; No. 5917), written informed consent for study participation was obtained before a patient's request for labor epidural analgesia. Parturients were eligible to participate if they were American Society of Anesthesiologists physical status II, spoke English, weighed less than 115 kg, were in active labor with a single fetus, had cervical dilation 5 cm or less, and had not received IV analgesics within 60 min before epidural administration. Patients with allergies to local anesthetics, fentanyl, or neostigmine also were excluded. The institutional review board initially approved the enrollment of 200 patients for a goal of 160 evaluable patients, but an amendment to increase the number of enrolled patients to 220 was approved in April 2013 due to the need to replace excluded or ineligible patients. Based on updated data used in our power analysis, our goal of 40 evaluable patients per group also was revised at that time to a minimum of 35 patients per group for the final analysis (fig. 1).

Patients were randomized in a balanced manner to one of four study groups *via* a computer-generated number allotment that was concealed in a sealed envelope. At the time of epidural labor analgesia request, an anesthesiologist not involved in the patient's care or data collection prepared the epidural study solution. All members of the patient's care team were blinded to the assignment and study drug. A lumbar epidural catheter was inserted after the administration of a combined subarachnoid and intravenous test dose with 45 mg lidocaine and 15 μg epinephrine. Patients were randomized to receive 15 ml bupivacaine, 1.25 mg/ml, mixed

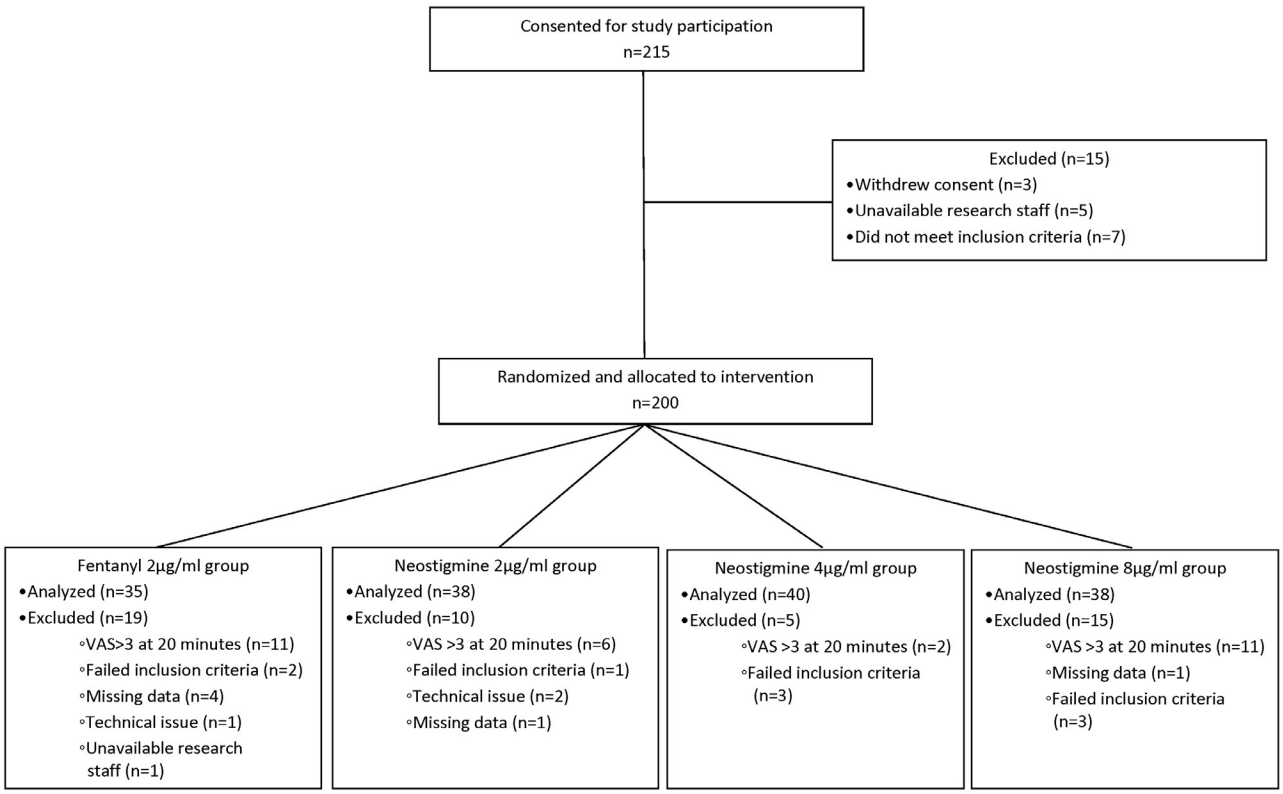

Fig. 1. Enrollment diagram. VAS = visual analog scale.

with one of four adjuvant medications: 2 μg/ml fentanyl or 2, 4, or 8 μg/ml phenol-free neostigmine methylsulfate (1 mg/ml, American Regent, USA; 10-ml multidose vial but discarded after single use). If the patient reported a verbal pain score greater than 3 on a 0 to 10 scale at 20 min after epidural injection, she was excluded from the study and the epidural catheter was replaced or managed by the anesthesiologist at his or her discretion.

After the initial dosing of the epidural catheter with the study solution to establish labor analgesia, a PCEA infusion pump was programmed and initiated with the assigned solution for maintenance analgesia with the following parameters: basal rate of 6 ml/h; PCEA bolus of 5 ml with a 10-min lockout interval; maximum dose of 30 ml/h. Patients with breakthrough pain were treated with a 5- to 10-ml bupivacaine, 2.5-mg/ml bolus, at the discretion of the anesthesiologist to achieve adequate labor analgesia. Patients reporting inadequate labor analgesia after receiving a bupivacaine bolus or patients requiring more than one bolus dose per hour were excluded from the study.

Pain was assessed on a 0 to 10 verbal scale at the following time points: before epidural catheter placement, immediately after combined subarachnoid/intravenous test dose, every 5 min for 20 min after initial epidural bolus of the study solution, and then every 2 h until delivery. In addition, the following parameters were recorded every 2 h until delivery: dermatomal level of sensory blockade to pinprick testing, degree of motor block according to a 0 to 3 scale described by Bromage,[13] maternal self-report of sedation (0 to 10), intensity of nausea (0 to 10), pruritus (0 to 10), and sleepiness (0 to 10), an observer's assessment of maternal alertness[14] (1 to 5), and presence of shivering. Maternal hypotension (20% change or greater from baseline and/or requiring treatment), maternal bradycardia (maternal heart rate less than 60 beats/min or greater than 20% decrease from the patient's baseline heart rate), fetal heart rate abnormalities, mode of delivery, and 1- and 5-min Apgar scores also were recorded. The total volume of study solution administered, the number of PCEA demand boluses, and the number and volume of anesthesiologist-administered bupivacaine 2.5-mg/ml bolus doses were recorded after termination of the PCEA infusion. After delivery, patients also were asked to rate their overall degree of epidural labor analgesia using a 1 to 5 verbal score (1 = not satisfied at all, 5 = extremely satisfied).

Written informed consent for study participation initially was obtained from 160 patients. The initial goal of 40 evaluable patients per group was based on preliminary data from a previous study evaluating epidural bupivacaine use with and without the addition of epidural neostigmine.[12] An estimated mean bupivacaine use of 11.0 ± 3.2 ml/h was used to detect a 20% difference between any groups with an effect size of 0.8, power 0.8, and alpha 0.05. Replacement of unevaluable patients was based sequentially on the randomization assignment of the previously excluded patients after enrollment of the initial 160 patients was completed. We obtained permission from the institutional review board to increase the number of enrolled patients because we did not have enough evaluable patients after 200 patients consented initially. Study enrollment occurred over a 5-yr period (October 2008 to November 2013), with intermittent pauses in enrollment due to neostigmine shortages, researcher availability for enrollment, and technical issues. Final analysis of the previous neostigmine study by Ross *et al.*[12] revealed a mean bupivacaine use of 11.9 ± 3.0 ml/h. With these new data, a sample size of 35 evaluable patients per group, instead of 40 patients, would be adequate to detect a 20% difference between any groups. Due to the prolonged enrollment of the study and intermittent difficulties obtaining neostigmine, the study was terminated in November 2013 when a minimum of 35 evaluable patients per group were enrolled and completed the study.

Data Analysis

Data are presented as mean ± SD or median with quartiles as appropriate. The primary outcome was defined as hourly bupivacaine use during labor. A sample size of 35 patients per group was chosen to detect a clinically meaningful difference of 20% in hourly bupivacaine use among groups ($\alpha = 0.05$; $1 - \beta = 0.20$). Groups were compared for the primary outcome by one-way ANOVA. Pain scores and maternal side effects were intended to be analyzed by repeated-measure ANOVA methods, but assumptions of ANOVA were violated and were therefore analyzed with linear mixed effect modeling. Other variables were compared with the Student's *t* test, chi-square analysis, Mann–Whitney U rank sum test, or Fisher exact test as appropriate. $P < 0.05$ was considered significant.

Statistical analyses were conducted with SigmaStat version 3.0 for Windows (SPSS Inc., USA, and then acquired by IBM [USA] in 2009). Descriptive statistics were calculated for all variables and compared among groups, such that mean ± SD was used for normally distributed variables; median [interquartile range] for data that were not normally distributed or for data with outliers or ordinal data; and number (percentage) for categorical data. Kolmogorov–Smirnov (with Lilliefors correction) test was used to test for normality of data distribution of each variable.

Results

Written informed consent for study participation was obtained from a total of 215 patients before their request for labor epidural analgesia. Data from 151 evaluable patients were included in the final analysis with a minimum of 35 patients per group (fig. 1). The most common reason for patient exclusion was visual analog scale pain score greater than 3 at 20 min after epidural placement.

Demographic information, labor characteristics, or neonatal outcomes did not differ among the 151 evaluable patients in the four study groups (table 1). There was no difference in median hourly bupivacaine use in PCEA, supplemental boluses, or their combination (fig. 2). The

Table 1. Demographics, Labor Characteristics, and Neonatal Outcomes of Laboring Patients

	Fentanyl, 2 μg/ml	Neostigmine, 2 μg/ml	Neostigmine, 4 μg/ml	Neostigmine, 8 μg/ml	*P* Value
Sample size, n	35	38	40	38	
Age, yr	27 ± 6	28 ± 6	27 ± 6	28 ± 5	0.68
BMI, kg/m^2	31 ± 5	31 ± 5	30 ± 5	30 ± 4	0.89
Parity	1 [0–1]	0 [0–1]	0 [0–1]	0.5 [0–1]	0.89
Estimated gestational age, wk	40 ± 1	40 ± 1	40 ± 1	40 ± 1	0.99
Cervical dilation at epidural placement, cm	3.8 [2.3–4.0]	3.0 [2.1–3.9]	3.0 [2.0–3.8]	3.0 [2.0–3.5]	0.82
Epidural placement to cervix complete, min	235 [192–373]	268 [167–452]	330 [221–517]	258 [154–366]	0.26
Epidural placement to delivery, min	322 [242–484]	415 [202–579]	396 [286–703]	303 [193–520]	0.29
Total study analgesia duration, min	410 ± 309	424 ± 290	480 ± 295	406 ± 322	0.69
Percent requiring cesarean delivery, %	14 (5/35)	24 (9/38)	15 (6/40)	21 (8/38)	0.67
Percent requiring bupivacaine bolus for labor analgesia, %	57 (20/35)	53 (20/38)	60 (24/40)	55 (21/38)	0.93
Patient satisfaction score, 1–5	4 [3–5]	4 [3–5]	4 [4–5]	5 [3–5]	0.82
Neonatal weight, g	3,424 ± 383	3,437 ± 485	3,403 ± 449	3,448 ± 430	0.97
1-min Apgar score	7 ± 2	8 ± 2	8 ± 2	8 ± 2	0.93
5-min Apgar score	9 ± 0	9 ± 1	9 ± 1	9 ± 0	0.83

Descriptive statistics were calculated for all variables such that mean ± SD was used for normally distributed variables; median [interquartile range] for data that were not normally distributed or for data with outliers or ordinal data; and number or percentage for categorical data. ANOVA, chi-square analysis, Mann–Whitney U rank sum test, and Fisher exact test were applied as appropriate. For all analyses, *P* was set at 0.05 for statistical significance.

BMI = body mass index.

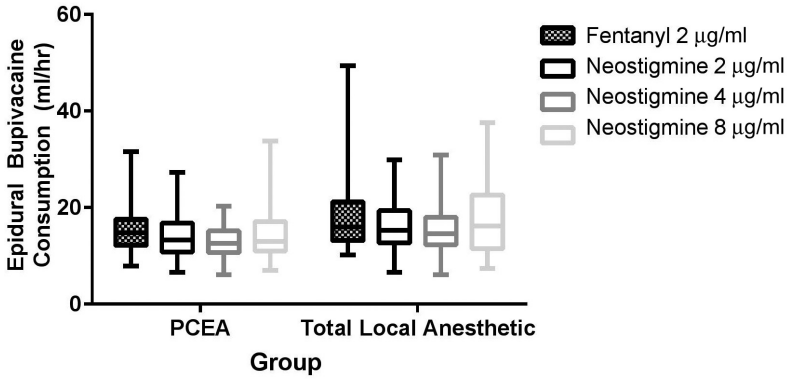

Fig. 2. Median local anesthetic consumption between groups. Median hourly bupivacaine consumption of parturients with an epidural for labor analgesia. *Box* indicates 25th and 75th percentile; *bars* indicate minimum and maximum values; and *middle line* in box indicates median consumption (ml/h). PCEA = patient-controlled epidural analgesia.

median hourly total bupivacaine consumption of patients in the fentanyl group was 16 ml/h, and in neostigmine 2, 4, and 8 μg/ml groups was 15.3, 14.6, and 16.2 ml/h, respectively (*P* = 0.55). The median hourly bupivacaine consumption of patients from only the PCEA pump was 14.8 ml/h in the fentanyl group and 13.3, 12.6, and 13.0 ml/h in the 2, 4, and 8 μg/ml epidural neostigmine groups, respectively (*P* = 0.25). The duration of total study epidural labor analgesia was nonsignificant among groups (*P* = 0.69). In addition, there was no difference among groups in number of patients requiring additional bupivacaine boluses for improved labor analgesia (*P* = 0.93).

Mean pain scores during labor did not differ between the groups over time (*P* = 0.36; fig. 3). Pain scores improved in all four groups after epidural placement. Overall patient satisfaction with labor analgesia did not differ among

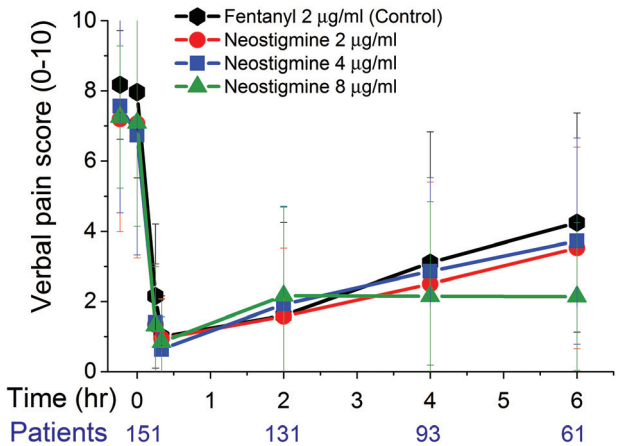

Fig. 3. Mean verbal pain scores (0 to 10) ± SD over time during labor.

groups ($P = 0.82$). The overall median satisfaction score was 4.0 (very satisfied) with a 1 to 5 scale. Patients in the epidural fentanyl group had a median satisfaction score of 4.0, whereas patients in the 2, 4, and 8 μg/ml epidural neostigmine groups had median satisfaction scores of 4.0, 4.0, and 4.5, respectively.

Labor progress did not differ among groups, nor did the cesarean delivery rate or neonatal outcomes (table 1). We also performed an intention-to-treat analysis of all patients, including those patients who were withdrawn from the study, for neonatal Apgar scores and mode of delivery. We found no significant difference in Apgar scores at 1 and 5 min ($P = 0.84$ and $P = 0.39$, respectively) or cesarean delivery rate ($P = 0.84$).

Epidural neostigmine at any of the doses studied did not cause greater intensity scores than epidural fentanyl of undesired side effects such as maternal nausea ($P = 0.66$), sedation ($P = 0.64$), shivering ($P = 0.40$), or degree of motor blockade ($P = 0.33$) (table 2). Average maximum pruritus scores of patients in the epidural fentanyl group were significantly greater than patients receiving epidural neostigmine ($P = 0.001$). We also examined whether the side effects of patients in the epidural fentanyl group (2 μg/ml) differed significantly from patients in the three epidural neostigmine groups (2, 4, and 8 μg/ml) at the time of epidural placement and over time. The four groups did not differ in the incidence of motor blockade, maternal self-assessment of nausea, maternal self-assessment of sleepiness, or pruritus over time (data not presented, fig. 4). Due to the significant decline in the number of patients in each group over time as patients underwent successful deliveries, the time scale for figure 4 has been limited to 6 h.

Discussion

Study solutions of epidural bupivacaine with varying doses of neostigmine (2 to 8 μg/ml) provide similar hourly epidural bupivacaine requirements to solutions of epidural bupivacaine with 2 μg/ml fentanyl in PCEA for labor. Within the definition of minimum clinically meaningful difference, epidural neostigmine was indistinguishable from epidural fentanyl as an analgesic adjunct to epidural bupivacaine.

Although a control group without epidural fentanyl was not included in this study, the use of epidural fentanyl at this concentration (2 μg/ml) is common and well documented to reduce local anesthetic use while still providing adequate labor analgesia.[15] The routine use of local anesthetic alone for epidural labor analgesia, without the addition of an adjuvant opioid, is uncommon in current practice in the United States.[16]

Because we found no significant difference in local anesthetic consumption for labor analgesia between neostigmine and fentanyl or among different doses of neostigmine, we were therefore unable to perform a subanalysis on the clinical dose response for epidural neostigmine. This is in contrast to the clear dose response seen for neostigmine to reverse neuromuscular blockade by its action on acetylcholinesterase. Neuraxial neostigmine may act in part by inhibiting meningeal acetylcholinesterase, thereby increasing the cerebrospinal fluid concentration of acetylcholine, resulting in increased bioavailability of acetylcholine in cholinergic spinal neurons.[17] However, the lack of dose response suggests a plateau effect on the blockade of meningeal acetylcholinesterase locally at the studied concentrations of epidural neostigmine, suggesting that neostigmine concentrations greater than 2 μg/ml under these infusion conditions are not needed and lower concentrations may be effective.

The study design also may have prohibited us from finding a significant difference in bupivacaine consumption between the three epidural neostigmine groups. The study was designed as a test of superiority, with the sample size deliberately constrained to ensure that the study only had 80% power to detect a difference in total bupivacaine consumption per hour between groups. Thus, we would not be able to detect a difference in bupivacaine consumption less than 20% between groups.

In addition, the concentration of bupivacaine (0.125%) used for labor PCEA in our study may have caused patients to reach the plateau phase of sensory blockade at the basal infusion rate. Thus, additional epidural adjuvants such as fentanyl or neostigmine may not have contributed to improving pain scores or reducing bupivacaine consumption.

Patients enrolled in either the epidural fentanyl or epidural neostigmine groups were very satisfied with their labor analgesia. This is likely because the protocol excluded poorly functioning epidurals with visual analog scale pain scores greater than 3 at 20 min after placement. Because labor pain relief was adequate and similar among all groups, satisfaction

Table 2. Side Effect Profile of Epidural Fentanyl *versus* Neostigmine

	Fentanyl, 2 μg/ml	Neostigmine, 2 μg/ml	Neostigmine, 4 μg/ml	Neostigmine, 8 μg/ml	*P* Value
Average maximum nausea score (0–10)	1 ± 2	2 ± 3	2 ± 3	1 ± 3	0.66
Average maximum sedation score (0–10)	4 ± 3	3 ± 3	3 ± 3	3 ± 3	0.64
Average maximum shivering score (0–10)	0 ± 0	0 ± 1	0 ± 0	0 ± 0	0.40
Average maximum pruritus score (0–10)	1 ± 2	0 ± 0	0 ± 0	0 ± 0	0.001*
Average maximum Bromage score (0–3)	1 ± 1	1 ± 1	1 ± 1	1 ± 1	0.33

All statistical variables are mean ± SD.

*Statistically significant ($P < 0.05$).

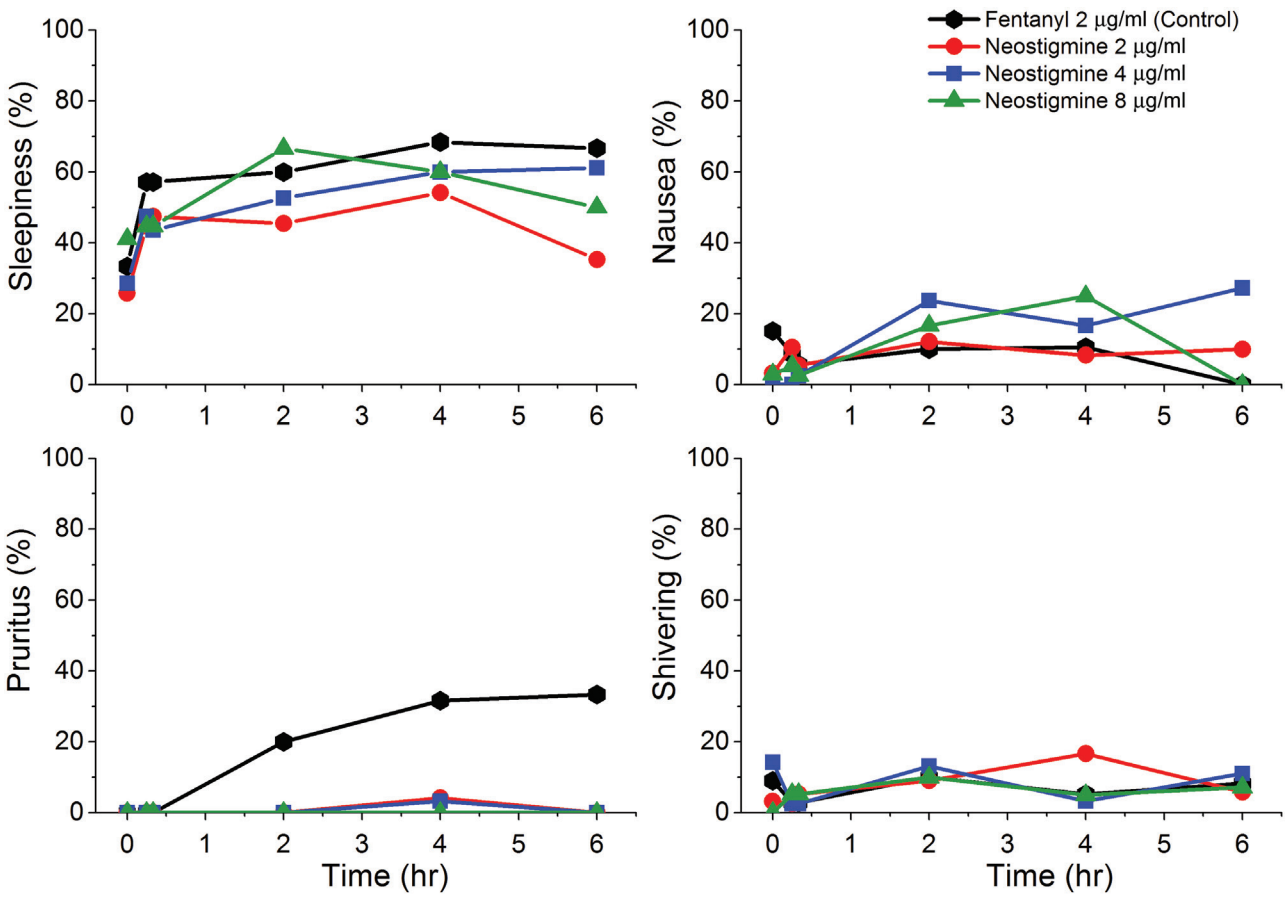

Fig. 4. Percent incidence of nonzero verbal scores of maternal sleepiness, nausea, pruritus, and shivering after initiation of patient-controlled epidural analgesia for labor.

scores were high. Pain scores increased overall in all four groups over time, likely related to labor advancement, epidural migration, and/or greater incidence of dysfunctional labor as time progressed. Overall labor satisfaction scores also may be influenced by concurrent delivery outcomes other than pain scores such as neonatal outcomes, duration of pushing, or need for forceps or vacuum delivery.

The maternal and neonatal outcomes in our study are consistent with previous smaller studies in obstetric patients,[18] showing no adverse effects on neonatal Apgar scores, maternal heart rate or blood pressure, or mode of delivery. Pruritus scores were significantly greater in the epidural fentanyl group, although the clinical importance of pruritus on a subjective basis is questionable due to the low mean reported scores. Other maternal side effects, such as motor block, nausea, and sedation, also were not significant among groups, suggesting that epidural neostigmine at these doses is well tolerated. Our study did not specifically examine fetal heart rate variability as a labor outcome, as this was felt to be logistically difficult due to the long duration of the infusion for labor analgesia and the potential for intermittent changes in the fetal heart rate tracing related to labor progression. We did record the fetal heart rate before and after epidural analgesia and every 2 h subsequently until delivery similar to the Ross *et al.* study,[12] and we found no significant difference between groups (data not shown). In addition, when performing an intention-to-treat analysis that included patients who were withdrawn from the study, we found no difference in neonatal Apgar scores or mode of delivery. The data from our study suggest that epidural neostigmine does not provide any clinical advantages or disadvantages over epidural fentanyl in terms of the overall side effect profile for labor analgesia.

Although neostigmine may be a more expensive alternative to fentanyl for epidural local anesthetic infusions, epidural neostigmine may be useful in a small number of clinical scenarios. Neostigmine may be a useful alternative in patients with extreme sensitivity (pruritus or vomiting) to opioids such as fentanyl. Neostigmine also can be used as a nonopioid adjunct alternative in women with a history of addiction or those who wish to avoid any opioid use for psychologic reasons. Neostigmine also may be used as an adjuvant for women who take buprenorphine or those with chronic opioid exposure secondary to chronic pain or addiction with potential significant dysregulation of opioid and pain receptors.

Major disadvantages of neostigmine include the fact that it remains an investigational drug by the U.S. Food

and Drug Administration for epidural use, the relatively small number of obstetric patients in the literature exposed to neuraxial neostigmine,[4,10,12,18–30] and the lack of clinical effect as measured by local anesthetic consumption in this study. Epidural neostigmine has not been shown to have adverse effects on maternal vital signs, maternal sedation, Apgar scores, or fetal heart rate tracings in this and previous studies, but there may be unrecognized or unusual side effects, given the overall small sample size in the literature to date.

In conclusion, we found that laboring parturients receiving epidural neostigmine in differing concentrations (2, 4, and 8 μg/ml) had similar hourly bupivacaine consumption and mean pain scores during labor compared with parturients receiving epidural fentanyl (2 μg/ml). Also, patients receiving either epidural fentanyl or epidural neostigmine combined with bupivacaine for epidural labor analgesia were satisfied equally at delivery. Although previous studies have demonstrated an improvement in postoperative analgesia in both adults and children with epidural neostigmine compared with epidural local anesthetic alone,[31,32] we were unable to show a clinical difference with epidural neostigmine compared with epidural fentanyl when combined with bupivacaine for labor analgesia. Although future studies are needed to further evaluate the clinical safety of neostigmine as well as the clinical effect of lower doses of epidural neostigmine on labor analgesia, the likelihood of futures studies are lessened by the intermittent inability to obtain neostigmine from the manufacturer due to production shortages, the cost of neostigmine, and lack of evidence showing a significant clinical effect compared with epidural fentanyl.

Research Support

Support was provided solely from institutional and/or departmental sources.

Competing Interests

The authors declare no competing interests.

Reproducible Science

Full protocol available at: jbooth@wakehealth.edu. Raw data available at: jbooth@wakehealth.edu.

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Neurophysiologic Correlates of Ketamine Sedation and Anesthesia

A High-density Electroencephalography Study in Healthy Volunteers

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ABSTRACT

Background: Previous studies have demonstrated inconsistent neurophysiologic effects of ketamine, although discrepant findings might relate to differences in doses studied, brain regions analyzed, coadministration of other anesthetic medications, and resolution of the electroencephalograph. The objective of this study was to characterize the dose-dependent effects of ketamine on cortical oscillations and functional connectivity.

Methods: Ten healthy human volunteers were recruited for study participation. The data were recorded using a 128-channel electroencephalograph during baseline consciousness, subanesthetic dosing (0.5 mg/kg over 40 min), anesthetic dosing (1.5 mg/kg bolus), and recovery. No other sedative or anesthetic medications were administered. Spectrograms, topomaps, and functional connectivity (weighted and directed phase lag index) were computed and analyzed.

Results: Frontal theta bandwidth power increased most dramatically during ketamine anesthesia (mean power ± SD, 4.25 ± 1.90 dB) compared to the baseline (0.64 ± 0.28 dB), subanesthetic (0.60 ± 0.30 dB), and recovery (0.68 ± 0.41 dB) states; $P < 0.001$. Gamma power also increased during ketamine anesthesia. Weighted phase lag index demonstrated theta phase locking within anterior regions (0.2349 ± 0.1170, $P < 0.001$) and between anterior and posterior regions (0.2159 ± 0.1538, $P < 0.01$) during ketamine anesthesia. Alpha power gradually decreased with subanesthetic ketamine, and anterior-to-posterior directed connectivity was maximally reduced (0.0282 ± 0.0772) during ketamine anesthesia compared to all other states ($P < 0.05$).

Conclusions: Ketamine anesthesia correlates most clearly with distinct changes in the theta bandwidth, including increased power and functional connectivity. Anterior-to-posterior connectivity in the alpha bandwidth becomes maximally depressed with anesthetic ketamine administration, suggesting a dose-dependent effect. **(Anesthesiology 2017; 127:58-69)**

KETAMINE is a general anesthetic with unique features at the molecular, neural, and behavioral levels. Unlike the intravenous and inhaled anesthetics in common clinical use, ketamine is thought to work primarily by antagonizing *N*-methyl-D-aspartate (NMDA) receptors[1–4] and hyperpolarization-activated, cyclic-nucleotide-gated channel 1,[5,6] without strong agonist effects on synaptic γ-aminobutyric acid (GABA) receptors. Furthermore, electroencephalographic characteristics of ketamine anesthesia are distinct from those associated with anesthetics that act primarily *via* GABA-receptor agonism. For example, coherent frontal alpha oscillations are observed with propofol-induced unconsciousness[7,8] that are believed to result from thalamocortical hypersynchrony[9] and that possibly inhibit corticocortical communication.[8] These same patterns are noted with ether-based volatile anesthetics during surgical

What We Already Know about This Topic

- Ketamine is a unique anesthetic with neural effects that are distinct from more commonly-used γ-aminobutyric acid agonists
- Although various electroencephalography studies of ketamine have been performed, none have assessed spectral power and connectivity at subanesthetic and anesthetic doses

What This Article Tells Us That Is New

- Ketamine had dose-dependent effects on spectral power, functional connectivity, and directed connectivity
- Anesthetic doses of ketamine resulted in markedly increased theta power across the cortex as well as increased gamma and delta power
- Increased anterior-posterior connectivity in the theta bandwidth and decreased connectivity in the alpha bandwidth were specific for ketamine anesthesia

This article is featured in "This Month in Anesthesiology," page 1A. P.E.V. and T.B.-B. contributed equally to this article. Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site (www.anesthesiology.org). This article has a video abstract.

 Anesthesiology 2017; 127:58-69

levels of unconsciousness.[10] Ketamine anesthesia, however, is not associated with these electroencephalographic characteristics.[11,12] During anesthetic induction with ketamine, an increase in gamma power has been consistently reported,[10,12,13] and when ketamine is administered in the presence of propofol or ether-based volatile anesthetics, there is a power shift to the beta bandwidth.[14,15] These effects may be due to anti-NMDA-mediated disinhibition of pyramidal neurons[16] and hyperpolarization-activated, cyclic-nucleotide-gated channel 1 inhibition.[15]

Previous studies[11–13] have examined the electroencephalographic effects of ketamine, but the precise neurophysiologic correlates remain unclear for a number of reasons. For example, ketamine has often been studied in the presence of other concomitantly administered sedative and anesthetic medications,[13,14,17] potentially altering or obfuscating neurophysiologic signatures specific to ketamine. Additionally, electroencephalogram studies to date have been constrained by either regionally limited analyses or low-resolution acquisition.[11–13] Studies have also focused on either subanesthetic[18,19] or anesthetic dosing,[11–13] without being able to directly compare dose-dependent electroencephalographic characteristics. Last, there is a dearth of information regarding recovery from ketamine-induced anesthesia, as studies have tended to focus on the beginning of surgical cases, with GABAergic anesthetics often coadministered or administered directly after ketamine induction. Improving the understanding of ketamine-specific effects on the electroencephalogram may provide insights into the neuroscientific correlates of consciousness, particularly given the unique molecular, neural, and phenomenologic features of ketamine that differ from other anesthetics. Thus, in this study, we used spectral and connectivity analyses of high-density electroencephalographic recordings to characterize neurophysiologic changes associated with ketamine as a single agent during subanesthetic administration, anesthetic dosing, and a recovery period. We hypothesized that ketamine would induce distinct, dose-dependent effects on spectral and functional connectivity patterns that would distinguish between these arousal states.

Materials and Methods

This study was approved by the University of Michigan Medical School Institutional Review Board, Ann Arbor, Michigan (HUM00061087), and written informed consent was obtained from all participants before the study. All study procedures were conducted at the University of Michigan Medical School, Ann Arbor, Michigan. Ten volunteers were recruited from September 2015 through January 2016 using recruitment flyers posted throughout the medical school and University Hospital of the University of Michigan, Ann Arbor, Michigan. Phone screening was conducted by a member of the research team to review inclusion and exclusion criteria before enrollment.

Submitted for publication November 13, 2016. Accepted for publication March 31, 2017. From the Department of Anesthesiology (P.E.V., T.B.-B., U.L., D.L., H.K., E.J., V.T., A.M.M., B.S.K., P.P., G.A.M.), Center for Consciousness Science (P.E.V., T.B.-B., U.L., D.L., H.K., G.A.M.), and Neuroscience Graduate Program (G.A.M.), University of Michigan Medical School, Ann Arbor, Michigan; and the Department of Anesthesiology, Northwestern University Feinberg School of Medicine, Chicago, Illinois (A.B.P.).

Study Population

Participants were considered eligible if they were American Society of Anesthesiologists physical status class I, between the ages of 20 and 40 yr, had a body mass index less than 30, and had no predictors of a difficult airway. Candidates were excluded from participation if they had cardiovascular disease, cardiac conduction abnormalities, hypertension, obstructive sleep apnea, asthma, ongoing respiratory illness, gastroesophageal reflux, history of drug use (or positive drug screen before the experiment), family history of problems with anesthesia, neurologic disorders, psychiatric disorders, or current pregnancy. The number of volunteers recruited was based on previous studies that investigated neurophysiologic correlates of anesthetic-induced unconsciousness.[7,20]

Anesthetic Protocol

Experiments were conducted between the hours of 8:00 AM and 1:00 PM for all participants except for one (participant No. 2, studied between 4:00 PM and 7:00 PM). Before initiation of the experiment, a full medical and anesthetic history was obtained, and a physical examination was performed. All participants fasted from food and drink for 8 h before the experiment. Peripheral intravenous catheters were placed, and American Society of Anesthesiologists standard monitors were applied before drug administration. At least two anesthesiologists were present for the full duration of the experiment. All participants underwent the following stepwise protocol, with electroencephalogram data collection throughout each period:

1. a 5-min eye-closed resting period before ketamine administration (baseline condition)
2. subanesthetic ketamine infusion (0.5 mg/kg) over 40 min (subanesthetic condition) with eyes closed, followed by 8 mg ondansetron for nausea and vomiting prophylaxis
3. break for completion of questionnaire (data not reported here)
4. anesthetic (1.5 mg/kg) bolus dose (anesthetic condition) with eyes closed
5. recovery period (recovery condition) with eyes closed

Participants were instructed to keep their eyes closed throughout each of these recording periods. Rather than using target-controlled infusions, we chose dosing strategies that were directly relevant to clinical care for either depression (0.5 mg/kg over 40 min) or anesthetic induction (1.5 mg/kg bolus) to enhance the translational relevance of this study. Participants were monitored through both loss of consciousness

(LOC) and return of consciousness (ROC) using a previously described protocol[20]; we acknowledge that our definition of LOC relates to consciousness of the environment and does not exclude the possibility of endogenous experiences such as dreams or hallucinations. In brief, volunteers held an object in each hand that makes a sound when squeezed. An audio loop then started playing that instructed participants, in a random manner, to squeeze their right or left hand. This audio loop would deliver a command every 30 s, and the loop played before, during, and after anesthetic ketamine bolus administration. LOC was marked when subjects ceased to respond to two audio commands in a row. A scopolamine patch (1.5 mg) was also placed after ROC for further nausea and vomiting prophylaxis. Participants were then evaluated and monitored after the conclusion of the experiment. Additional antiemetics were administered if needed. Participants were discharged once they were awake, alert, and responsive; had no significant nausea or vomiting; were able to ambulate with minimal assistance; and had a responsible adult to accompany them home. Follow-up calls were made to patients both the evening after the experiment and the next day.

Electroencephalography Analysis

Data Acquisition. Electroencephalogram data were acquired with 128-channel HydroCel nets, Net Amps 400 amplifiers, and Net Station 4.5 software (Electrical Geodesics, Inc., USA). The electroencephalogram was digitized continuously at 500 Hz with a vertex reference. Per manufacturer recommendations, channel impedances were kept at less than 50 kΩ, and the net was wrapped with gauze to optimize contact between the electrodes and scalp.

Spectral Processing and Analysis. Data processing was performed with Chronux (http://chronux.org/)[21,22] and custom MATLAB (MathWorks, USA) scripts and toolboxes. All visual inspection was performed by one of the investigators (T.B.-B.) trained in dense electroencephalography review. After each recording session, the data were bandpass-filtered at 0.5 to 55 Hz (eegiltnew, firfilt plugin, zero-phase, Hamming-windowed finite impulse response filter, 3,301 points (6.6 s), 0.5 Hz transition band, 0.5 and 55 pass-band edges, –6 dB cutoff frequencies: 0.25 and 55.25) to decrease the influence of low-frequency drift and high-frequency artifacts. Electrodes on the lowest parts of the face and head were removed, leaving 98 remaining channels. Bad channels were then detected and removed using visual inspection and the *rejchan* and *clean rawdata* functions, with 86 to 91 channels retained. The channels were then average-referenced to the mean of the voltage across all remaining channels. Conditions of interest—the baseline eyes-closed period (5 min), subanesthetic infusion (0 to 38 min), anesthetic bolus dose (from LOC to 5 min after LOC, 3.8 min for one volunteer), and recovery period (5 min, collected 8 to 15 min after ROC)—were extracted from the data for each participant. These specific time epochs were chosen to include data relatively preserved from artifact and to represent stable neurophysiologic periods for each of the four experimental conditions. Large artifacts were removed after visual inspection and the *rejcont* function. Remaining periods of continuous data were segmented into 3-s epochs. Data epochs with remaining large artifacts were removed *via* a combination of visual inspection and the *rejkurt*, *rejtrend*, and *eegthresh* functions. For each participant, the remaining epochs from the four periods of interest were submitted to independent component analysis (runica function, infomax, extended). Independent components representing eye blink, lateral eye, muscle, facial electromyography, focal channel noise, and focal trial noise were removed from all epochs using visual inspection and the following component classification plugins: SASICA (semi-automated selection of independent components of the electroencephalogram for artifact correction),[23] IC-MARC (independent components of electroencephalogram into multiple artifact classes),[24] and ADJUST (automatic electroencephalogram artifact detection based on the joint use of spatial and temporal features).[25] All remaining epochs for each period and each participant were then visually inspected again to remove any remaining epochs with excessive artifacts. For the four conditions (baseline, subanesthetic, anesthetic, and recovery), the mean remaining trial counts were 54 (SD = 17), 574 (SD = 48), 66 (SD = 8), and 73 (SD = 11), respectively. The previously removed channels in each data set were interpolated to the 98-channel subset. Spectral power was computed with multitaper spectral analyses (*mtspecgramc* function; time window: 3 s, overlap: 0.5 s, number of tapers: 3, time-bandwidth product: 5, spectral resolution: 0.25 Hz). The median absolute power (10*log10[μV^2/Hz]) was calculated for each of the four experimental periods at each of five frequency bands (delta: 1 to 4 Hz, theta: 4 to 8 Hz, alpha: 8 to 13 Hz, beta: 13 to 30 Hz, gamma: 30 to 48 Hz) for all 98 channels and then for eight frontal channels centered on the Fz site (figure in Supplemental Digital Content 1, http://links.lww.com/ALN/B458). The final data presented in the spectrograms are the sequential, 3-s epochs (grand-averaged across all participants) that remained after the data-cleaning steps described above were implemented. The data were temporally sequential but not necessarily contiguous given that some epochs were removed after cleaning and artifact removal. Images presented are log-transformed (10*log10 transform of the grand average of the single-subject data). The recovery period data for one participant were unavailable, and therefore recovery period calculations were completed for only nine participants.

Topographic Analysis. The topographic maps of spectral power for each experimental condition and electroencephalogram bandwidth were constructed using the topoplot function in the MATLAB (MathWorks) toolbox EEGLAB.[26]

Connectivity Processing and Analysis. The selected continuous data epochs from the spectral analyses were used for connectivity analysis without channel interpolation or independent component analysis; this was done to preserve the phase information of the original signals.[27,28] Additionally, eight occipital channels were removed due to excessive artifact presence, which was deemed necessary for connectivity

analyses, leaving 90 remaining channels. The functional connectivity between electroencephalogram channels was examined across all experimental conditions. The undirected and directed connectivity were measured with debiased weighted phase lag index (wPLI)[29] and directed phase lag index (dPLI),[30] respectively. The wPLI is a measure of how the instantaneous phases of two electroencephalogram signals are phase-locked to each other. If the instantaneous phase of one signal is consistently ahead or behind of the other signal, the phases are considered locked, and wPLI = 1. However, if the signals randomly alternate between a phase lead and a phase lag relationship, there is no phase locking, and wPLI = 0. The directionality of functional connectivity between two electroencephalogram signals was determined with the asymmetry of the phase lead/lag relationship. If the instantaneous phase of one signal consistently leads that of the other signal, dPLI = 1, and with the inverse case, dPLI = −1. If there is no a bias in the phase lead/lag relationship, then dPLI = 0. In practice, both measures are robust to the volume conduction problem of scalp electroencephalography. Electroencephalogram signals were divided into 1-min-long epochs with 50% overlap, which was further divided into 2-s nonoverlapping subepochs. For each subepoch, the cross-spectral density was estimated using the multitaper method, with time-bandwidth product = 2 and the number of tapers = 3,[21,22] and from these repetitions the averaged wPLI and dPLI values at variable frequencies were estimated using a custom-written function adapted from the Fieldtrip toolbox.[31] To remove the bias of the measures for a given electroencephalogram data set, the shuffled-data method was used. A series (N = 20) of shuffled signal pairs were generated for each pair of electroencephalogram signals, and the wPLI and dPLI measures were calculated with the shuffled data, the mean of which was subtracted from the raw wPLI and dPLI values as the final estimation of undirected and directed connectivity.[32] Final baseline and recovery time epochs were chosen as 2-min segments selected in the middle of each respective recording. Subanesthetic epochs were selected as two sequential 2-min epochs (noted in the figures as Subanes-1 and Subanes-2) that were representative of the observed spectral power during the subanesthetic infusion period. Anesthetic epochs (noted in the figures as Anes-1 and Anes-2) were chosen and displayed in the same manner.

Statistical Analysis

We tested for differences between the experimental periods (baseline, subanesthetic, anesthetic, and recovery) by entering the single-subject electroencephalogram-derived values into a linear-mixed model analysis in SPSS 22 (IBM, USA). Linear-mixed model analysis allowed for statistical comparisons in the event of partially missing data from a given participant (as was the case with missing recovery period data from one volunteer, noted above). Each experimental period served as the fixed factor with participant data serving as a correlated random effect. We used restricted maximum likelihood estimation and diagonal covariance structure with heterogenous variances and zero correlation between elements. Direct pairwise comparisons were adjusted using Bonferroni's method. We did not model repeated covariance effects, and a P value less than 0.05 was considered statistically significant. The average of median connectivity values was reported for connectivity data, as has been previously described methodologically.[7] All variability estimates are in SD.

Results

All study participants successfully completed subanesthetic and anesthetic dosing protocols; 6 of 10 (60%) experienced nausea and vomiting after ketamine anesthesia, and 5 of 10 (50%) required additional antiemetic treatment. One volunteer (1 of 10, 10%) briefly required a chin lift and jaw thrust to maintain airway patency shortly after LOC. No adverse clinical events were otherwise noted. All 10 participants experienced LOC after the 1.5 mg/kg ketamine bolus; the mean time between bolus administration and LOC was 1.23 (±0.35) min, and LOC lasted an average of 10.68 (±3.51) min.

Dose-dependent Global Effects of Ketamine—Spectral and Topographic Analysis

Prominent changes were apparent across theta, alpha, and gamma bandwidths. There was a marked increase in theta power during ketamine anesthesia (figs. 1 and 2, and Supplemental Digital Content 2, http://links.lww.com/ALN/B459, for individual participants), and this power localized to both frontal and posterior channels (fig. 2). There was a reduction in posterior alpha power from baseline through anesthetic dosing, and no increase or anteriorization of alpha power was noted with either ketamine dose (fig. 2). Increased gamma power was also apparent during the anesthetic epoch compared to other states (figs. 1 and 2).

Frontal Channel Cluster—Ketamine-induced Changes across Brain States

Frontal channels are of particular interest given their accessibility and use in the operating room. During the subanesthetic infusion, a gradual dissipation of alpha power was noted compared to baseline (mean power ± SD, 0.46 ± 0.34 and 0.88 ± 0.80 dB, respectively), and alpha power remained decreased during the periods of ketamine anesthesia (0.49 ± 0.22 dB) and recovery (0.34 ± 0.38 dB); $F(3,26) = 3.679$, $P < 0.05$ (fig. 3, A and B). With anesthetic dosing, there was a significant increase in theta bandwidth power (4.25 ± 1.90 dB) compared to baseline (0.64 ± 0.28 dB), subanesthetic (0.60 ± 0.30 dB), and recovery (0.68 ± 0.41 dB) periods; $F(3,26) = 41.01$, $P < 0.001$ (fig. 3, A and B; table 1). Theta power was not otherwise significantly modulated during nonanesthetic periods, and relative increases in theta power during ketamine anesthesia were higher than that of any other bandwidth (table 1).

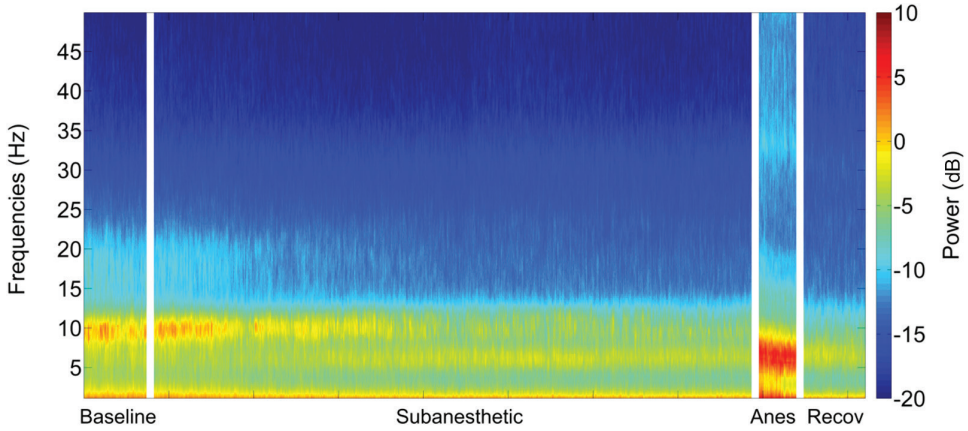

Fig. 1. Group-level absolute power spectrogram (0.05- to 55-Hz bandpass filter). There is a marked increase in theta bandwidth power during ketamine anesthesia. Anes = anesthetic period; Recov = recovery period.

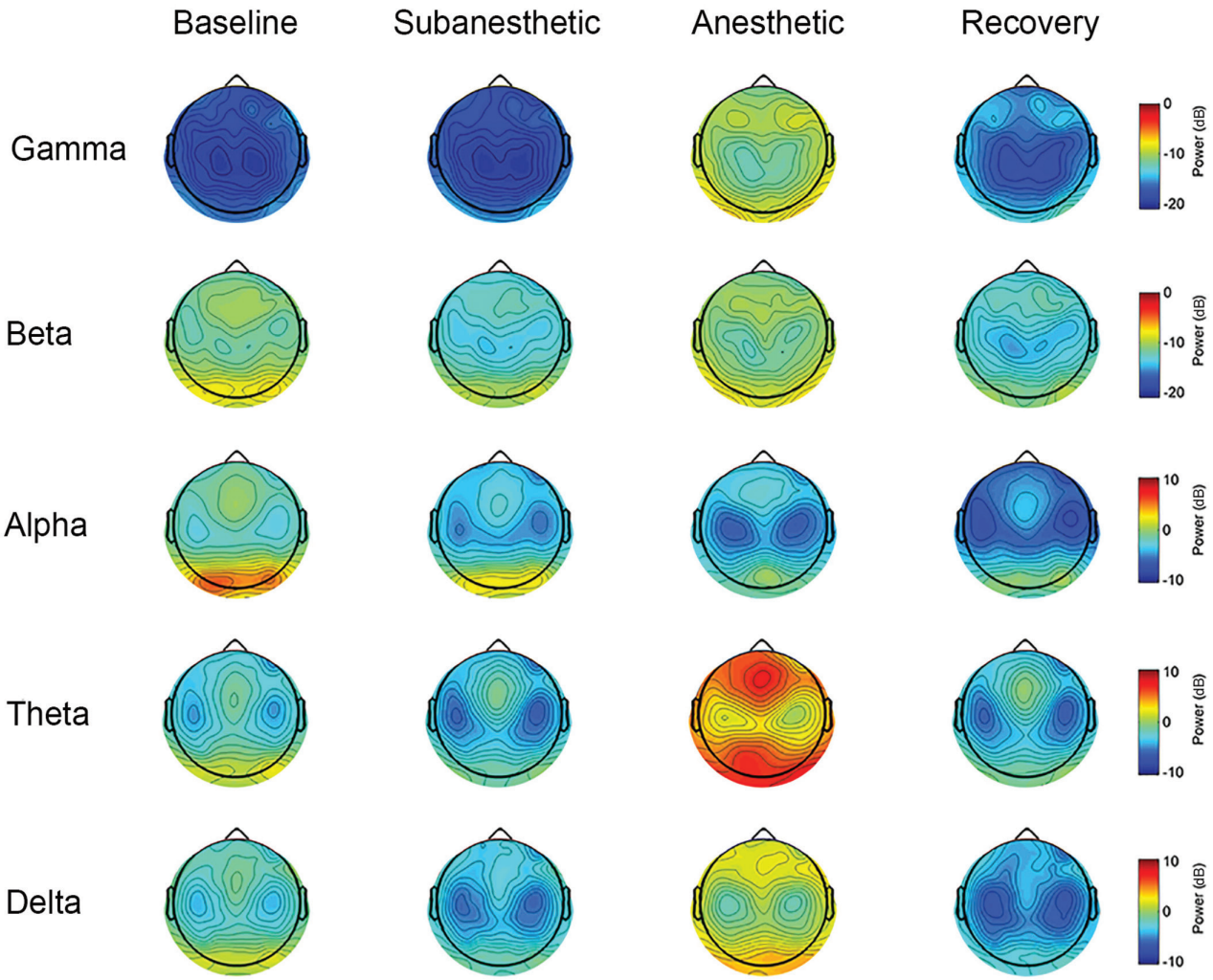

Fig. 2. Topomaps from retained trials for each condition. During ketamine anesthesia, theta power increases in frontal and posterior channel clusters. Posterior alpha power decreases sequentially during subanesthetic and anesthetic ketamine dosing, and no anteriorization of alpha power is noted during anesthetic dosing (delta, 1 to 4 Hz; theta, 4 to 8 Hz; alpha, 8 to 13 Hz; beta, 13 to 30 Hz; and gamma, 30 to 48 Hz).

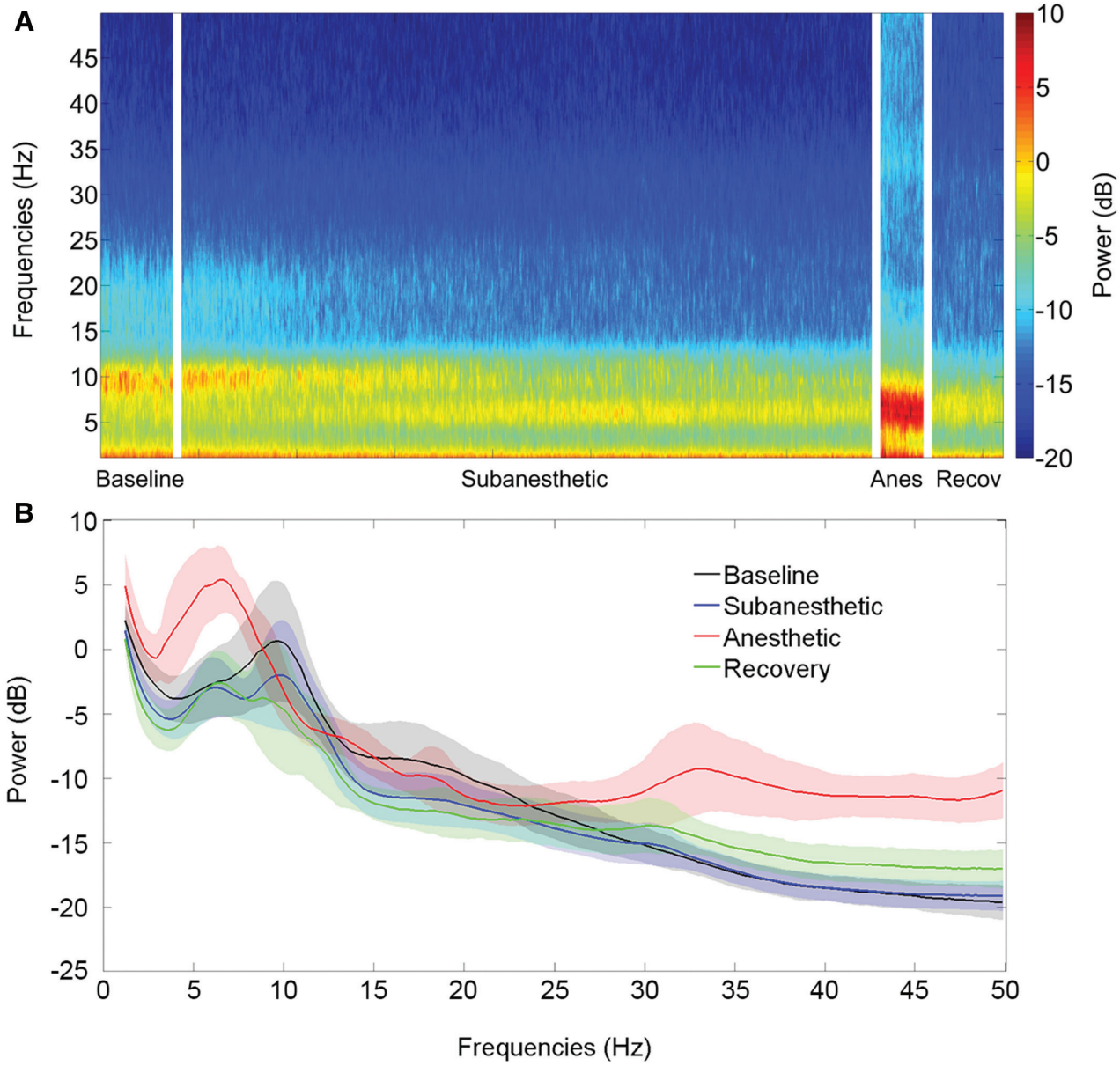

Fig. 3. Frontal channel, group-level spectrogram, and line graph (see figure in Supplemental Digital Content 1, http://links.lww.com/ALN/B458, for electrode placement location). (*A*) Spectrogram depicts marked increase in frontal channel theta power during the anesthetic period. Increased gamma bandwidth power compared to baseline is noted as well. (*B*) Line graph demonstrates a shift to theta bandwidth power during ketamine anesthesia. *Shaded regions* represent ±1 SD. Anes = anesthetic period; Recov = recovery period.

Functional Connectivity Analyses

Given the significant increases in theta power and decreases in alpha power with progressive ketamine dosing, we undertook connectivity analyses in these bandwidths across each condition. Reduced functional connectivity in the alpha bandwidth has also been previously demonstrated with anesthetic ketamine dosing,[11] further motivating our choice of bandwidths for analysis. Although we noted marked increases in gamma bandwidth power, electromyographic artifact might contribute to gamma activity despite our data cleaning.[33,34] Thus, we did not examine gamma connectivity in this study. Furthermore, subanesthetic ketamine has been shown to increase gamma power in other paradigms,[35,36] whereas increased theta power seems to correlate specifically to the anesthetized state.[19,37,38]

Weighted Phase Lag Index—Theta Phase Locking during Ketamine Anesthesia

Figure 4 presents the global and regional wPLI changes across each experimental condition. Figure 4A shows the average median wPLI between anterior and posterior regions in the time and frequency domains (see Supplemental Digital

Table 1. Mean Power Values across Ketamine Doses and Electroencephalogram Bandwidths—Frontal Channels

	Mean Power ± SD, dB			
Bandwidth	Baseline	Subanesthetic Dose	Anesthetic Dose	Recovery
Gamma (> 30 Hz)	0.02 ± 0.00	0.02 ± 0.00	0.09 ± 0.06	0.03 ± 0.01
Beta (13–30 Hz)	0.09 ± 0.04	0.06 ± 0.02	0.08 ± 0.03	0.06 ± 0.02
Alpha (8–13 Hz)	0.88 ± 0.80	0.46 ± 0.34	0.49 ± 0.22	0.34 ± 0.38
Theta (4–8 Hz)	0.64 ± 0.28	0.60 ± 0.30	4.25 ± 1.90	0.68 ± 0.41
Delta (1–4 Hz)	0.72 ± 0.23	0.46 ± 0.13	1.39 ± 0.70	0.36 ± 0.12

Absolute power values across conditions and frequency bandwidths in frontal channels are shown. Linear-mixed modeling analysis was used for statistical comparisons; $P < 0.001$ for all bandwidths except alpha ($P < 0.05$).

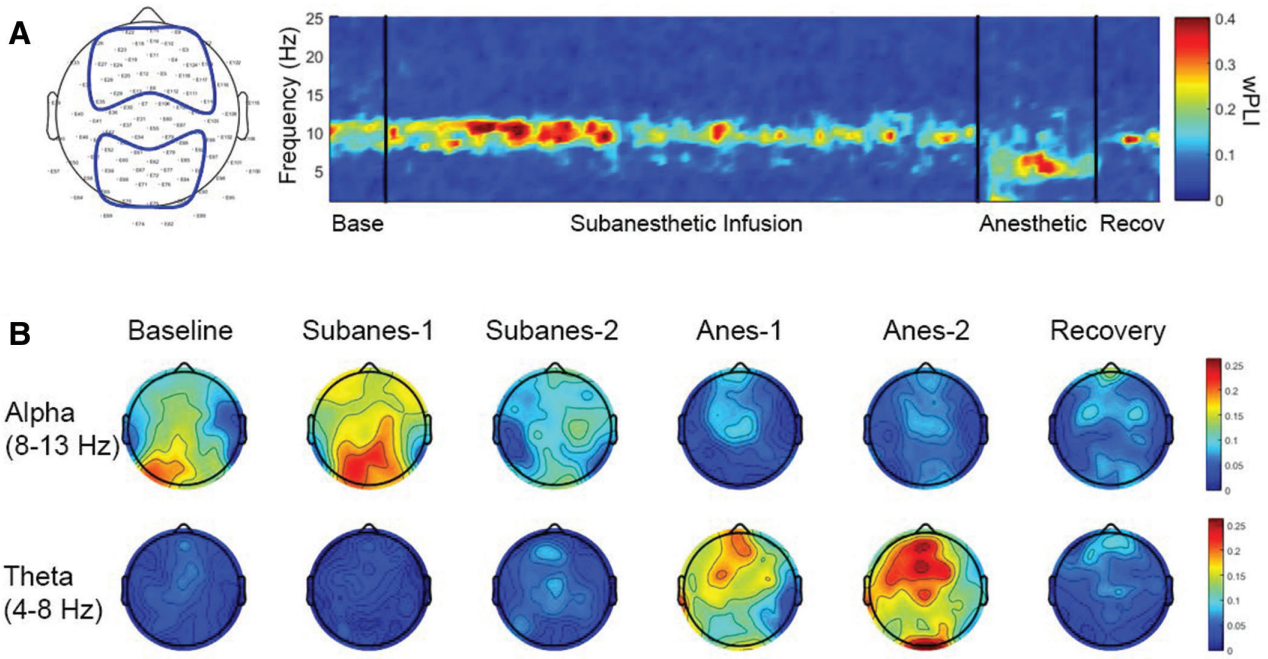

Fig. 4. Weighted functional connectivity changes, as assessed by weighted phase lag index (wPLI). (*A*) Group level connectogram of the mean wPLI between anterior and posterior regions. The *vertical black lines* separate the baseline, subanesthetic, anesthetic, and recovery periods. The time periods during subanesthetic and anesthetic recordings were rescaled; the *horizontal axis* indicates number of epochs (1-min long with 50% overlapping). (*B*) Scalp topography of the mean wPLI at alpha (8 to 13 Hz) and theta (4 to 8 Hz) for the six periods studied. Anes-1 = first anesthetic period examined; Anes-2 = second anesthetic period examined; Base = baseline period; Recov = recovery period; Subanes-1 = first subanesthetic period examined; Subanes-2 = second subanesthetic period examined.

Content 3, http://links.lww.com/ALN/B460, for individual participants). Alpha wPLI was dominant at baseline and increased during the initial phase of the subanesthetic infusion (fig. 4A). During the anesthetic period, however, wPLI connectivity strength shifted to the theta bandwidth (fig. 4A). Figure 4B presents the topographic changes of global wPLI networks for the theta and alpha bandwidths across experimental conditions. Within the anterior region, theta wPLI was highest during the anesthetic period (mean wPLI ± SD; 0.2349 ± 0.1170) compared to all other states; $F(5,44) = 7.996$, $P < 0.001$ (fig. 3B). Theta wPLI was also highest during ketamine anesthesia between anterior and posterior regions (0.2159 ± 0.1538); $F(5,44) = 4.192$, $P < 0.01$ (fig. 3B). Alpha wPLI within the anterior region remained relatively unchanged throughout each study condition, $F(5,44) = 0.697$, $P = 0.628$, although significant changes were noted between anterior and posterior regions, with mean wPLI lowest (0.0833 ± 0.1083) during the anesthetic period; $F(5,44) = 3.147$, $P < 0.05$ (fig. 4B).

Directed Phase Lag Index—Directional Connectivity Changes during Ketamine Anesthesia

Figure 5 demonstrates how the directionality of functional connectivity changed across the experimental conditions. Figure 5A shows the average median dPLI between anterior and posterior electrodes in the time and frequency domains (see Supplemental Digital Content 3, http://links.lww.com/ALN/B460, for individual participants). An anterior-to-posterior directionality

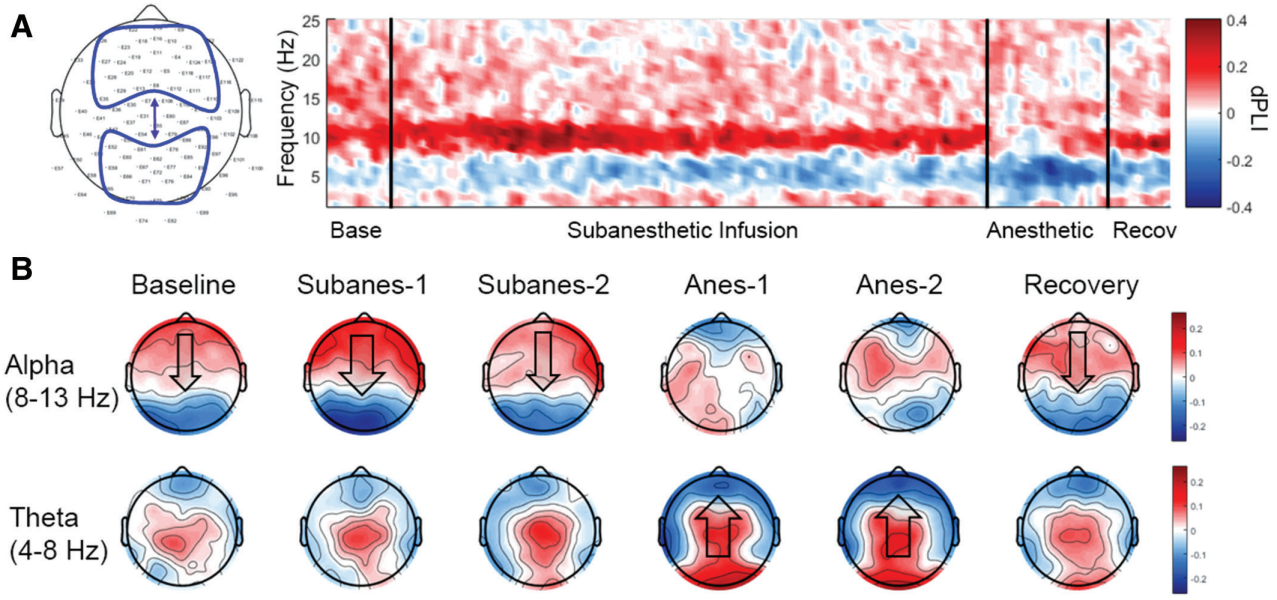

Fig. 5. Directional functional connectivity changes, as assessed by directed phase lag index (dPLI), across all conditions in the alpha and theta bandwidths. (*A*) Group-level connectogram of the mean dPLI between anterior and posterior regions. The *vertical black lines* separate the baseline (Base), subanesthetic, anesthetic, and recovery periods. Time on the *horizontal axis* was rescaled; epochs are 1-min long with 50% overlap. (*B*) Scalp topograph of the mean dPLI (in each channel and with all other channels) at alpha and theta for the six studied conditions. Anes-1 = first anesthetic period examined; Anes-2 = second anesthetic period examined; Recov = recovery period; Subanes-1 = first subanesthetic period examined; Subanes-2 = second subanesthetic period examined.

(the positive dPLIs denoted with red color) was observed in the alpha bandwidth during the baseline and subanesthetic periods. Theta shows a consistently opposite directionality from posterior-to-anterior regions (the negative dPLI denoted with blue color). Figure 5B shows the frequency-specific and state-specific topographies of electroencephalographic directionality. Alpha dPLI was maximally reduced between anterior and posterior channels during ketamine anesthesia (mean dPLI ± SD, 0.0282 ± 0.0772); $F(5,44) = 2.484$; $P < 0.05$ (fig. 4B). Alpha dPLI was also examined between prefrontal and frontal channels and was again maximally reduced during ketamine anesthesia (−0.0224 ± 0.0400); $F(5,44) = 4.137$; $P < 0.01$ (fig. 4B). Compared to baseline (0.0427 ± 0.0717), direct pairwise comparisons (with Bonferroni correction) revealed a statistically significant reduction in alpha dPLI only during each of the anesthetic epochs (Anes-1, −0.0170 ± 0.0481, $P < 0.05$; Anes-2, −0.0224, ± 0.0400, $P < 0.05$). Thus, an overall maximally reduced anterior-to-posterior connectivity pattern was demonstrated with alpha dPLI during ketamine anesthesia among these channel regions. Although a posterior-to-anterior pattern was noted with theta dPLI during ketamine anesthesia (fig. 5B), these associations did not reach statistically significant differences between anterior and posterior channels, $F(5,44) = 0.177$, $P = 0.970$, or for prefrontal and frontal channels, $F(5, 44) = 0.768$, $P = 0.578$.

Discussion

This study characterized ketamine-induced electroencephalographic changes during subanesthetic, anesthetic, and recovery periods (relative to baseline) in healthy volunteers using high-density electroencephalography. During subanesthetic ketamine administration, spectral power gradually (and modestly) shifts from the alpha bandwidth to the theta bandwidth, with anterior-to-posterior connectivity (as measured by alpha dPLI) maintained. During ketamine anesthesia, however, the power shifts dramatically to the theta bandwidth, theta wPLI increases in anterior and posterior regions, and anterior-to-posterior alpha connectivity (as measured by alpha dPLI) is significantly reduced. Upon recovery, these connectivity patterns return to near-baseline levels in each bandwidth. Ketamine anesthesia was also associated with increased gamma and delta power, as previously reported,[10,12,13] and frontal gamma power remained slightly elevated compared to baseline.

Our results demonstrate increased theta power correlating with ketamine anesthesia. Of note, increased theta oscillatory activity during ketamine anesthesia was first documented more than five decades ago. In the seminal human study that first examined the pharmacologic effects of ketamine in 1965, Domino *et al.*[37] remarked, "Characteristic thetalike waves were recorded at dosage levels which produced coma; these were distributed over most of the cerebral hemisphere." Increased theta activity has since been documented during anesthetic ketamine administration.[13,15,38] A strictly receptor- and channel-based explanation for this theta resonance may be difficult to describe, because ketamine has a considerably diverse array of molecular targets.[1,2,5,6,39–42] A network-level consideration of the phenomenon might provide insight regarding the consequences for information processing. In the

awake state, theta and alpha frequencies interact in a circular, reciprocating pattern of "information flow" between anterior and posterior brain regions.[43,44] Although oscillatory amplitude and phase are theoretically independent, amplitude (and thus power) may associate with phase to facilitate interactions with other neuronal populations within a network.[45] As alpha power and anterior-to-posterior connectivity become maximally depressed during ketamine anesthesia, increased theta power and phase locking between the anterior and posterior regions may represent large-scale network disequilibrium. This is, however, a speculative interpretation, and additional studies are needed to further explore the neurobiologic relevance of such connectivity patterns. From a clinical perspective, the distinct appearance of theta power during anesthesia may be informative for clinicians who routinely administer ketamine. The appearance of increasing theta power could alert clinicians to anesthetic ketamine dosing, which may be particularly helpful in settings where subanesthetic dosing is desired (as in the treatment of depression).[46,47] As the global and frontal spectrograms in this study both illustrated marked increases in theta power during ketamine anesthesia, the use of frontal electroencephalogram channels alone may be sufficient to monitor for this theta signature.

It is notable that ketamine anesthesia is associated with a maximal depression of anterior-to-posterior connectivity in the alpha bandwidth. Inhibition of frontal-to-parietal connectivity (with certain measures) has correlated with general anesthesia in humans across a diverse range of anesthetics,[12,32,48,49] and our group has previously demonstrated reduced alpha directional connectivity during ketamine anesthesia with low-resolution electroencephalography.[11] Recently, disruptions in NMDA– and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid–mediated frontoparietal connectivity patterns have been demonstrated during subanesthetic ketamine administration in healthy volunteers,[35] who remained conscious during these experiments. This raises the possibility that disrupted frontal-to-parietal connectivity may not strictly correlate with anesthetic-induced unconsciousness. An alternative explanation, however, is that such connectivity was not maximally depressed during these subanesthetic infusion periods. Findings from our study demonstrate that alpha directional connectivity remained relatively unchanged during subanesthetic dosing, decreased dramatically during anesthetic ketamine administration, and returned to baseline levels at recovery. The connectograms (figs. 4A and 5A) demonstrate significant functional connectivity in the alpha bandwidth during baseline and subanesthetic states—despite a gradual reduction of power—and this connectivity diminishes significantly during ketamine anesthesia. Thus, there may be a dose-dependent relationship between connectivity and loss of consciousness. In fact, Untergehrer *et al.*[50] illustrated dynamic fluctuations in functional connectivity patterns, whereby maximum functional connectivity occurs during varying time intervals on a continuous scale, with surrogates of information transfer times being fastest during unconsciousness. Accordingly, connectivity is a dynamic process and may need to cross certain real-time, continuous thresholds to correlate with varying levels of consciousness.

Multiple and unique strengths of this study are worth highlighting. No other sedative, opioid, or hypnotic medications were administered; thus, electroencephalographic effects of ketamine were not confounded by coadministration of other psychoactive medications. Additionally, an advanced-data cleaning process was used for spectral analysis. In addition to visual inspection, data from each time epoch underwent independent component analysis blind-source separation, and independent components representing eye blink, eye muscle, facial muscle, channel noise, and single-trial artifact were removed. Analytic comparisons were also made across multiple states—we were able to compare effects of ketamine before administration, at both subanesthetic and anesthetic doses, and during recovery. High-density electroencephalography was utilized, allowing for high-resolution and multiregional analyses. Last, functional connectivity based on phase relationships was also assessed with the use of wPLI and dPLI. To our knowledge, this is the first study to combine all of these methodologic strengths for the electroencephalographic analysis of ketamine in humans (table 2).

Table 2. Related Human Studies Examining the Effects of Ketamine on Electroencephalographic and Magnetoencephalographic Recordings

Study	High-density Data Acquisition	Subanesthetic Dosing	Anesthetic Dosing	Recovery Period	Ketamine Only	Connectivity/ Coherence Analysis
Domino *et al*., 1965[37]		*	*	*	*	
Schüttler *et al*., 1987[38]		*	*	*	*	
Kochs *et al*., 1996[19]		*	*	*	*	
Lee *et al*., 2013[12]			*		*	*
Blain-Moraes *et al*., 2014[11]			*		*	*
Muthukumaraswamy *et al*., 2015[35]	*	*		*	*	*
Rivolta *et al*., 2015[36]	*	*			*	*
Akeju *et al*., 2016[13]			*			*
Vlisides *et al.,* 2017 (current article)	*	*	*	*	*	*

*Presence of the specified methodologic consideration for each given study.

There are significant limitations to the study as well. Administering the subanesthetic ketamine infusion immediately before anesthetic dosing may influence neurophysiologic patterns observed during the anesthetic period. Our spectral data demonstrate a gradual reduction in alpha bandwidth power and increased theta power during the subanesthetic infusion. Whether this preceding subanesthetic exposure modulates subsequent neurophysiology and network connectivity during anesthetic dosing is unclear. Additionally, although a 128-channel electroencephalogram system was used, 86 to 91 channels were ultimately retained for spectral analyses after removal of artifact and bad channels. These artifact-removal strategies may also yield spectral patterns that differ from a real-world, clinical setting. For example, ocular artifact removal may have attenuated changes in delta power that might otherwise be present.[51] As we wanted to optimize electroencephalogram data for connectivity analyses, we did not employ the same artifact-removal strategies (*e.g.*, independent component analysis) as the spectral analyses, because they may interfere with phase-synchronization data.[27,28] Thus, spectral and connectivity data results emerged from different data-processing steps. The administration of both ondansetron and scopolamine could potentially have modulated central neurophysiology, but we regarded this as unlikely, and both of these medications were ultimately warranted for participant comfort. Participants were instructed to keep their eyes closed during each experimental period, but sporadic eye opening was noted throughout the recording sessions. This could have impacted certain epochs of the neurophysiologic data acquisition and analysis.

There are also significant limitations to consider regarding connectivity analyses. Although connectivity measures (in this case, wPLI and dPLI) are used as surrogates for functional interactions across the cortex, this is merely an assumption. Our recent data in nonhuman primates confirm a breakdown of corticocortical information transfer during ketamine anesthesia,[52] but different connectivity measures or experimental conditions can yield disparate results.[43] The phase lag index can also be associated with significant intersubject variability, particularly given the complex neural processes associated with these phase-based methods; as a result, phase lag index is often not amenable to statistical averaging or traditional artifact reduction strategies.[53] In terms of the general interpretation of directional connectivity results, it must be kept in mind that these measures reflect a large spatial scale and a long temporal scale, with an unclear relationship to the underlying neural spiking networks. As an intuitive example, the Dow Jones Industrial Average samples the performance of only a select number of publically traded companies. As a single value that rises or falls, it does not capture the rich dynamics and widespread stock exchange on the trading-room floor. Similarly, directed connectivity measures sample features that might reflect large-scale cortical interactions, but they do not capture the neuronal dynamics and widespread information exchange in cortical and subcortical systems. With that being said, changes in directed connectivity measures and surrogates of information transfer observed in the brain during anesthetic-induced unconsciousness have been shown to reflect fundamental network properties.[45]

In summary, this study used high-resolution electroencephalographic data to characterize ketamine-induced changes across multiple doses and associated levels of consciousness. Notably, increased theta power and phase locking occur between anterior and posterior regions during ketamine anesthesia, returning to baseline upon recovery. Anterior-to-posterior connectivity in the alpha bandwidth was maximally suppressed during ketamine anesthesia, and this connectivity is also restored to baseline levels during recovery. This constellation of power and connectivity results (increased theta power, as seen during rapid-eye-movement sleep, coupled with loss of anterior-to-posterior alpha connectivity, as seen during anesthesia) might contribute to the unique qualities of ketamine anesthesia.

Acknowledgments

The authors thank Yumeng Li, M.S., Center for Statistical Consultation and Research, University of Michigan, Ann Arbor, Michigan, for statistical consultation.

Research Support

Supported by grant Nos. T32GM103730 (to Drs. Vlisides and Mashour) and R01GM111293 (to Dr. Mashour) from the National Institutes of Health, Bethesda, Maryland, and by the Department of Anesthesiology, University of Michigan, Ann Arbor, Michigan.

Competing Interests

The authors declare no competing interests.

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GNAQ TT(-695/-694)GC Polymorphism Is Associated with Increased Gq Expression, Vascular Reactivity, and Myocardial Injury after Coronary Artery Bypass Surgery

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ABSTRACT

Background: Angiotensin II receptor type 1–mediated activation of the α-subunit of the heterotrimeric Gq protein evokes increased vasoconstriction and may promote hypertrophy-induced myocardial damage. The authors recently identified a TT(-695/-694)GC polymorphism in the human Gq promoter, the GC allele being associated with an increased prevalence of cardiac hypertrophy. In this article, the authors tested whether the TT(-695/-694)GC polymorphism is associated with differences in (1) myocardial Gq protein expression, (2) vascular reactivity, and (3) myocardial damage after coronary artery bypass grafting.

Methods: Gq protein expression was measured in right atrial muscle from 55 patients undergoing coronary artery bypass grafting as were skin perfusion changes (n = 18; laser Doppler imaging), saphenous vein ring vascular reactivity (n = 50, organ bath) in response to angiotensin II, and myocardial damage (227 patients undergoing coronary artery bypass grafting), as assessed by postoperative cardiac troponin I concentration.

Results: Myocardial Gq expression was greater in GC/GC genotypes (GC/GC *vs.* TT/TT: 1.27-fold change; $P = 0.006$). Skin perfusion after intradermal angiotensin II injection decreased only in GC/GC genotypes ($P = 0.0002$). Saphenous vein rings exposed to increasing angiotensin II concentrations showed an almost doubled maximum contraction in GC/GC compared with individuals with the TT/TT genotype ($P = 0.022$). In patients undergoing coronary artery bypass grafting, baseline cardiac ejection fraction was different (GC/GC: 55 ± 13%; GC/TT: 54 ± 14%; TT/TT: 48 ± 15%; $P = 0.037$) and postoperative peak cardiac troponin I was greater in patients with the GC/GC (11.5 ± 13.8 ng/ml) than in patients with the GC/TT (9.2 ± 9.2 ng/ml) or patients with the TT/TT genotype (6.6 ± 4.8 ng/ml, $P = 0.015$).

Conclusions: The GC/GC genotype of the TT(-695/-694)GC polymorphism is associated with increased Gq protein expression, augmented angiotensin II receptor type 1–related vasoconstriction, and increased myocardial injury after coronary artery bypass grafting, highlighting the impact of Gq genotype variation. **(Anesthesiology 2017; 127:70-7)**

LEFT ventricular hypertrophy is an adaptational response to an increased load that involves increased protein synthesis and cardiomyocyte size. However, with pathologic conditions such as hypertension or after myocardial infarction, maladaptive cardiac hypertrophy can result in tissue fibrosis and is associated with a greater mortality due to heart failure and arrhythmia.[1,2] Moreover, left ventricular hypertrophy is associated with changes in the density, structure, and coronary vasodilator capacity so that the cross-sectional diameter of endomyocardial capillaries and coronary reserve are decreased even in the absence of detectable coronary atherosclerosis.[3,4]

Interestingly, activated mutants of the G-protein α-subunit Gq promote myocardial hypertrophy.[5] In line with these studies, knockout of Gq or the functionally similar G protein G11 in cardiomyocytes abolished pressure overload–induced myocardial hypertrophy.[6] Activation of the Gq pathway *via* angiotensin II and the angiotensin II receptor type 1[7] results in activation of phospholipase c beta, which hydrolyses the plasma membrane phosphatidylinositol

What We Already Know about This Topic

- Previous studies have demonstrated angiotensin II receptor type 1–mediated activation of the α-subunit of the heterotrimeric Gq protein evokes increased vasoconstriction and may promote hypertrophy-induced myocardial damage
- This study determined whether a TT(-695/-694)GC polymorphism in the human Gq promoter is associated with differences in (1) myocardial Gq protein expression, (2) vascular reactivity, and (3) myocardial damage after coronary artery bypass grafting

What This Article Tells Us That Is New

- The GC/GC genotype of the TT(-695/-694)GC polymorphism is associated with increased Gq protein expression, augmented angiotensin II receptor type 1–related vasoconstriction, and increased myocardial injury after coronary artery bypass grafting

Submitted for publication November 8, 2016. Accepted for publication March 13, 2017. From Klinik für Anästhesiologie und Intensivmedizin (U.H.F., S.K., T.K., J.P.), Die Blutdruck Praxis, Dorsten (A.M.), Klinik für Thorax- und kardiovaskuläre Chirurgie (H.J., M.T.), and Institut für Pharmakogenetik (W.S.), Universität Duisburg-Essen, Essen; and Universitätsklinikum Essen (U.H.F., S.K., T.K., J.P., H.J., M.T.), Essen, Germany.

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4,5-bisphosphate to generate the second messengers inositol 1,4,5-trisphosphate, a regulator of the intracellular calcium response, and diacylglycerol, an activator of protein kinase C subtypes, thereby evoking an diverse array of cellular responses, *e.g.*, vasoconstriction and cardiac hypertrophy.[8]

In patients undergoing coronary artery bypass grafting, recovery of contractile function after reperfusion is depressed, and the serum concentration of cardiac troponin I (cTnI), a marker of myocardial damage, is increased to a much greater extent in hypertrophied hearts subjected to global ischemia. This finding suggests greater susceptibility to ischemia–reperfusion injury of hypertrophied hearts, especially in patients with coronary artery disease in whom coronary flow reserve is diminished independently of stenosis severity.[9–11]

We previously characterized the promoter of the *GNAQ* gene encoding the Gq subunit of heterotrimeric G proteins and identified a novel functional TT(-695/-694)GC promoter polymorphism resulting in increased gene transcription.[12] Allele frequencies are different between ethnic groups, with a GC allele frequency of 0.52 in white,[12] 0.67 in African American,[13] and 0.81 in Chinese populations.[14]

Accordingly, we now tested in an *a priori* analysis whether the TT(-695/-694)GC polymorphism is associated with differences in (1) myocardial muscle Gq protein expression, (2) vascular reactivity, and (3) myocardial damage after coronary artery bypass grafting.

Materials and Methods

Gq Expression Analysis

Following ethics committee approval and written informed consent from all patients, right atrial appendages were obtained as part a former study investigating Gq mRNA expression before cardiopulmonary bypass in patients undergoing coronary artery bypass grafting between 2006 and 2007.[12] Immediately after sampling, specimens were transferred into carbogenated Tyrode solution, quickly frozen in liquid nitrogen, and stored at –80°C. After the collection of appendages from a sufficient number of patients, tissues were split in liquid nitrogen, and the remaining samples were stored in liquid nitrogen for membrane preparations (n = 55). Membranes were prepared as follows: 100 mg tissue was washed in phosphate-buffered saline, minced with a scalpel, and homogenized in 1 ml ice-cold buffer H (300 mM sucrose, 25 mM HEPES; pH 7 with Tris) and a complete Protease Inhibitor Cocktail (Roche Applied Sciences, Germany). Samples were centrifuged at 1,000*g* for 20 min. The supernatant was centrifuged at 80,000*g* (Beckman, Fullerton, USA). After the supernatant was discarded, the pellet was resuspended in 40 μl buffer H. Membrane proteins (30 μg protein per lane) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by Western Blot analysis with an anti-$Gq_{/11}$ antibody (Upstate, USA) or, after stripping the blot, anti-Actin (Santa Cruz Biotechnology, USA). Films were scanned and signals were quantified by densitometry (NIH Image, Scion, USA). To compare Gq expression between different genotypes, the average signal intensity was multiplied by the number of pixels in that area and corrected for the Gβ signal present in the same lane (calculated the same way).

Blood Sampling for Cardiac Biomarker Analysis and DNA Genotyping

Venous blood samples were drawn from each patient the day before coronary artery bypass grafting surgery and postoperatively at 6, 12, 24, 48, 72, and 96 h and were analyzed for cTnI in an accredited laboratory with a specific two-side immunoassay (Dimension Flex; Dade Behring GmbH, Germany). The detection range for cTnI was 0.04 to 40 ng/ml, requiring further dilutions if necessary. The assay's reference interval was 0.00 to 0.05 ng/ml. A cTnI value greater than 0.1 ng/ml was considered abnormal. All laboratory measurements were made without knowledge of *GNAQ* genotypes.

Genomic DNA was extracted from whole blood via the use of standard techniques. A 368-bp polymerase chain reaction fragment was amplified with primer Gq_Se4 (5′-CCCCCTGCCCCGATTGCCA-3′) and Gq_AS4 (5′-GGGTCTGGCCCCGACTTCG-3′), as described previously,[12] with a slowdown polymerase chain reaction technique including 5% dimethyl sulfoxide.[15] Genotypes of the TT(-695/-694)GC polymorphism were determined by restriction with *Nae*I (New England Biolabs, Germany), separation on a 2.5% agarose gel, and visualization under ultraviolet illumination.

Assessment of Skin Microcirculation by Laser Doppler Imaging

Skin microcirculation experiments had been performed previously as part of a study addressing the effects of angiotensin II receptor type 1 receptor antagonism on various vasoconstrictors (data collection between 2004 and 2005)[16] and were analyzed retrospectively to assess the influence of the Gq TT(-695/-694)GC polymorphism. Eighteen white male volunteers (age: 29 ± 4 yr, mean ± SD; *GNAQ* genotype: n = 5 GC/GC, n = 7 GC/TT, n = 6 TT/TT) were studied. All participants were nonsmokers and healthy on the basis of their medical history, physical examination, electrocardiogram results, and routine clinical chemistry screening, and they had a body mass index of 25 kg/m^2 or less. Each volunteer provided written informed consent, and the study was approved by the University of Duisburg-Essen Medical School Ethics Committee (Duisburg, Germany).

A laser Doppler image scanner (Moor LDI; Moor Instruments Ltd., UK) was used to assess skin perfusion, as described previously.[16] To summarize in brief, before intradermal injections, the volar surface of the arm was scanned to assess resting blood flow at each injection site. Then, 0.01 ml saline was injected intradermally followed by angiotensin II

(10^{-16} and 10^{-14} mol/0.01 ml) or a second injection of saline. The double-injection technique has been applied in several studies and shows a high interday reproducibility.[16]

Measurements of Vascular Function In Vitro

Following written informed consent and local ethics committee approval, saphenous vein remnants were obtained during coronary artery bypass grafting between 2006 and 2007 from 50 white patients without venous pathology immediately after the last coronary anastomosis had been completed. Saphenous vein remnants were preserved in oxygenated modified Krebs–Henseleit solution (NaCl 118 mM, KCl 4.69 mM, $CaCl_2$ 2.5 mM, $MgSO_4$ 1.04 mM, $NaHCO_3$ 25 mM, D-glucose 11.1 mM, and HEPES 21.8 mM, pH 7.40) until use. Each piece of saphenous vein was cut into four rings of approximately 5 mm width. The rings were mounted between two L-shaped, stainless-steel hooks in organ baths filled with 10 mL oxygenated Krebs–Henseleit solution of 37°C (pH 7.4). Each preparation was secured to an isometric force transducer (FMI, Germany) via a silk thread, and force was recorded with a dedicated computer system (VitroDat; FMI). Each ring was subjected to a pretension of 10 mN, which was maintained throughout the experiment. After an equilibration period of 60 min in the organ bath, the rings were primed and tested for viability by exposing them twice to KCl (final concentration: 40 mM). Cumulative concentration–response curves were then constructed for angiotensin II (10^{-9} to 10^{-5} M; Sigma, USA) with 1-h intervals. The vasoconstrictive responses to different angiotensin II concentrations were calculated as a percentage of the maximum KCl-induced contraction.

Assessment of GNAQ *Polymorphism-related Myocardial Damage after Coronary Artery Bypass Grafting*

Extending another study[17] following approval by the University of Duisburg-Essen medical faculty's ethics committee and informed written consent, we analyzed in a genotype-dependent manner myocardial injury by using cTnI and included 227 white patients between 2007 and 2013 with single- or multivessel coronary artery disease. Patients were assessed after recruitment on the day before coronary artery bypass grafting. Of 268 patients screened initially, 15 refused to participate, 12 eventually underwent combined bypass and cardiac valve repair surgery, and another 14 patients were excluded due to missing data or DNA. None of the patients underwent previous cardiac surgery, and all clinical, laboratory, and angiographic data were obtained from the patients' medical records.

For coronary artery bypass grafting, anesthesia was induced with etomidate (0.3 mg/kg), sufentanil (1 μg/kg), and rocuronium (0.6 mg/kg) and maintained by the administration of isoflurane (end-tidal concentration: 0.6 to 1.0%) and sufentanil (1 to 4 μg/kg), as required. During cardiopulmonary bypass, isoflurane was given *via* a vaporizer connected to the oxygenator's gas supply. Coronary artery bypass grafting was performed *via* a midline sternotomy with moderate hypothermia, aortic cross-clamping, and cardioplegia by Bretschneider solution. The primary endpoint was myocardial injury as assessed by serial cTnI serum concentrations more than 96 h after surgery.

Statistical Analyses

The *GNAQ* polymorphism was tested for conformation with Hardy–Weinberg expectations, and no evidence for a deviation was detected. Descriptive statistics are summarized for categorical variables as frequencies (%) and compared between groups by use of the Fisher exact test. Continuous variables are expressed as means ± SD and were compared between groups with ANOVA. All statistical analyses were two-tailed and performed with SPSS, version 22.0 (SPSS, USA). Because no data regarding linear endpoints (cTnI) with *GNAQ* genotypes are available, an *a priori* power analysis was not possible. Study sample sizes were therefore used based on previous experiences showing a 1.5-fold increased genotype-related Gq mRNA expression and intracellular signal transduction in GC/GC genotypes compared with TT/TT genotypes.[12]

Data from the laser Doppler scanner were analyzed offline after the completion of each experiment with Moor Software V.3.01 (Moor Instruments Ltd.). To assess the net effects of angiotensin II, the values for resting blood flow and saline at each injection site were subtracted from the values obtained for the agonists. All values were presented as mean changes of perfusion units ± SD. Vascular responses to angiotensin II (Doppler scanner and vein rings) were analyzed by two-way ANOVA with the factors genotype and drug dose and the Tukey *post hoc* test. Serum cTnI of patients was analyzed by two-way (genotype × time) ANOVA for repeated measures with the Tukey *post hoc* test for multiple comparisons. In addition, analysis of covariance including ejection fraction as a covariate was performed. The peak serum cTnI was compared by ANOVA. Investigators of skin microcirculation, vascular reactivity, myocardial damage, and Gq expression were blind as to the TT(-695/-694)GC genotypes. Differences were regarded statistically significant with an *a priori* alpha error $P < 0.05$.

Results

GC/GC Genotype Increased Cardiac Gq Expression

We measured Gq protein expression by Western Blot analysis using membrane preparations from human right atrial specimens (fig. 1, upper panel). Densitometric quantification of Gq protein expression in human right atrial specimens (n = 55) yielded a highly significant fold change of 1.27 for GC/GC *versus* TT/TT genotype carriers (fig. 1; $P = 0.006$).

Skin Perfusion

To investigate whether increased Gq expression in GC/GC genotype carriers translates into enhanced vasoconstriction

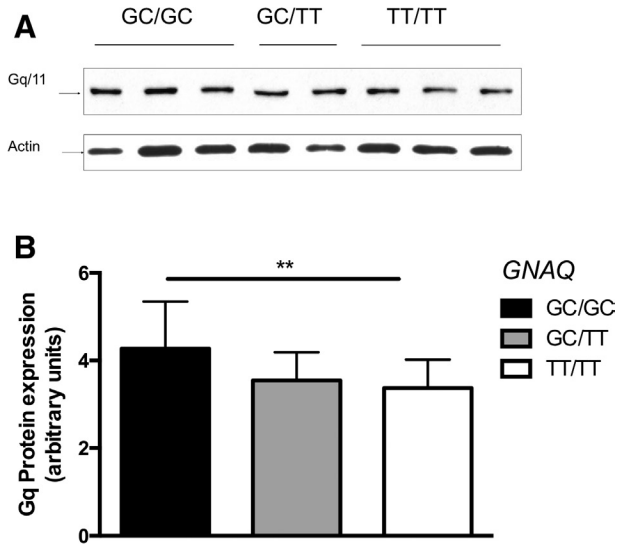

Fig. 1. Genotype-dependent Gq expression. (*A*) Lysate from cell membranes of right atrial specimens from patients with different *GNAQ* genotypes. Displayed is one representative blot probed with a Gq/11 antibody and, after stripping the blot, with an actin antibody as a control. (*B*) Relative quantification of Gq/11 expression by densitometry (mean ± SD) from experiments in right atrial specimens (n = 55: GC/GC, n = 24; GC/TT, n = 20; TT/TT, n = 11). $^{**}P < 0.01$ ANOVA.

after Gq activation *via* angiotensin receptor stimulation, skin perfusion changes were analyzed after intradermal injection of angiotensin II (10^{-16} and 10^{-14} M) in 18 healthy individuals (GC/GC, n = 5; GC/TT, n = 7; TT/TT, n = 6). Although baseline skin perfusion before injections was similar in different genotypes, angiotensin II evoked vasoconstriction with both angiotensin II concentrations only in GC/GC genotypes. In contrast, GC/TT genotypes showed a shifted dose–response curve with detectable vasoconstriction only after 10^{-14} M angiotensin II, and perfusion was almost unchanged in TT/TT-homozygous individuals ($P = 0.0002$ for comparison of genotypes, $P = 0.0003$ for GC/GC *vs.* TT/TT, and $P = 0.007$ for GC/TT *vs.* TT/TT; fig. 2A).

Angiotensin II–mediated Vasoconstriction in Isolated Human Saphenous Vein Rings

Angiotensin II–induced vasoconstriction was analyzed in vein rings obtained from patients undergoing coronary artery bypass grafting exposed to increasing angiotensin II in an organ bath. Angiotensin II resulted in an almost doubled maximum contraction in GC/GC homozygous compared with TT/TT carriers (62.9 ± 25.9% *vs.* 35.4 ± 21.7% of maximum KCl-evoked contraction, respectively, $P = 0.022$ for comparison of genotypes; fig. 2B).

Myocardial Injury

Baseline characteristics of the patients are presented in table 1. Genotype distribution (GC allele frequency 0.54) was comparable with that of healthy blood donors,[12] arguing against an association of *GNAQ* genotypes with increased

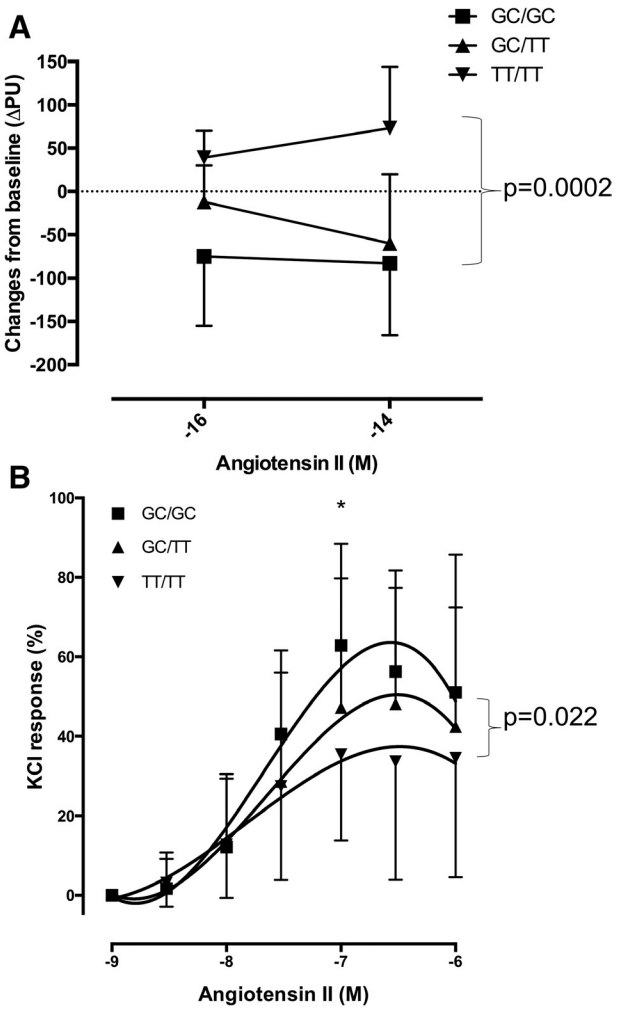

Fig. 2. Vascular response after intradermal angiotensin II injection. (*A*) Mean changes (±SD) in skin perfusion (expressed as changes from baseline of arbitrary perfusion units [ΔPU]) in response to angiotensin II, as stratified by *GNAQ* genotypes (GC/GC, n = 5; GC/TT, n = 7; TT/TT, n = 6). *P* value represents comparison of genotypes from two-way ANOVA (Tukey multiple comparison test yielded significant results for GC/GC *vs.* TT/TT and GC/TT *vs.* TT/TT). (*B*) Dose–response curves of contraction of saphenous vein rings in response to increasing angiotensin II concentrations (1 nM to 1 μM) according to *GNAQ* genotypes (GC/GC, n = 12; GC/TT, n = 28; TT/TT, n = 10). Curves were drawn with the use of a nonlinear fit-model (±SD) and a polynomial third-order equation. *P* value represents comparison of genotypes from two-way ANOVA ($^{*}P = 0.01$ for *post hoc* comparison for GC/GC *vs.* TT/TT).

susceptibility for coronary artery disease. Genotypes did not differ with regard to their demographics, risk factors, comorbidities, and medications. However, preoperative cardiac ejection fraction was significantly greater in patients with the GC/GC genotype (table 1). Intraoperative data such as bypass time, aortic cross-clamp time, and number of bypass grafts were all similar between different genotype carriers.

All patients presented postoperative increases of cTnI. Although preoperative cTnI did not differ between

Table 1. Perioperative Patient Characteristics of Patients Undergoing Coronary Artery Bypass Grafting

GNAQ Genotype	GC/GC	GC/TT	TT/TT	*P* Value
No. of patients	64	119	44	
Age, yr	68 ± 9	67 ± 9	68 ± 8.8	0.748
Body weight, kg	85 ± 16	83 ± 13	83 ± 17	0.395
Smoking				
Current	7 (11)	16 (13)	7 (16)	
Former	36 (56)	61 (51)	21 (48)	0.488
Preoperative creatinine serum concentration, mg/dl	1.2 ± 0.2	1.3 ± 0.5	1.3 ± 0.4	0.189
Systolic blood pressure, mmHg	135 ± 17	135 ± 21	134 ± 20	0.305
Diastolic blood pressure, mmHg	73 ± 11	75 ± 12	73 ± 12	0.909
Cardiac ejection fraction, %	55 ± 13	54 ± 14	48 ± 15	0.037
NYHA classification				
I-II	33 (52)	80 (67)	24 (55)	—
III	28 (44)	32 (27)	20 (46)	—
IV	3 (5)	7 (6)	0 (0)	0.385
Peripheral arterial disease	8 (13)	20 (17)	6 (14)	0.840
Left main coronary artery stenosis >50%	13 (20)	35 (30)	10 (23)	0.640
Preoperative cTnI >0.1 μg/l	4 (7)	12 (10)	3 (7)	0.826
No. grafts	3 ± 1	3 ± 1	3 ± 1	0.146
Internal mammary artery graft	63 (98)	106 (89)	42 (96)	0.362
Mitral valve insufficiency (moderate or severe)	6 (9)	10 (8)	4 (9)	0.935
Cardiopulmonary bypass time, min	126 ± 43	126 ± 42	127 ± 48	0.878
Aortic cross-clamp time, min	85 ± 26	83 ± 31	85 ± 33	0.937
Medication				
ASA	49 (79)	94 (82)	39 (89)	0.217
Clopidogrel	12 (19)	25 (22)	7 (16)	0.726
β-Blocker	52 (81)	99 (83)	34 (77)	0.672
Statins	38 (61)	83 (72)	29 (66)	0.510
ACEI/ARB	51 (80)	91 (76)	33 (75)	0.552
Diuretics	25 (38)	59 (50)	23 (52)	0.135
Calcium antagonists	12 (19)	27 (23)	11 (25)	0.426

Data are presented as means ± SD or no. (%).

ACEI = angiotensin-converting enzyme inhibitor; ARB = angiotensin receptor blocker; ASA = acetylsalicylic acid; cTnI = cardiac troponin I; NYHA = New York Heart Association.

genotypes, postoperative cTnI was greatest in patients with the GC/GC genotype, followed by those with GC/TT and TT/TT genotypes, suggesting a gene–dose effect ($P = 0.034$ for comparison of genotypes; fig. 3). *Post hoc* analysis revealed a mean difference over time of 3.2 ng/ml between GC/GC and TT/TT genotypes ($P = 0.032$). Analysis of covariance including ejection fraction as a covariate did not change the results (mean difference over time between GC/GC and TT/TT genotype: 3.1 ng/ml; $P = 0.011$), thus demonstrating an independent association of the *GNAQ* polymorphism with cTnI. Peak cTnI after coronary artery bypass grafting almost doubled in GC/GC genotypes (11.5 ± 13.8 ng/ml) compared with heterozygous GC/TT (9.2 ± 9.2 ng/ml) and homozygous TT/TT genotypes (6.6 ± 4.8 ng/ml; $P = 0.015$).

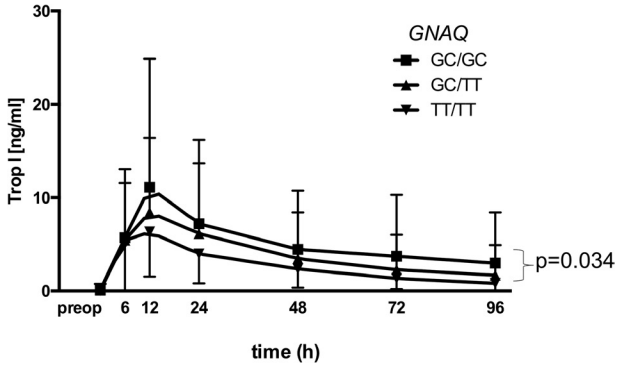

Fig. 3. Postoperative serum troponin I concentration (TropI) more than 96 h (mean ± SD) after coronary artery bypass grafting according to genotypes GC/GC (n = 64), GC/TT (n = 119), and TT/TT (n = 44). Peak cTnI was greater in GC/GC carriers compared with GC/TT and TT/TT genotypes ($P = 0.015$).

Discussion

Here we show that the substitution of TT for GC at positions -695/-694 of the dinucleotide polymorphism of the *GNAQ* gene promoter is associated with increased myocardial Gq protein expression. Moreover, we provide functional data showing that this polymorphism is functionally active: GC/GC genotype carriers show enhanced vasoconstrictor responses to the Gq activator angiotensin II in two different systems, skin capillary perfusion in volunteers and isolated

human saphenous veins *in vitro*. Finally, our data identify this dinucleotide polymorphism as a genetic risk factor for myocardial damage after coronary artery bypass grafting. These data, therefore, strongly support the clinical relevance of this *GNAQ* polymorphism.

Perioperative myocardial injury is attributed to transient myocardial ischemia–reperfusion and surgical injury and the acute inflammatory response associated with cardiopulmonary bypass.[18] Moreover, angiotensin II serum concentrations are increased during and after cardiopulmonary bypass and have been suggested to be involved in postoperative hypertension, potentially resulting in myocardial ischemia after surgery.[19,20]

The renin–angiotensin–aldosterone system is responsible for peripheral as well as central effects of vasoconstriction, and these effects are transmitted *via* angiotensin receptors.[7] In humans, attenuation of angiotensin II–mediated Gq signaling by angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers is a cornerstone of heart failure therapy.[21] It seems that chronic activation of the heterotrimeric G proteins Gq and G11 and their downstream signaling pathways is necessary and sufficient for myocardial hypertrophy. We and others have proposed that genetic variants may be associated with altered perioperative myocardial injury after coronary artery bypass grafting[22–25] but no data yet exist for the cardiac hypertrophy-related Gq pathway.

We previously characterized the *GNAQ* promoter and identified a dinucleotide TT(-695/-694)GC promoter polymorphism where both nucleotides always are exchanged simultaneously.[12] We also have shown that the GC allele displays increased binding to the transcription factor Sp-1 and is associated with enhanced promoter activity, increased Gq transcription, increased Gq-mediated intracellular signal transduction, and an increased prevalence of left ventricular hypertrophy.[12,26] This provided the first evidence that effects observed in transgenic mice may translate to the situation in human hearts.

However, because mRNA concentrations do not necessarily evoke corresponding changes of protein concentration, we extended our analyses and measured myocardial Gq protein along with functionally relevant phenotypes. Our current findings demonstrate that Gq protein expression is greatest in GC/GC genotype carriers, and this increased Gq expression translated into a measurable phenotype impacting on or reflecting perioperative myocardial injury. Measuring genotype-dependent differences of angiotensin II–induced vasoconstriction in different systems we could show enhanced vasoconstriction in GC/GC genotypes compared with GC/TTs or TT/TTs, suggesting a gene–dose effect.

Various signaling events are important both for the development and decompensation of left ventricular hypertrophy, and these involve cardiac paracrine and/or autocrine mediators like endothelin-1, norepinephrine, and/or angiotensin II, all of which act on cognate G protein-coupled receptors expressed in the myocardium.[27] Studies in transgenic mice show that the cardiomyocyte-specific overexpression of some of these G protein-coupled receptors, such as α_1-adrenergic and angiotensin type-1 receptors, or activated mutants of their coupled G-protein α -subunit Gq result in myocardial hypertrophy.[5]

Clinically, myocardial hypertrophy becomes evident and potentially has prognostic relevance especially in patients with coronary artery disease, implying reductions in coronary flow reserve in these patients.[28] Moreover, there is evidence that increased coronary microvascular tone, such as by α-adrenergic vasoconstriction, occurs more often in hearts with pathologic left ventricular hypertrophy, thereby reducing coronary blood flow with the risk of myocardial ischemia.[29]

Because global ischemia in hypertrophied hearts evokes increased troponin I concentrations,[10,11] we also investigated whether genotype-related differences in Gq expression are associated with altered perioperative myocardial damage after coronary artery bypass grafting surgery. Baseline characteristics of *GNAQ* genotypes showed greater ejection fraction in GC-allele carriers whereas factors, in particular with regard to clinical risk factors for perioperative myocardial damage, did not differ between genotypes across the study cohort. However, although the percentage of patients with angiotensin-converting enzyme inhibitors or angiotensin receptor blockers was not different between genotypes and baseline arterial blood pressure also showed no difference, GC-homozygous patients may have received increased doses of those drugs to lower arterial blood pressure. Although, unfortunately, data collection about exact previous drug dosages was beyond the scope of our study, future investigations also should take into account this information.

Perioperative myocardial damage, as assessed by postoperative cTnI, was greatest in GC/GC-homozygous patients followed by GC/TT and TT/TT genotypes, again consistent with a gene–dose effect. One possible explanation for our observation could be that increased Gq expression in GC/GC genotype carriers results in left ventricular hypertrophy and decreased coronary reserve, rendering these individuals more susceptible to the detrimental effects of cardiac surgery. This hypothesis is supported by experiments in rats, where recovery of contractile function is depressed and lactate dehydrogenase or creatinine kinase activity was increased in hypertrophied hearts subjected to global ischemia, also suggestive of greater susceptibility to ischemia–reperfusion injury.[30–32]

Interestingly, a cross-talk between Gq and Gs signaling pathways has been proposed,[33] and increased Gq expression has been shown to decrease cAMP production through Gs protein ubiquitination and its proteasomal degradation.[34,35] Although cAMP represents a critical regulator for left ventricular contractile function, facilitated Gs degradation and depressed cAMP production by increased Gq expression in GC-allele carriers may represent a novel mechanism for Gq-induced cardiac dysfunction after coronary artery bypass grafting.

Our results may have clinical implications. Although we have shown previously that remote ischemic preconditioning protects the heart from ischemic damage,[36] two large multicenter trials failed to show a cardioprotective effect of remote ischemic preconditioning.[37,38] Given that a certain *GNAQ* genotype is associated with altered postoperative cTnI, one could argue that the remote ischemic preconditioning effect may be detectable in selected genotype carriers only. Although we can only speculate on this topic, future studies are necessary to investigate a potential interaction of remote ischemic preconditioning with the *GNAQ* polymorphism.

Another potential clinical implication is the therapy with a vasodilator, *e.g.,* therapy with vascular endothelial growth factor, a treatment option for heart failure. Here, vascular endothelial growth factor gene therapy in patients with coronary artery disease hitherto has not demonstrated a clinical benefit.[39] However, it might be speculated from our data that only certain individuals, such as GC-homozygous patients for the TT(-695/-694)GC polymorphism, may benefit from vasodilator therapy because those individuals have a high level of coronary vasoconstriction. Those individuals also may benefit from other postoperative vasodilator therapies, such as with endothelin-receptor blockers. However, these questions were beyond the scope of the present study.

Some limitations must be addressed. First, we speculate that increased myocardial damage observed in GC/GC carriers during coronary artery bypass grafting surgery may be due to left ventricular hypertrophy. Although we did not measure hypertrophy-related echocardiographic parameters, this was not tested directly. However, we already had shown in our previous study that the polymorphism is indeed associated with left ventricular hypertrophy[12] and therefore assume the same mechanism for the current study. Second, we were not able to perform a reasonable *a priori* power analysis because this is the first analysis of *GNAQ* genotypes regarding postoperative myocardial damage as well as vasoconstriction response after angiotensin II stimulation. Therefore, our results should be regarded as a pilot study, and future studies may take these results into account to calculate an appropriate *a priori* power analysis.

In conclusion, our results demonstrate that the functionally relevant *GNAQ* TT(-695/-694)GC promoter polymorphism evokes increased myocardial Gq expression, enhanced vasoconstrictor responses in skin and isolated veins after angiotensin II stimulation, and increased perioperative myocardial damage after coronary artery bypass grafting. Thus, our data shed new light on the role of genetically evoked altered Gq expression in ischemic heart disease as well as for human vasomotor responses and may help to identify patients at greater risk for myocardial injury after coronary artery bypass grafting.

Research Support

Support was provided solely from institutional and/or departmental sources.

Competing Interests

The authors declare no competing interests.

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A Systematic Review and Meta-analysis Examining the Impact of Incident Postoperative Delirium on Mortality

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ABSTRACT

Background: Delirium is an acute and reversible geriatric syndrome that represents a decompensation of cerebral function. Delirium is associated with adverse postoperative outcomes, but controversy exists regarding whether delirium is an independent predictor of mortality. Thus, we assessed the association between incident postoperative delirium and mortality in adult noncardiac surgery patients.

Methods: A systematic search was conducted using Cochrane, MEDLINE/PubMed, Cumulative Index to Nursing and Allied Health Literature, and Embase. Screening and data extraction were conducted by two independent reviewers. Pooled-effect estimates calculated with a random-effects model were expressed as odds ratios with 95% CIs. Risk of bias was assessed using the Cochrane Risk of Bias Tool for Non-Randomized Studies.

Results: A total of 34 of 4,968 screened citations met inclusion criteria. Risk of bias ranged from moderate to critical. Pooled analysis of unadjusted event rates (5,545 patients) suggested that delirium was associated with a four-fold increase in the odds of death (odds ratio = 4.12 [95% CI, 3.29 to 5.17]; I^2 = 24.9%). A formal pooled analysis of adjusted outcomes was not possible due to heterogeneity of effect measures reported. However, in studies that controlled for prespecified confounders, none found a statistically significant association between incident postoperative delirium and mortality (two studies in hip fractures; n = 729) after an average follow-up of 21 months. Overall, as study risk of bias decreased, the association between delirium and mortality decreased.

Conclusions: Few high-quality studies are available to estimate the impact of incident postoperative delirium on mortality. Studies that controlled for prespecified confounders did not demonstrate significant independent associations of delirium with mortality. **(Anesthesiology 2017; 127:78-88)**

DELIRIUM is a fluctuating, neuropsychiatric geriatric syndrome that represents a decompensation of cerebral function and can result in acute and reversible cognitive decline.[1] Causes of delirium are multifactorial and can be related to acute physical stressors, such as surgery.[2] More than 51-million surgeries occur annually in North America,[3] and in some high-risk surgical populations up to 50% of patients may develop postoperative delirium.[2,4,5]

Despite the growing body of evidence that associates delirium with mortality,[6–9] the causal relationship of delirium with mortality is difficult to ascertain due to the high risk of confounding bias. Many of the strongest risk factors for postoperative delirium, such as advanced age, comorbidity, preexisting cognitive dysfunction, and high-risk surgery, are also independent risk factors for mortality.[7,10] Because delirium is a disease state and not an intervention, causal inference depends on the conduct and reporting of high-quality observational studies.

What We Already Know about This Topic

- Although the occurrence of delirium in the perioperative period is associated with increased mortality, it is not clear whether delirium *per se* is an independent predictor of mortality.
- A meta-analysis of the extant literature on perioperative delirium in patients undergoing noncardiac surgery was performed. Importantly, the risk of bias, particularly with respect to confounding variables that may independently contribute to mortality, in each of the reviewed studies was determined.

What This Article Tells Us That Is New

- Patients who develop delirium are at increased risk of death.
- However, in the studies with reduced bias and adequate control for confounding, an independent association between delirium and mortality was not apparent.

Studies to date have produced conflicting results regarding the association between postoperative delirium and mortality in the perioperative setting. A recent study conducted

Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site (www.anesthesiology.org).

Submitted for publication October 4, 2016. Accepted for publication March 21, 2017. Department of Anesthesiology and Pain Medicine (G.M.H., M.M.L., D.I.M.), Faculty of Medicine (K.W., J.D.), Regenerative Medicine Program (M.M.L.), Clinical Epidemiology Program (D.I.M.), and The Ottawa Hospital Research Institute (M.M.L., D.I.M.), University of Ottawa, Ottawa, Ontario, Canada.

 Anesthesiology 2017; 127:78-88

by Gottschalk *et al.*[11] in elderly patients with hip fracture demonstrated that incident postoperative delirium was not independently associated with mortality. In contrast, Dubljanin-Raspopović *et al.*[12,13] found that, in a similar population of patients with hip fracture, postoperative delirium was an independent predictor of mortality. The divergent findings may be at least partly explained by the differing approach to control for confounding. Although both studies included variables to account for age, sex, and American Society of Anesthesiology (ASA) score, Gottschalk *et al.*[11] additionally controlled for preexisting cognitive impairment, as well as several postoperative variables. Dubljanin-Raspopović *et al.*[12,13] did not account for baseline cognitive function, which is the strongest known predictor of delirium[14,15] and an independent predictor of postoperative mortality.[16,17] This comparison exemplifies the potential fragility of the delirium–mortality association depending on choice of confounders included in adjusted models.

Existing systematic reviews have examined the association of delirium with mortality in mixed patient populations; however, none of these studies focused specifically on surgical patients who develop incident postoperative delirium.[8,18] Furthermore, to our knowledge, no existing review uses a systematic approach to account for the multiple sources of confounding known to be pertinent to the delirium–mortality relationship in perioperative patients. Therefore, we conducted a systematic review to specifically examine the independent association of incident postoperative delirium with mortality in adult noncardiac surgery patients.

Materials and Methods

We carried out this systematic review and meta-analysis of prospective observational studies following recommendations of the Meta-Analysis of Observational Studies in Epidemiology group.[19] The protocol for the systematic review was registered with the International Prospective Register of Systematic Reviews (CRD42015029805, http://www.crd.york.ac.uk/PROSPERO/display_record.asp?ID=CRD42015029805) and was conducted in accordance with Cochrane Collaboration guidelines.[20] This manuscript is reported as per the Preferred Reporting Items for Systematic Reviews and Meta-Analysis.[21]

Search Strategy

Cochrane, MEDLINE, Cumulative Index to Nursing and Allied Health Literature, and Embase databases were systematically searched using a strategy designed in consultation with an information specialist. The search strategy was then reviewed and finalized using the peer review of electronic search strategy checklist.[22] Key words for delirium (*i.e.,* delirium, delirious, acute confusion, cognitive dysfunction, and cognitive impairment) were combined with surgery-specific key words (*i.e.,* postoperative complications, postoperative care, postsurgery, noncardiac surgery, surgical patients, and hip fractures) and mortality key words (*i.e.,* hospital mortality and death; see Supplemental Digital Content 1, http://links.lww.com/ALN/B434, which outlines our full search strategy). Abstracts and other gray literature were excluded, because methodologic descriptions would be insufficient to assess the risk of bias and validity of study findings. The bibliographies of the included studies were hand searched to identify any additional articles that met our inclusion criteria. There were no language restrictions. Our search was restricted to articles after January 1981, because a formal nomenclature to differentiate delirium from dementia was first established with the Diagnostic and Statistical Manual of Mental Disorders (3rd edition) in 1980.[23]

Inclusion and Exclusion Criteria

Eligible studies were included if they met the following criteria: (1) adults (>18 yr of age) undergoing noncardiac surgery; (2) incident postoperative delirium (new-onset delirium that occurs during the postoperative course) was prospectively identified using a validated instrument or diagnosed prospectively based on Diagnostic and Statistical Manual of Mental Disorders criteria; and (3) reported quantitative data (*i.e.,* event rates, risk ratios [RRs], odds ratios [ORs], or hazard ratios [HRs]) to measure the association between delirium and mortality. Studies were excluded if: (1) there were cardiac surgery patients, because the risk factors for delirium (*e.g.* cardiopulmonary bypass) and nature of clinical care (*e.g.,* routine intensive care unit admission after surgery) differ significantly between cardiac and noncardiac surgical populations); (2) surgery-specific subgroups and their outcome data could not be extracted independent of other types of patients (*e.g.,* noncardiac surgery patients combined with nonsurgical or cardiac surgical patients); (3) the majority of patients had preexisting (*i.e.,* not incident postoperative) delirium; or (4) the subgroup with incident delirium and the patient outcome data could not be extracted independent of preexisting delirium cases (*i.e.,* present before surgery).

Selection of Included Studies

Titles and abstracts of identified studies were independently screened in duplicate (G.M.H., K.W., J.D.). Study screening and selection, as well as data collection, were performed using DistillerSR (Evidence Partners, Canada). Relevant abstracts were selected and the full-text articles reviewed. Any disagreements were resolved by consensus decision in discussion with the senior team members (D.I.M., M.M.L.). Study design, demographic data, exposure, and outcome data were extracted. A calibrating exercise was performed to ensure that interrater agreement was high for both the study selection and data extraction. After the data extraction, authors were contacted to verify missing data and offer clarifications as needed.

Assessment of Risk of Bias

Risk of bias was assessed in duplicate by the primary author and senior author using the method outlined in the Cochrane Risk of Bias Tool for Non-Randomized Studies.[24] The risk of bias was assessed as low, moderate, high, or critical for each of confounding bias, selection bias, measurement bias (outcome or exposure), missing data bias, and selection bias. Any disagreement was resolved by consensus.

Statistical Analysis

For the unadjusted analysis, we included any study that reported the effect of incident delirium on mortality and extracted the number of events relative to the total number of participants in the delirium and control groups (*i.e.,* crude event rates).

For the primary adjusted analysis, we extracted quantitative data (*i.e.,* ORs, RRs, and HRs) that were adjusted for prespecified key confounders reflecting the association between incident delirium and mortality. In keeping with Witlox *et al.*,[8] our primary analysis included only studies that adjusted for age, sex, comorbidity, and baseline cognitive status. Because ASA score describes illness severity and predicts both delirium and mortality, studies controlling for ASA score were considered to account for comorbid illness. To identify additional perioperative confounders, we searched the literature for reviews or key articles that described risk factors for both postoperative mortality and postoperative delirium.[10,14,25,26] We then identified key variables that predicted both delirium and mortality. Based on this search, the type and urgency of surgery were also identified as key perioperative confounding variables for delirium and mortality. Therefore, these variables were included in our list of required adjusted variables for a study to be included in our the primary analysis (table 1). Based on best-practice recommendations, control for confounding was determined to be inadequate if the key variables were not included in the final adjusted model, despite clinical and epidemiologic grounds for their inclusion.[27–30] We also planned a secondary adjusted analysis, in which we included measures of association (*i.e.,* ORs, RRs, and HRs) that were adjusted for any confounders.

Where possible, we performed a meta-analysis for the primary outcome of mortality. Pooled-effect outcomes were calculated using inverse variance methods with random-effects models and expressed as ORs and 95% CIs. Heterogeneity was assessed using the I^2 statistic. Statistical analyses were performed in STATA 10.0 (StataCorp LLC, Texas). Figures were created in RevMan 5.3 (The Cochrane Collaboration, Denmark). *P* values of less than 0.05 were considered statistically significant.

Table 1. Key Confounders in the Delirium–Mortality Relationship

Key Confounders
Age
Sex
Comorbidity (*e.g.,* ASA)
Previous cognitive impairment
Surgery type
Surgery urgency

ASA = American Society of Anesthesiology.

Results

Our search identified 4,968 citations, of which 445 citations were selected for a full-text review. After full-text review, a total of 34 studies met our eligibility criteria (see Supplemental Digital Content 2, http://links.lww.com/ALN/B435, which lists all of the studies that met our primary, secondary, and tertiary analyses); 2 studies met criteria for our primary analysis, and 6 studies met criteria for secondary analysis (table 2). The three most common reasons for excluding a citation after full-text review were as follows: (1) conference abstract only citation; (2) the definition of delirium was not validated or it was reported as an outcome variable (not an exposure); or (3) no mortality data were reported. Thirty four of the included studies were published in English, one in Korean,[31] and one in Spanish.[32] A Preferred Reporting Items for Systematic Reviews and Meta-Analysis flowchart outlining the search results is shown in figure 1.[33]

Of the 34 studies (n = 7,738 patients) identified through our search, 21.5% of patients developed incident postoperative delirium, and 10.8% of patients died after surgery. Of those patients found to be delirious, 21.8% died compared with an 8.7% mortality rate for nondelirious patients. The mortality outcome ascertainment time frame varied between studies, including in-hospital mortality (8 studies; n = 1,274), 30 days to 6 months (13 studies; n = 2,413), and more than 6 months (13 studies; n = 4,051). For studies that reported multiple mortality outcome ascertainment time frame variables, we used the longest time frame reported for our analysis.

Risk of Bias

Overall and categorical risk of bias for each included study in the primary and secondary analyses are summarized in table 3. There was 80% agreement between raters across all of the studies and risk of bias domains. At no time did any disagreement on ratings for a given domain for a given study differ by more than 1 level (*e.g.,* if one rater said moderate, the other rater would have said low or serious, not critical). Lack of control for confounding and bias related to the selection of the reported result were the two categories that resulted in the high and critical risks of bias found. As a result, there were 2 studies at a moderate risk of bias, 6 with high risk of bias, and 26 studies that were of a critical risk of bias.

Impact of Incident Postoperative Delirium on Outcomes

Of the studies that met our inclusion criteria, there were 2 studies (n = 729) that adjusted for our prespecified key confounders (fig. 2).[11,34,35] Both studies were conducted in patients who were undergoing emergency hip fracture surgery. A pooled analysis of these two studies was not possible, because one citation reported an adjusted HR[11] and one

Table 2. Studies Included in the Primary and Secondary Analysis

First Author (yr)	Study Design	Surgery Type	Surgery Urgency	Study Size, n	Age (Mean), yr	Sex, % Women	ASA, ≤2	Delirium Diagnosis	Baseline Cognitive Impairment (Proportion)	Mortality (Length of Follow-Up)*	Crude OR (Unless Otherwise Specified) (95% CI)	Adjusted OR (Unless Otherwise Specified) (95% CI)	Primary/ Secondary Analysis	Event Rates (for Mortality)
Gottschalk (2015)[11]	Prospective cohort	Orthopedics (hip fracture)	Emergency	459	81.3	73	0.16	CAM	Dementia (0.26)	49 mo (mean F/U)	(HR) 1.65 (95% CI: 1.32 to 2.06)	HR = 1.2 (0.93–1.54)	Primary	Delirious: 127/151 Nondelirious: 213/308
Radinovic (2014)[35]; Radinovic (2015)[34]	Prospective cohort	Orthopedics (hip fracture)	Emergency	270	78.1	74	0.19	CAM	GDS >6 (0.31), SPMSQ (mean = 5.7)	30 d	3.47[b] (95% CI: 1.29 to 10.83)	0.46 (0.13–1.65)	Primary	Delirious: 21/143 Nondelirious: 6/127
Veiga (2013)[37]	Prospective cohort	General surgery (hepatectomy)	Elective	70	59‡	50	0.33	ICDSC	N/A	6 mo	13.78 (95% CI: 3.4 to 55.6)	9.33 (1.35–64.61)	Secondary	Delirious: 9/17 Nondelirious: 4/53
Abelha (2013)[36]§; Veiga (2012)[38]	Prospective cohort	Major noncardiac (mixed)	Elective	562	66‡	37	0.34	ICDSC	N/A	6 mo	4.26† (95% CI: 2.37 to 7.53)	2.562 (1.36–4.82)	Secondary	Delirious: 28/89 Nondelirious: 46/473
Dubljanin-Raspopović (2012)[13]; Dubljanin-Raspopović (2015)[12]	Prospective cohort	Orthopedics (hip fracture)	Emergency	344	78.2	80	0.58	CAM	Cognitive impairment (SPMSQ) <3 (0.11)	12 mo‖	4.67 (95% CI: 2.97 to 7.34)	2.31 (1.36–3.90)	Secondary	Delirious: 28/43 Nondelirious: 59/301
Bickel (2008)[39]	Prospective cohort	Orthopedics (hip surgery)	Mixed (elective = 0.72, fracture = 0.28)	200	73.8	69.5	N/A	CAM	MMSE (average = 27.1)	38 mo	4.8 (95% CI: 2.1 to 10.8)	1.7 (0.6–5.0)	Secondary	Delirious: 15/41 Nondelirious: 17/159
Furlaneto (2007)[42]	Prospective cohort	Orthopedics (hip fracture)	Emergency	85	80.26	83.5	N/A	CAM	Dementia (0.43)	48 mo	(HR) 1.83 (no CIs reported)	HR = 1.28 (0.66–2.47)	Secondary	Delirious: 15/25# Nondelirious: 24/60#
Nightingale (2001)[40]; Holmes (2000)[41]	Prospective cohort	Orthopedics (hip fracture)	Emergency	316**	80.3††	78††	N/A	Geriatric mental state (AGECAT); delirium rating scale	N/A	2 yr	3.32† (1.99–5.56)	HR = 2.404‡‡ (1.66–3.48)	Secondary	Delirious: 62/108§§ Nondelirious: 60/208§§

*Data include the longest mortality frame reported. †Data were calculated using STATA 10.0 (OR with Cornfield approximation using cci command). ‡Data show the median. §Data are from Abelha *et al.* 2013.[36] ‖Data are from Dubljanin-Raspopović *et al.* 2012.[13] #Data were calculated using the predicted percentages reported in the text (Furlaneto *et al.* 2007).[42] **Data only included well and delirious patients. ††Data were calculated from table 1 in Holmes (2000).[41] ‡‡Data are from Holmes (2000).[41] §§Data are from Nightingale (2001),[40] the same population as Holmes (2000).[41]

AGECAT = Automated Geriatric Examination for Computer Assisted Taxonomy; CAM = confusion assessment method; F/U = follow-up; GDS = geriatric depression scale; HR = hazard ratio; ICDSC = Intensive Care Delirium Screening Checklist; MMSE = Mini–Mental State Examination; N/A = not applicable; OR = odds ratio; SPMSQ = Short Portable Mental Status Questionnaire.

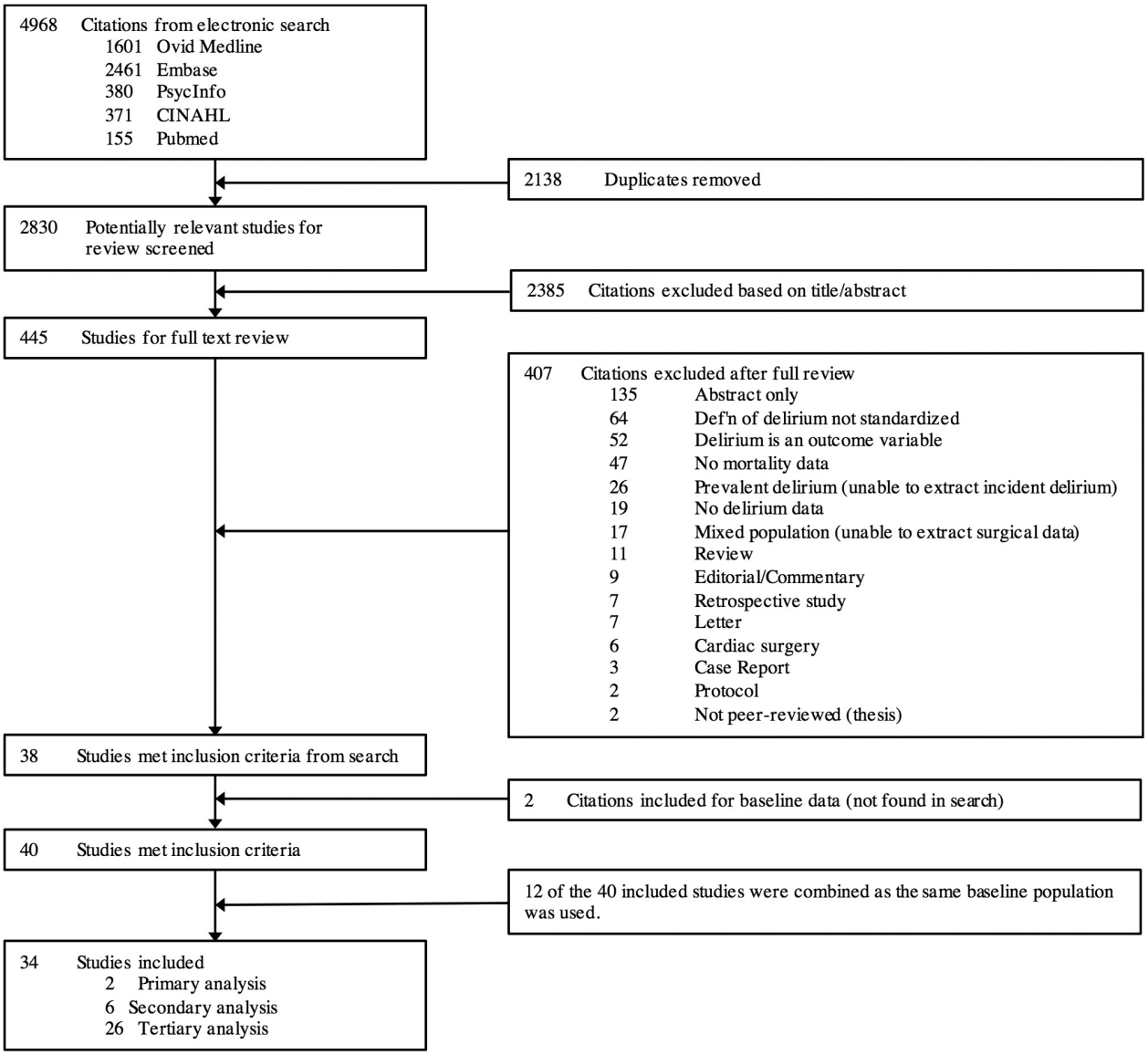

Fig. 1. Identification, review, and selection of articles included in the systematic review. CINAHL = Cumulative Index to Nursing and Allied Health Literature.

reported an adjusted OR.[34] Neither of these studies found a statistically significant association between incident postoperative delirium and mortality after an average follow-up of 21 months (range, 30 days to 49 months). Their adjusted effect estimates were HR at 1.2 (95% CI, 0.93 to 1.54)[11] and OR at 0.46 (95% CI, 0.13 to 1.65),[34,35] respectively.

There were six additional studies[12,13,36–42] (n = 1,577) that calculated adjusted effect estimates to assess the effect of postoperative delirium on mortality, but these authors did not include all of our predefined key confounders in their adjusted effect estimate (table 3). The six adjusted studies were conducted in orthopedic hip fracture patients,[12,13,40–42] hip surgery,[39] general surgery,[37] and a mixed surgical population.[36,38] Given the heterogeneity of the adjusted effect measure types reported, it was not possible to conduct a pooled analysis. Four studies found that delirium was an independent predictor of mortality,[12,13,36,38,40,41] whereas two studies[39,42] did not (fig. 3). These studies presented an average follow-up of 26 months (range, 6 to 48 months).

Twenty seven[11–13,32,34–61] of the 34 studies (n = 5,545) presented unadjusted event rates available for pooled analysis (fig. 4). Seven studies were not included in the pooled analysis because two studies[62–64] had no event rates and five studies[31,65–68] had zero values in their two-by-two tables, making it impossible to obtain an OR.[69] The 27 studies used for pooled analysis had a mean follow-up of 12.3 months (range, 1 to 60 months), and 355 of 1,199 patients with delirium (29.6%) had an increased risk of death compared with 440 of 4,352 control subjects (10.1%). The pooled OR suggested that incident postoperative delirium was associated with an unadjusted four-fold increase in the odds of mortality (OR = 4.12 [95% CI, 3.29 to 5.17]; I^2 = 24.9%).

Table 3. Risk of Bias Assessment Showing the Methodologic Quality of the Studies Included in the Primary and Secondary Analyses and Confounding Variables Included in the Delirium–Mortality Effect Estimate (Primary and Secondary Analyses Only)

	Risk of Bias Assessment							Confounding Variables Included in the Delirium–Mortality Effect Estimate						
Analysis (First Author)	Bias due to Confounding	Bias due to Selection of Participants into the Study	Bias due to Measurement of Outcomes or Exposure	Bias due to Missing Data	Bias due to Measure-ment of Outcomes	Bias due to Selection of the Reported Result	Overall Risk of Bias	Age	Sex	Comorbidity (*e.g.*, ASA)	Previous Cognitive Impairment	Surgery Type	Surgery Urgency	Adjusted OR/HR (95% CI)
Primary analysis														
Gottschalk (2015)[11]	🟡	🟢	🟢	🟢	🟢	🟡	🟡	●	●	●	●	●	●	HR = 1.2 (0.93–1.54)
Radinovic (2015/2014)[34,35]	🟡	🟡	🟢	🟡	🟢	🟡	🟡	●	●	●	●	●	●	OR = 0.46 (0.13–1.65)
Secondary analysis														
Dubljanin-Raspopovic (2015/2012)[12,13]*	🔴	🟡	🟢	🟡	🟡	🟡	🔴	●				●	●	OR = 2.31 (1.36–3.90)
Abelha (2013)[36]; Viega (2012)[38]	🔴	🟡	🟢	🟢	🟢	🟡	🔴	●	●	●		●		OR = 2.562 (1.36–4.82)
Viega (2013)[37]	🔴	🟢	🟢	🟡	🟢	🟡	🔴	●				●		OR = 9.33 (1.35–64.61)
Bickel (2008)[39]	🔴	🟢	🟢	🟢	🟢	🟡	🔴	●	●	●	●	●		OR = 1.7 (0.6–5.0)
Furlaneto (2007)[42]†	🔴	🟢	🟢	🟡	🟢	🟡	🔴			●	●	●	●	HR = 1.28 (0.66–2.47)
Nightingale (2001)[40]; Holmes (2000)[41]‡	🔴	🟡	🟢	🟡	🟢	🟡	🔴	●	●	●		●	●	HR = 2.404 (1.66–3.48)

*Data used backward regression (without controlling for sex or ASA) and only included significant variables in the model. †Univariate analysis was performed with confounders and then included significant variables into the model. ‡It is not clear how concurrent diagnoses of delirium/dementia were handled.

Risk of bias rating scale: low = 🟢, moderate = 🟡, serious = 🔴, critical = ●.

ASA = American Society of Anesthesiologists; HR = hazard ratio; OR = odds ratio.

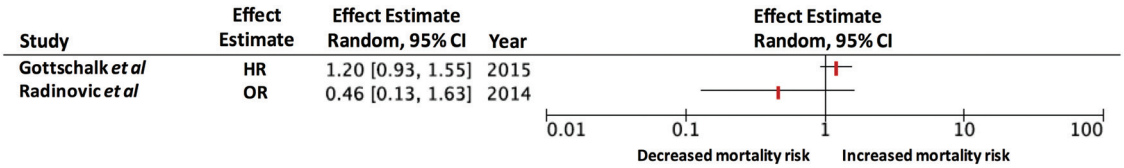

Fig. 2. Primary analysis: forest plot of adequately adjusted event rates (all key confounders included in the statistical model). Note that the point estimates and lower CI values shown in this figure are identical to values found in the articles. Given the variation in statistical techniques used to obtain adjusted odds ratios (ORs), the upper CI value in this figure may not be identical to reported values found in the individual studies (see Supplemental Digital Content 2, http://links.lww.com/ALN/B435, which lists all of the studies that met our primary, secondary, and tertiary analyses). HR = hazard ratio.

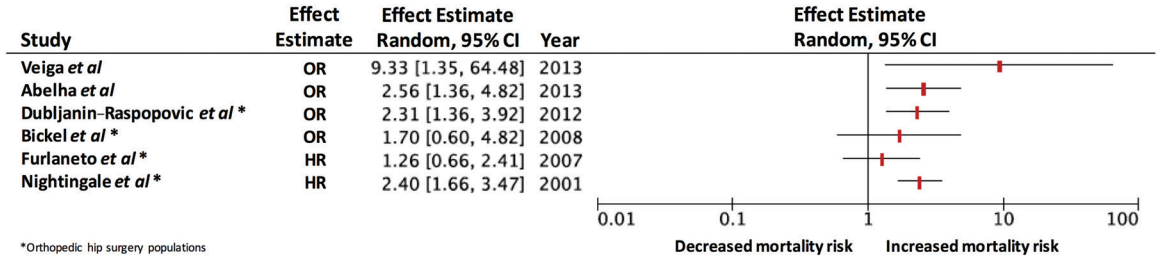

Fig. 3. Secondary analysis: forest plot of inadequately adjusted event rates (not all of the key confounders included in the statistical model). Note that the point estimates and lower CI values shown in this figure are identical to values found in the articles. Given the variation in statistical techniques used to obtain adjusted odds ratios (ORs), the upper CI value in this figure may not be identical to reported values found in the individual studies (see Supplemental Digital Content 2, http://links.lww.com/ALN/B435, which lists all of the studies that met our primary, secondary, and tertiary analyses). HR = hazard ratio.

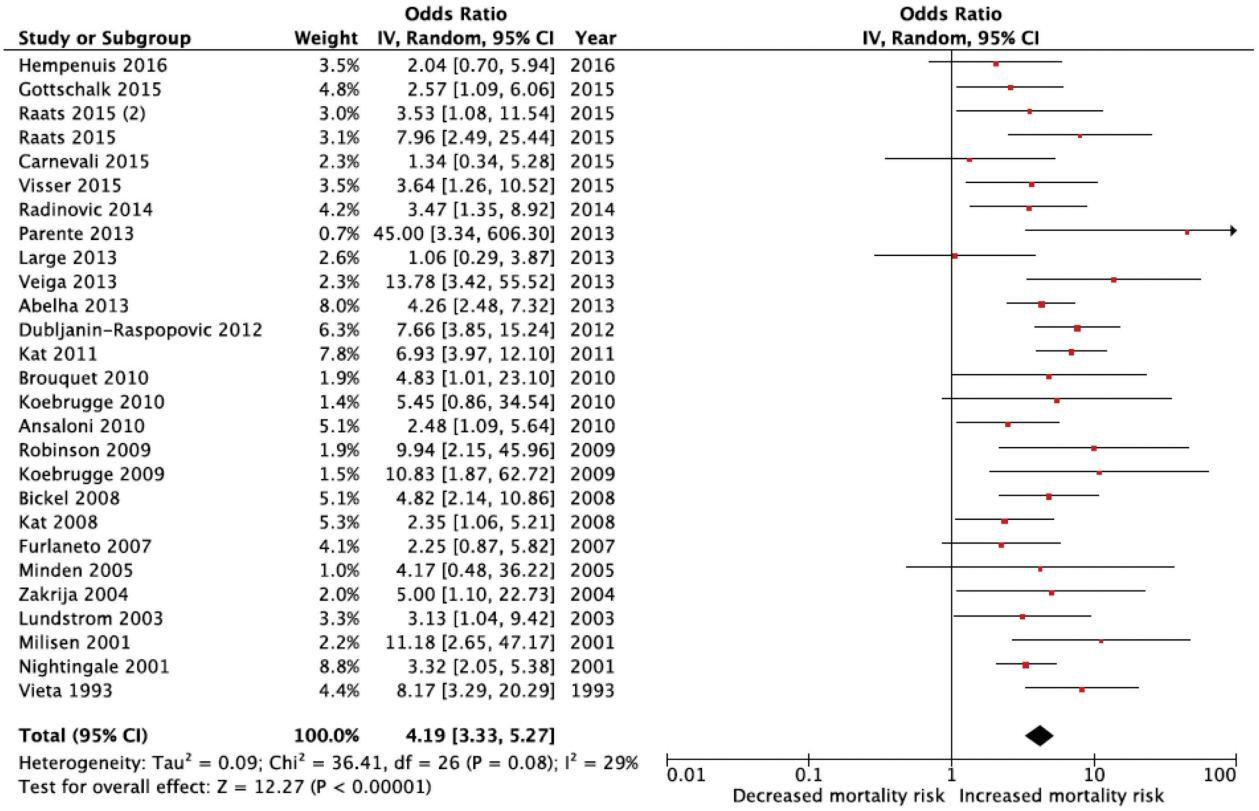

Fig. 4. Tertiary analysis: forest plot of unadjusted event rates available for pooled analysis. Note THAT The point estimates and lower CI values shown in this figure are identical to values found in the articles. Given the variation in statistical techniques used to obtain adjusted odds ratios, the upper CI value in this figure may not be identical to reported values found in the individual studies (see Supplemental Digital Content 2, http://links.lww.com/ALN/B435, which lists all of the studies that met our primary, secondary, and tertiary analyses). df = degrees of freedom.

Discussion

On an unadjusted basis, death is far more common in patients who become delirious after surgery. However, based on our findings there is currently insufficient evidence to support a causal relationship between delirium and postoperative mortality. Because inspection of forest plots when studies were grouped by risk of confounding bias demonstrated a decrease in the effect size estimates for delirium as control for confounding improved, this suggests that, within the perioperative population, either the true effect of postoperative delirium on mortality risk may be substantially smaller than previously reported, or delirium may simply be an indicator of underlying factors that predispose a patient to an increased risk of death rather than a true independent risk factor. We found only two studies that adjusted for our predefined key confounding variables, and in both studies no significant association was found between incident postoperative delirium and mortality.

The major strength of this study is that we sought to investigate the independent nature of delirium as an exposure on mortality in a fashion specific to the perioperative setting. This systematic review and meta-analysis is, to our knowledge, the first study of its kind to systematically synthesize data on the impact of incident delirium on mortality in perioperative patients. Furthermore, our protocol was registered *a priori* and designed in keeping with best-practice methods, which should limit the risk of bias in our results. The present study also has limitations. First, no included study was at low risk of bias. Second, although this study was restricted to noncardiac surgical patients, the surgical populations remained heterogeneous. Third, the mortality outcome windows were variable. The variable duration of mortality follow-up from the surgical period may have altered the causative impact that a perioperative delirious episode would have on mortality; however, given a recent study by Smith *et al.*[70] that reinforced that early mortality risk stratification is consistent over the first postoperative year, we believed that it was appropriate not to stratify by outcome ascertainment window despite the variations in follow-up duration between studies. Fourth, we were unable to use data on the duration of the delirium given the heterogeneity and paucity of our data (inconsistently reported by 9 of 34 studies). Finally, the cause of death was not examined in our review; however, such data could help to explain a possible causal relationship between delirium and mortality and should be considered in future prospective studies.

We focused only on mortality as an outcome because mortality is reliably measured, is of importance to multiple stakeholders in the perioperative setting, and confounding variables in the delirium–mortality relationship are relatively well defined. Other outcomes are also relevant to patients, clinicians, and the healthcare system; however, a methodologically sound analysis of other outcomes (*e.g.,* complications, length of stay, discharge disposition, or quality of recovery) was not possible due to limitations in measurement of these outcomes and unclear sources of confounding.

Delirium is common after surgery, particularly in older patient populations.[7] At baseline, patients who develop delirium tend to differ substantially from patients who do not become delirious, and these differences (*e.g.,* advanced age, comorbidity burden, baseline cognitive status, surgical indication and urgency, and sex) are also consistently associated with an increased risk of death. Therefore, the delirium–mortality relationship is likely to be highly confounded. Because of this confounded relationship, any attempt at identifying an independent association between delirium and mortality requires careful control of these factors. In the two studies that we identified with adequate confounder control,[11,34,35] no significant independent association of delirium on postoperative mortality was identified. In contrast, Witlox *et al.*[8] examined the risk of delirium on postdischarge mortality among all of the hospitalized patients. In their primary analysis that included effect estimates from seven studies (three of which included surgical patients) that controlled for the confounders age, sex, comorbidity or illness severity, and baseline dementia, they found a significant increase in mortality risk (pooled HR = 1.95 [95% CI, 1.51 to 2.52]) associated with delirium. However, their result must be interpreted in consideration of additional sources of bias, such as combining substantially heterogeneous populations, combining both prevalent and incident delirium, and a lack of control for confounders specific to the perioperative setting. In fact, none of the surgical studies included in the primary analysis by Witlox *et al.*[8] met our *a priori* criteria for adequate confounder control, mainly due to a lack of control for surgery-specific confounders. A secondary analysis from Witlox *et al.*[8] that combined unadjusted effect estimates from 17 strictly surgical studies found a pooled OR of 2.94 (95% CI, 2.30 to 3.75) associating delirium with mortality, a finding that is in keeping with the unadjusted pooled OR found in our study. Therefore, we suggest that the divergence of our findings from those of Witlox *et al.*[8] are accounted for by an approach to confounder control that was specifically defined for perioperative patients in our study and/or potential differences between the pathophysiology of postoperative delirium in medical *versus* surgical patients. In fact, there is some evidence suggesting that delirium in patients with hip fractures is more likely to result in complete recovery than other forms of delirium.[71]

Although our findings do not support an independent association between postoperative delirium and mortality, this finding is not conclusive. First, only two of 34 studies that we identified had adequate control for confounding based on a minimum set of required variables. Our six predefined confounding variables likely represent a set of factors that are necessary but not fully sufficient to control for confounding in the delirium–mortality relationship. In addition, our inclusion criteria did not specify required methods for confounder definitions, handling of quantitative variables, or statistical methods that would be preferred in low risk-of-bias observational studies. Next, studies in

our primary analysis included only patients undergoing hip surgery; therefore, we are unable to generalize our findings to other noncardiac surgery populations and, in particular, to patients undergoing elective surgery. Finally, the two studies included in our analysis featured two different outcome ascertainment periods (30 days *vs.* 49 months), and although neither found a significant difference in mortality, they each reported a different directional association (short-term follow-up study-adjusted OR = 0.46; long-term follow-up study-adjusted HR = 1.2). Therefore, if the relationship between incident delirium and postoperative mortality is to be understood in a fashion that allows for causal inference and evidence-based clinical care, appropriately powered multicentered studies of relevant patient populations with a reliable delirium definition, complete capture of long-term mortality, granular control for confounding using best-practice methods in observational research, and a time-to-event analysis will be needed.

Until such studies are available, clinicians should consider the following when interpreting our results. Although our article suggests that delirium may not independently change the risk of mortality, there are many other reasons that clinicians might seek to prevent delirium in the perioperative setting. Delirium can be a frightening and unpleasant experience for patients and their families. In addition, we have not assessed the impact of delirium on other important outcomes. Finally, many interventions used to decrease delirium risk (*e.g.*, orientation, mobilization, and opioid sparing analgesia,) would likely positively impact other geriatric-specific risks.

The available literature does not support an independent association between delirium and mortality after noncardiac surgery. However, unadjusted results indicate that patients who develop delirium are at an increased risk of death. As the risk of bias decreased, the association between delirium and mortality decreased; and in the lowest risk-of-bias studies, no association was present. Therefore, given the increasing population of older patients presenting for surgery, low risk-of-bias studies are urgently needed to solidify our understanding of the delirium–postoperative mortality relationship.

Acknowledgments

The authors acknowledge Sascha Davis, Information Specialist (Learning Services, The Ottawa Hospital, Ottawa, Ontario, Canada), for her assistance in developing and executing our search.

Research Support

Support was provided solely from institutional and/or departmental sources.

Competing Interests

Dr. McIsaac acknowledges salary support and use of DistillerSR from The Ottawa Hospital Department of Anesthesiology. Dr. Hamilton is a Canadian Frailty Network Highly Qualified Person. The authors declare no competing interests.

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High Concentrations of Tranexamic Acid Inhibit Ionotropic Glutamate Receptors

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ABSTRACT

Background: The antifibrinolytic drug tranexamic acid is structurally similar to the amino acid glycine and may cause seizures and myoclonus by acting as a competitive antagonist of glycine receptors. Glycine is an obligatory co-agonist of the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors. Thus, it is plausible that tranexamic acid inhibits NMDA receptors by acting as a competitive antagonist at the glycine binding site. The aim of this study was to determine whether tranexamic acid inhibits NMDA receptors, as well as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid and kainate subtypes of ionotropic glutamate receptors.

Methods: Tranexamic acid modulation of NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, and kainate receptors was studied using whole cell voltage-clamp recordings of current from cultured mouse hippocampal neurons.

Results: Tranexamic acid rapidly and reversibly inhibited NMDA receptors (half maximal inhibitory concentration = 241 ± 45 mM, mean ± SD; 95% CI, 200 to 281; n = 5) and shifted the glycine concentration–response curve for NMDA-evoked current to the right. Tranexamic acid also inhibited α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (half maximal inhibitory concentration = 231 ± 91 mM; 95% CI, 148 to 314; n = 5 to 6) and kainate receptors (half maximal inhibitory concentration = 90 ± 24 mM; 95% CI, 68 to 112; n = 5).

Conclusions: Tranexamic acid inhibits NMDA receptors likely by reducing the binding of the co-agonist glycine and also inhibits α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid and kainate receptors. Receptor blockade occurs at high millimolar concentrations of tranexamic acid, similar to the concentrations that occur after topical application to peripheral tissues. Glutamate receptors in tissues including bone, heart, and nerves play various physiologic roles, and tranexamic acid inhibition of these receptors may contribute to adverse drug effects. **(Anesthesiology 2017; 127:89-97)**

TRANEXAMIC ACID (TXA) is an antifibrinolytic drug that is used widely to reduce blood loss resulting from a variety of hemorrhagic causes, including trauma, postpartum hemorrhage, and surgical procedures.[1–8] TXA is a synthetic analog of the endogenous amino acid lysine, which binds to the lysine binding site of plasminogen.[9] TXA blocks the conversion of plasminogen to plasmin and the degradation of fibrin blood clots, thereby producing hemostatic effects.[9]

TXA is administered either systemically or topically. Systemic administration to patients by intravenous injection produces concentrations in the cerebrospinal fluid of 30 to 200 μM and plasma of 0.6 to 2 mM.[7,10–14] In contrast, topical application of TXA directly to peripheral tissues during some surgical procedures would produce high localized concentrations (0.7 to 100 mg/ml, equivalent to 5 to 600 mM).[2] Topical application of TXA is becoming increasingly popular, as this method may reduce bleeding and produce fewer side effects than systemic administration.[2] Plasma concentrations after topical application generally are less than one tenth of the level after intravenous administration.[2,15–17]

What We Already Know about This Topic

- The antifibrinolytic drug tranexamic acid may cause seizures by acting as a competitive antagonist of glycine receptors.
- Glycine is an obligatory co-agonist of the *N*-methyl-D-aspartate receptors found in the brain and peripheral tissues.

What This Article Tells Us That Is New

- Tranexamic acid inhibits *N*-methyl-D-aspartate receptors likely by reducing the binding of the co-agonist glycine and also inhibits other ionotropic glutamate receptors. Receptor blockade only occurs at high concentrations, similar to those that occur after topical application to peripheral tissues.
- Inhibition of glutamate receptors in peripheral tissues may contribute to adverse effects observed at high concentrations.

TXA inhibition of receptors other than plasminogen may cause adverse effects including seizures and myoclonus.[14,18,19] TXA is structurally similar to the inhibitory

Corresponding article on page 6. This is a Coagulation 2016 article. I.L. and D.-S.W. contributed equally to this article.

Submitted for publication July 29, 2016. Accepted for publication March 31, 2017. From the Departments of Physiology (I.L., D.-S.W., K.K., C.D.M., B.A.O.) and Anesthesia (C.D.M., B.A.O.), University of Toronto; Department of Anesthesia, Keenan Research Centre for Biomedical Science and the Li Ka Shing Knowledge Institute, St. Michael's Hospital (C.D.M.); and Department of Anesthesia, Sunnybrook Health Sciences Centre (B.A.O.), Toronto, Ontario, Canada.

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neurotransmitter glycine and acts as a competitive antagonist at glycine receptors.[14] Concentrations of TXA in the low millimolar range reduce inhibitory neurotransmission (or disinhibition), which causes network hyperexcitability and seizure-like events in animal models.[14,19–21]

The effects of TXA on the major subtypes of excitatory ionotropic glutamate receptors that also modify brain network excitability have not been elucidated fully. These receptor subtypes include *N*-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, and kainate receptors.[22] TXA modulation of NMDA receptors is of particular interest because these receptors contain a high-affinity glycine binding site.[23] Binding of both glutamate and glycine is required for full activation of the NMDA receptors, and drugs that inhibit glycine binding reduce NMDA receptor function.[24] TXA could compete with glycine at the glycine binding of the NMDA receptor and thereby reduce receptor function. Others have shown that TXA, at low concentrations (1 to 5 mM), does not inhibit excitatory synaptic currents in amygdala slices.[25] However, the effects of high concentrations of TXA on glutamate receptors, such as those that occur during topical application, have not been elucidated. Glutamate receptors are expressed widely in peripheral tissues, including bone, heart, pancreas, and nerves, where they serve diverse physiologic roles.[26–32] Thus, it is of interest to determine whether TXA blocks ionotropic glutamate receptors.

For these proof-of-concept studies, currents generated by NMDA, AMPA, and kainate receptors were recorded from hippocampal neurons. The rationale is that these cells express high levels of ionotropic glutamate receptors,[33] and receptors in hippocampal neurons and peripheral tissues have similar structural, physiologic, and pharmacologic properties.[26,28,31] The results show that TXA reduces NMDA receptor function by acting as a competitive antagonist at the glycine binding site. Surprisingly, TXA also inhibits AMPA and kainate receptors. These results predict that TXA at high concentrations, applied topically to peripheral tissues, inhibits ionotropic glutamate receptors.

Materials and Methods

Cell Culture

All experimental procedures were approved by the Animal Care Committee of the University of Toronto (Toronto, Ontario, Canada). Primary cultures of hippocampal neurons were prepared from Swiss White mice (Charles River, Canada) as previously described.[34] In brief, fetal pups (embryonic day 18) were removed from maternal mice that had been euthanized by cervical dislocation. The hippocampi of each fetus were collected and placed on an ice-cooled culture dish. Neurons were then dissociated by mechanical trituration with a Pasteur pipette (tip diameter 150 to 200 μm) and plated on 35-mm culture dishes. The culture dishes were coated with collagen or poly-D-lysine (Sigma-Aldrich, Canada). The density of neurons per culture dish was approximately 1×10^6 cells. Two hours later, the medium was changed to a neurobasal medium supplemented with 2% B27 and 1% GlutaMAX (Life Technologies, USA). The medium was changed every 3 days. The low-density dissociated neurons were maintained in culture for 14 to 20 days before use. At this point in time, hippocampal neurons become appropriately polarized, develop extensive axonal and dendritic arbors, and form numerous, functional synaptic connections with one another, which resemble mature hippocampal neurons *in vivo*.[35] Culture dishes were prepared from at least two different mice for each experiment, and a maximum of two cells was recorded from each dish.

Whole Cell Voltage-clamp Recordings

Whole cell currents were recorded under voltage-clamp (–60 mV) conditions with an Axopatch 1D amplifier (Molecular Devices, USA) controlled with pClamp 8.0 software (Molecular Devices) *via* a Digidata 1322A interface (Molecular Devices). Patch pipettes with open-tip resistances of 2 to 3 MΩ were pulled from thin-walled borosilicate glass capillary tubes. Patch electrodes were filled with an intracellular solution containing (in mM) 140 cesium chloride, 10 HEPES, 11 EGTA, 2 magnesium chloride, 1 calcium chloride, 4 magnesium adenosine triphosphate, and 2 triethanolamine (adjusted to pH 7.3 with cesium hydroxide and to 285 to 295 mOsm with water). To record NMDA-evoked current, magnesium-free extracellular solution was used and contained (in mM) 140 sodium chloride, 1.3 calcium chloride, 2 potassium chloride, 25 HEPES, and 28 glucose (adjusted to pH 7.4 with sodium hydroxide and to 320 to 330 mOsm with water). To record AMPA- and kainate-evoked current, magnesium chloride (1 mM) was added to the extracellular solution. Tetrodotoxin (300 nM) was added to the extracellular solution to block voltage-sensitive sodium channels. A computer-controlled, multibarrelled perfusion system (SF-77B; Warner Instruments, USA) was used to apply the extracellular solution to neurons. The time interval between applications of agonists, including NMDA, AMPA, and kainate, was 2 min. This interval was sufficient to allow receptors to recover from desensitization. No randomization methods were applied. Currents were recorded before and during the application of TXA, and the experimenters were not blinded to the drug application conditions.

Drugs

TXA, NMDA, AMPA, kainate, and glycine were obtained from Sigma-Aldrich. Tetrodotoxin was purchased from Alomone Labs (Israel). Stock solutions of these reagents were prepared with distilled water.

Data Analysis

Currents were analyzed with pClamp 10 software (Molecular Devices). The concentration–response plots were fitted to the modified Michaelis–Menten equation: $I = I_{max}/[1 + (EC_{50}/c)^{nH}]$, where I is the current amplitude, EC_{50} is the concentration of agonist that produces currents with 50%

of the maximal amplitude, c is the concentration of agonist, and nH is the estimated Hill coefficient. The concentration–response plots for TXA inhibition were fitted to the following equation: $I = (IC_{50})^{nH}/[c^{nH} + (IC_{50})^{nH}]$, where IC_{50} is the concentration of TXA that inhibited 50% of the current amplitude.

Statistical Analyses

No statistical power calculation was conducted before the study, and the sample size was based on our previous experience with this experimental design. There were no missing data from the results presented in this manuscript. Results are presented as mean ± SD together with 95% CI of the mean. Statistical analysis was performed with GraphPad Prism 5 software (GraphPad Software Inc., USA). Differences between groups were determined with the Student's *t* test or a one-way ANOVA with a Dunnett multiple-comparison *post hoc* test. A two-tailed hypothesis test was used, and any *P* value less than 0.05 was considered significant.

Results

High Concentrations of TXA Inhibit NMDA Receptors

We first identified the concentration of NMDA that evoked 50% of the maximal current (EC_{50}) in hippocampal neurons as this information was used to design studies that examined TXA inhibition of NMDA receptors. Application of NMDA (3 to 3,000 μM) rapidly activated inward current, which increased in amplitude with increasing concentrations of NMDA in the presence of glycine (1 μM). The concentration–response plot for NMDA-evoked current revealed an EC_{50} value of 98 ± 44 μM (95% CI, 51 to 146; n = 4; fig. 1A).

Next, to study the inhibitory effects of TXA on NMDA receptors, NMDA was applied at a concentration close to the EC_{50} value (100 μM) together with glycine (1 μM), in the absence or presence of TXA. Low concentrations of TXA (1 mM, 3 mM) had no effect on the amplitude of the peak current. However, higher TXA concentrations (10 mM or greater) rapidly and reversibly inhibited the NMDA-evoked current (fig. 1B). TXA at the threshold concentration (10 mM) inhibited the NMDA current by 4.2 ± 5.4% (95% CI, −0.5 to 8.9; n = 5), and the maximum inhibition at 3 M was 97.1 ± 1.8% (95% CI, 95.5 to 98.7; n = 5). The concentration–response plot showed that the concentration of TXA required to inhibit 50% of the maximal peak current (IC_{50}) was 241 ± 45 mM (95% CI, 200 to 281; n = 5).

TXA Is a Competitive Antagonist at the Glycine Binding Site

To further probe the hypothesis that TXA decreases NMDA receptor function by competitively inhibiting glycine binding, we next examined the inhibitory effects of TXA (100 mM) on current evoked by NMDA (100 μM) with different concentrations of glycine in the extracellular solution. The extent of TXA-mediated inhibition decreased with increasing concentrations of glycine (fig. 2A), and a near-saturating concentration of glycine almost completely abolished TXA inhibition of NMDA-evoked current. Specifically, TXA inhibited NMDA currents by 59.1 ± 23.2%

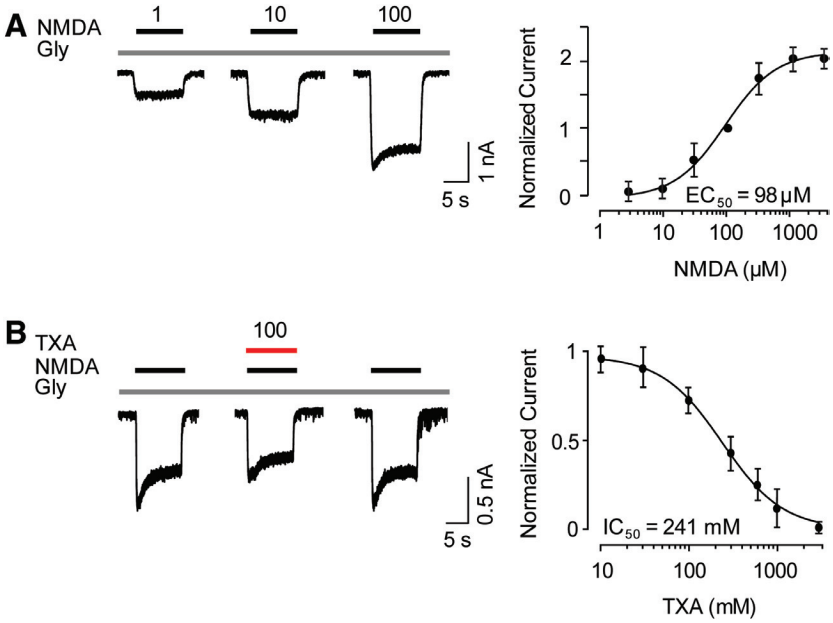

Fig. 1. TXA inhibits *N*-methyl-D-aspartate (NMDA)-evoked currents in hippocampal neurons. (*A*) Representative traces showing currents evoked by increasing concentrations of NMDA and the corresponding concentration–response plot (n = 4). (*B*) Representative traces and the corresponding concentration–response plot (n = 5) showing the inhibitory effects of tranexamic acid (TXA) on currents activated by NMDA (100 μM). Glycine (1 μM) was present continuously in the bath. All data are presented as mean ± SD.

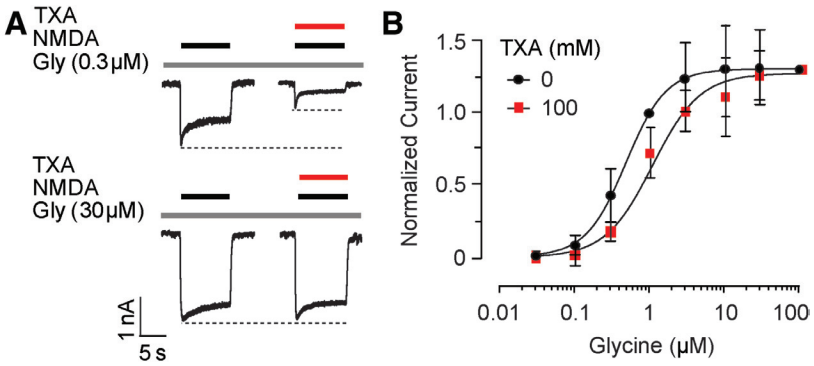

Fig. 2. Tranexamic acid (TXA) inhibition of *N*-methyl-D-aspartate (NMDA)-evoked currents decreases with increased glycine concentration. (*A*) Representative traces demonstrating decreased TXA (100 mM) inhibition of currents evoked by NMDA (100 μM) with an increased glycine concentration. (*B*) Glycine concentration–response plots for currents evoked by NMDA (100 μM) in the absence and presence of TXA. Glycine half maximal effective concentration was increased from 0.48 ± 0.09 μM (95% CI, 0.43 to 0.53) without TXA to 0.95 ± 0.19 μM (95% CI, 0.81 to 1.1) with TXA (P = 0.02, unpaired one-tailed Student's t test). All responses were normalized to the peak current evoked by NMDA (100 μM) and glycine (1 μM) without TXA. The sample sizes in the absence and presence of TXA were n = 10 and n = 8, respectively. All data are presented as mean ± SD.

(95% CI, 43.0 to 75.2; n = 8) in the presence of 0.3 μM glycine but by only 2.2 ± 3.7% (95% CI, −0.4 to 4.8; n = 8) when the glycine concentration was increased to 30 μM. We next constructed glycine (0.03 to 100 μM) concentration–response plots for NMDA current, recorded in the absence and presence of TXA (fig. 2B). TXA shifted the glycine concentration–response curve to the right without changing the maximum response. The glycine EC_{50} increased from 0.48 ± 0.09 μM (95% CI, 0.43 to 0.53; n = 10) to 0.95 ± 0.19 μM (95% CI, 0.81 to 1.1; n = 8) in the presence of TXA (P = 0.02), whereas the Hill coefficient was unchanged (control 1.5 ± 0.6; 95% CI, 1.1 to 1.9; n = 10 *vs.* TXA 1.6 ± 0.3; 95% CI, 1.5 to 1.7; n = 8, P = 0.9). These results are consistent with the hypothesis that TXA inhibits NMDA receptors by acting as a competitive antagonist at the glycine binding site.

The binding sites of NMDA and glycine interact allosterically such that glycine affinity increases with the binding of NMDA and, conversely, NMDA affinity increases with the binding of glycine.[36,37] These allosteric interactions predict that TXA blockade should decrease with increasing concentrations of NMDA (because of increased glycine binding). To test this prediction, the inhibitory effects of TXA (100 mM) on NMDA currents were studied under conditions in which the concentration of NMDA was varied whereas the concentration of glycine (1 μM) was fixed (fig. 3). For NMDA 1, 10, and 100 μM, TXA inhibited the currents by 66.1 ± 10.3% (95% CI, 57.9 to 74.3), 48.3 ± 12.7% (95% CI, 38.1 to 58.5), and 26.4 ± 2.5% (95% CI, 24.6 to 28.6), respectively (n = 6). As predicted, TXA inhibition decreased with increasing concentrations of NMDA.

Inhibition by TXA Is Not Use Dependent or Voltage Dependent

TXA inhibition should exhibit no use or voltage dependence if it acts as a competitive antagonist at the glycine binding site, rather than as a noncompetitive blocker of the open channel pore.[38] The extent of TXA (100 mM) inhibition was similar after repeated application of NMDA and TXA (fig. 4A), suggesting the block is not use dependent. Next, the concentration of TXA in the extracellular solution was increased to 300 mM (close to IC_{50} value). Neurons were perfused continuously with this solution. Three sequential applications of NMDA (100 μM) showed that inhibition of three current

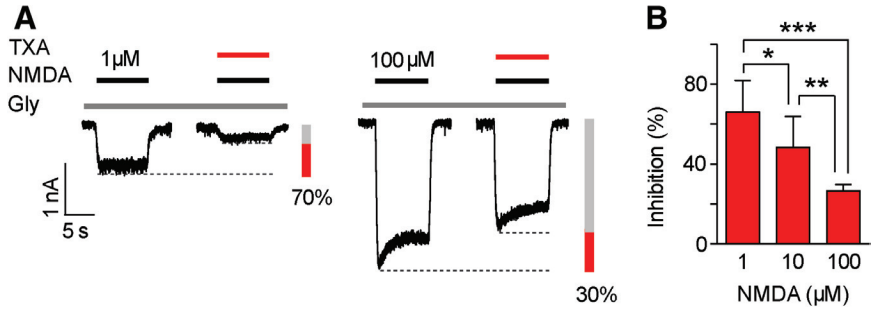

Fig. 3. Tranexamic acid (TXA) inhibition of *N*-methyl-D-aspartate (NMDA)-evoked currents is reduced with increasing NMDA concentrations. (*A*) Representative traces showing decreased effects of TXA (100 mM) on currents evoked by an increased concentration of NMDA. (*B*) Summarized data for the responses shown in (*A*) (n = 6). Glycine (1 μM) was present continuously in the bath. *P < 0.05, **P < 0.01, ***P < 0.001, one-way ANOVA with Dunnett multiple-comparison *post hoc* test. All data are presented as mean ± SD.

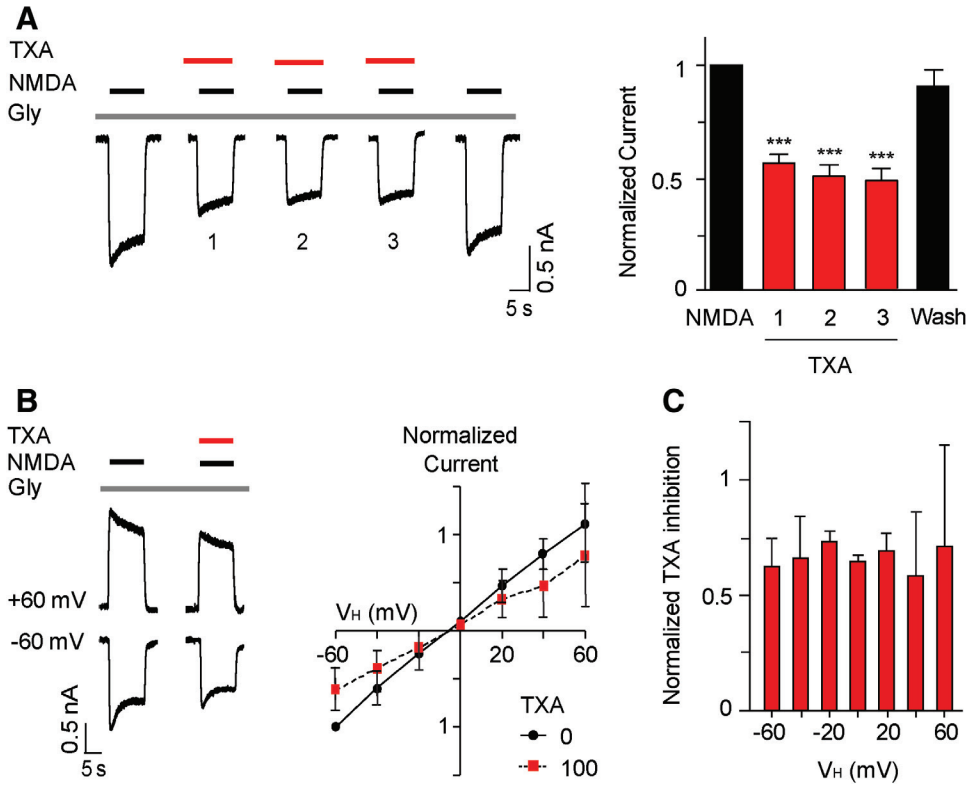

Fig. 4. Tranexamic acid (TXA) inhibition of *N*-methyl-D-aspartate (NMDA) currents is not use and voltage dependent. (*A*) Repeated application of TXA (100 mM) did not cause a statistically significant increase in blockade of currents evoked by NMDA (100 μM). Currents recorded during the sequential application of TXA are identified as 1, 2, and 3. The *bar graph* shows mean peak amplitude of the current recorded with and without coapplication of TXA (n = 5). ***$P < 0.001$ *versus* NMDA, one-way ANOVA with Dunnett multiple-comparison *post hoc* test. (*B* and *C*) Representative traces and summarized data showing the inhibitory effects of TXA (100 mM) on currents evoked by NMDA (100 μM) at different holding potentials (V_H). For current–voltage (*I–V*) plots in (*B*), the currents were normalized to the peak current induced by NMDA without TXA at a holding potential of –60 mV. The sample sizes for the *I–V* plot in the absence and presence of TXA were n = 9 and n = 7, respectively. In (*C*), note that TXA-induced inhibition of NMDA currents was similar for all holding potentials. $P = 0.8$, one-way ANOVA with Dunnett multiple-comparison *post hoc* test. For all recordings, glycine (1 μM) was continuously in the bath. All data are presented as mean ± SD.

pulses by TXA was similar (78.9 ± 10.3% with 95% CI, 69.9 to 87.9; 78.5 ± 9.6% with 95% CI, 70.1 to 86.9; and 79.8 ± 9.8% with 95% CI, 71.2 to 88.4; respectively, n = 5; $P = 0.5$, one-way ANOVA). These results further indicate that TXA inhibition of NMDA receptors was not use dependent. To determine whether TXA-mediated inhibition was voltage dependent, NMDA currents were recorded at different holding potentials in the absence or presence of TXA (100 mM) and current–voltage plots were constructed. TXA decreased the slope of the current–voltage plot but did not change the reversal potential (NMDA: –5.2 mV, +TXA: –5.5 mV, $P = 0.9$; fig. 4B). Moreover, the extent of TXA-induced inhibition of NMDA currents was equivalent at holding potentials between –60 and +60 mV ($P = 0.8$, fig. 4C). Thus, TXA inhibition of NMDA currents was not voltage dependent.

High Concentrations of TXA Inhibit AMPA and Kainate Receptors

Unlike NMDA receptors, AMPA receptors and kainate receptors do not require the binding of the co-agonist glycine to promote channel opening. Thus, we hypothesized that TXA would not inhibit AMPA and kainate receptor function. To test this hypothesis, AMPA and kainate were first applied to the neurons to determine EC_{50} values (fig. 5, A and B). Next, the inhibitory effects of TXA on currents evoked by AMPA (10 μM) and kainate (20 μM), applied at concentrations close to the EC_{50} values, were studied. Contrary to what was predicted, TXA caused concentration-dependent inhibition of both AMPA- and kainate-evoked currents, with IC_{50} values of 231 ± 91 mM (95% CI, 148 to 314; n = 5 to 6; fig. 5C) and 90 ± 24 mM (95% CI, 68 to 112; n = 5; fig. 5D), respectively. TXA blockade was rapid and was completely reversed after drug washout.

Discussion

The results show that TXA inhibited ionotropic glutamate receptors, but only at high millimolar concentrations. The efficacy of TXA inhibition of NMDA receptors depended on the concentrations of glycine and NMDA, and the blockade was neither use dependent nor voltage dependent. TXA also

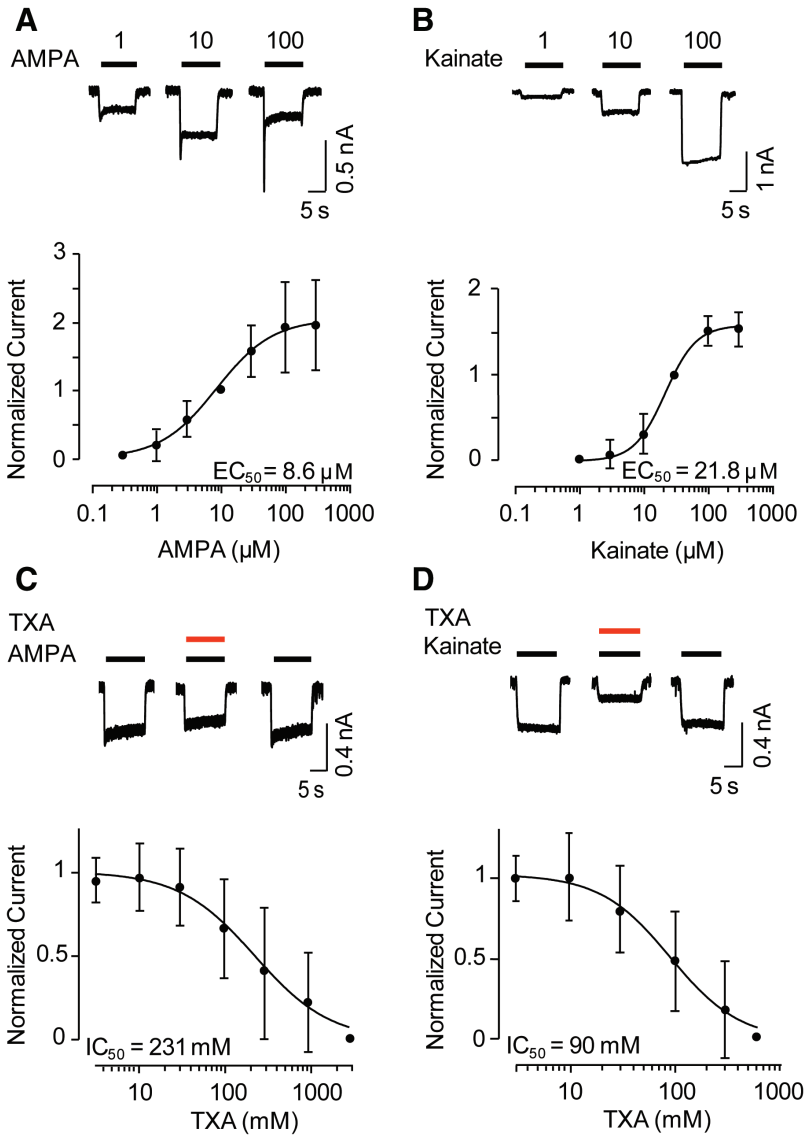

Fig. 5. Tranexamic acid (TXA) inhibits α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)- and kainate-evoked currents. (*A* and *B*) Representative traces and the concentration–response plots for currents evoked by (*A*) AMPA and (*B*) kainate. The half maximal effective concentration was 8.6 ± 4.0 μM (95% CI, 5.2 to 12.0) for AMPA responses and 21.8 ± 3.6 μM (95% CI, 18.3 to 25.3) for kainate responses. The sample sizes for 0.3 to 10, 30 to 100, and 300 μM AMPA were n = 6, n = 5, and n = 4, respectively. The sample size for kainate was n = 4. (*C* and *D*) Representative traces and the corresponding concentration–response plots demonstrating the inhibitory effects of TXA on currents induced by AMPA (10 μM, *C*) and kainate (20 μM, *D*). The sample sizes for 3 to 100 mM and 300 to 3,000 mM TXA on AMPA currents were n = 6 and n = 5, respectively. The sample sizes for TXA on kainate currents were n = 5. All data are presented as mean ± SD.

inhibited AMPA and kainate receptors at concentrations that are similar to those that inhibited NMDA receptors.

These results build on two previous studies that examined the effects of TXA on glutamate receptor function.[18,25] In one of these studies, electrophysiologic recordings showed that TXA did not inhibit excitatory currents in amygdala slices, but only low millimolar concentrations were studied.[25] More specifically, TXA concentrations less than 10 mM did not modify synaptic currents generated by glutamate receptors.[25] These findings are consistent with our results. In the second study, TXA, at concentrations up to 10 mM, did not bind to NMDA receptors in rat cortical tissue.[18] However, the experimental conditions used in the study did not favor TXA binding as the extracellular solution contained high concentrations of NMDA and glycine. TXA blockade of NMDA receptors is reduced under these conditions, possibly because of a competitive interaction between glycine and TXA at the NMDA receptor.

The results from our electrophysiologic studies do not discern whether TXA binds directly to NMDA receptors or indirectly reduces receptor function. However, several lines of evidence suggest that TXA inhibits NMDA receptors directly by preventing glycine from acting as a full co-agonist at these receptors. First,

high extracellular concentrations of glycine reduce TXA blockade. Second, TXA shifts the glycine concentration–response plot to the right without reducing the maximal NMDA-evoked current response. Third, TXA inhibition of NMDA receptors is not use dependent or voltage dependent, which suggests that TXA lacks the features of steric blockers, such as magnesium and ketamine, which occlude the open channel pore.[39–41]

Interestingly, increasing the concentration of NMDA reduced TXA inhibition of NMDA receptors. This result is consistent with evidence indicating that glutamate and glycine binding to the NMDA receptors is allosterically coupled.[36,37] Glutamate agonists increase [^{3}H]glycine binding, whereas glutamate antagonists decrease [^{3}H]glycine binding.[37] Reciprocally, competitive antagonists at the glycine binding site allosterically reduce glutamate binding.[36] Increasing the concentration of NMDA likely increases glycine affinity, thereby reducing TXA inhibition.

TXA also inhibits AMPA receptors and kainate receptors. These results were unexpected, given that the AMPA and kainate receptors lack a glycine binding site.[22] However, TXA inhibits other transmitter-gated ion channels that lack a glycine binding site, including γ-aminobutyric acid type A receptors.[14,18,25] It remains unknown how TXA inhibits AMPA and kainate receptors, and further studies are required to determine whether inhibition results from steric, allosteric, or other indirect mechanisms.

Although we examined the TXA sensitivity of glutamate receptors expressed in central neurons, the receptors expressed in peripheral tissues are more likely to be exposed to high millimolar concentrations of TXA. Nevertheless, ionotropic glutamate receptors in peripheral tissues and central neurons exhibit similar structural, kinetic, and pharmacologic properties, suggesting that the results are clinically relevant.[26,28,31] For example, in both central neurons and peripheral tissues, GluN1 and GluN2 subunits form functional NMDA receptors.[28,31] Also, current generated by glutamate receptors in bone and heart tissue exhibits kinetic and pharmacologic sensitivities that are similar to those of glutamate receptors in neurons.[26,28] For example, NMDA receptors in bone cells and neurons are inhibited by both magnesium ions and the high-affinity antagonist MK-801,[26] suggesting that receptors expressed in neurons and nonneuronal tissues likely exhibit similar sensitivity to TXA.

High concentrations of TXA that inhibit glutamate receptors could occur after topical application of the drug. Glutamate receptors expressed in peripheral tissues would be exposed to such high concentrations. For example, topical application of TXA is used commonly during orthopedic surgery.[17,42–45] NMDA receptors composed of GluN1 and GluN2A-D are expressed in osteoblasts and osteoclasts in bone.[28,31] As such, TXA inhibition of NMDA receptors could alter bone healing. Furthermore, receptor subunits of NMDA (GluN1 and GluN2), AMPA (GluA2 and GluA3), and kainate (GluK1 and GluK2) are expressed in the heart, pancreas, and gastrointestinal tissue.[28–30,46] Cardiac glutamate receptors, which are localized preferentially within the conducting system, the nerve terminals, and the intramural ganglia cells,[27] are involved in conducting impulses and rhythm control.[27,47] It is plausible that topical application of TXA to the heart's conducting system could cause dysrhythmias. Inhibition of NMDA receptors in human and mouse pancreatic islets increases secretion of insulin, so receptor inhibition by TXA could alter glucose levels.[48] Finally, inhibition of NMDA receptors in peripheral nerves alters nociception in both rodents and humans.[49]

Awareness of such potential "off-target" effects secondary to TXA effects on glutamate receptors will inform studies that seek to identify adverse effects of topically applied TXA in patients. Clinical studies first focused on the adverse consequences of antifibrinolytic effects of TXA, including deep vein thrombosis, stroke, and myocardial infarction.[50–53] Side effects due to off-target receptor effects only have been recognized recently. For example, seizure and myoclonus likely due to TXA inhibition of glycine receptors and γ-aminobutyric acid type A receptors and mechanistically based treatments for those seizures only have been identified recently.[19] By analogy, the physiologic roles of glutamate receptors expressed in peripheral tissues are only beginning to be understood. It will be worthwhile, in future studies, to determine whether TXA inhibition of such physiologic functions contributes to adverse effects of TXA.

In summary, high concentrations of TXA inhibit ionotropic glutamate receptors. These preclinical results will support future studies aiming to clarify the safety and consequences of applying TXA topically to nonneuronal tissues and peripheral nerves in patients.

Acknowledgments

The authors thank Ella Czerwinska, M.Sc., and Junhui Wang, M.D., Ph.D., Department of Physiology, University of Toronto, Toronto, Ontario, Canada, for their assistance with the cell cultures.

Research Support

This work was supported by operating grants from the Canadian Institutes of Health Research (CIHR), Ottawa, Ontario, Canada, to Dr. Orser (grant Nos.: 416838, 480143). Dr. Lecker was supported by the Frederick Banting and Charles Best Doctoral Scholarship from CIHR. K. Kaneshwaran was supported by the Kirk Weber Award in Anesthesia from the Department of Anesthesia, Sunnybrook Health Services Centre, Toronto, Ontario, Canada; and the Ontario Graduate Scholarship, Ontario, Canada.

Competing Interests

The authors declare no competing interests.

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Triggering Receptor Expressed on Myeloid Cells 2, a Novel Regulator of Immunocyte Phenotypes, Confers Neuroprotection by Relieving Neuroinflammation

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ABSTRACT

Background: Microglia can not only detrimentally augment secondary injury but also potentially promote recovery. However, the mechanism underlying the regulation of microglial phenotypes after stroke remains unclear.

Methods: Mice were subjected to middle cerebral artery occlusion for 60 min. At 3 days after reperfusion, the effects of activation and suppression of triggering receptor expressed on myeloid cells 2 on immunocyte phenotypes (n = 5), neurobehavioral scores (n = 7), infarct volumes (n = 8), and neuronal apoptosis (n = 7) were analyzed. *In vitro*, cultured microglia were exposed to oxygen–glucose deprivation for 4 h. Inflammatory cytokines, cellular viability (n = 8), neuronal apoptosis (n = 7), and triggering receptor expressed on myeloid cells 2 expression (n = 5) were evaluated in the presence or absence of triggering receptor expressed on myeloid cell-specific small interfering RNA or triggering receptor expressed on myeloid cells 2 overexpression lentivirus.

Results: Triggering receptor expressed on myeloid cells 2 expression in the ischemic penumbra peaked at 3 days after ischemia–reperfusion injury (4.4 ± 0.1-fold, $P = 0.0004$) and was enhanced in interleukin-4/interleukin-13–treated microglia *in vitro* (1.7 ± 0.2-fold, $P = 0.0119$). After oxygen–glucose deprivation, triggering receptor expressed on myeloid cells 2 conferred neuroprotection by regulating the phenotypic conversion of microglia and inflammatory cytokine release. Intraperitoneal administration of triggering receptor expressed on myeloid cells 2 agonist heat shock protein 60 or unilateral delivery of a recombinant triggering receptor expressed on myeloid cells 2 lentivirus into the cerebral ventricle induced a significant neuroprotective effect in mice (apoptotic neurons decreased to 31.3 ± 7.6%; infarct volume decreased to 44.9 ± 5.3%). All values are presented as the mean ± SD.

Conclusions: Activation or up-regulation of triggering receptor expressed on myeloid cells 2 promoted the phenotypic conversion of microglia and decreased the number of apoptotic neurons. Our study suggests that triggering receptor expressed on myeloid cells 2 is a novel regulator of microglial phenotypes and may be a potential therapeutic target for stroke.
(Anesthesiology 2017; 127:98-110)

STROKE is the second leading cause of death worldwide. The incidence and mortality of stroke increase considerably with age, and the majority of survivors suffer from disabilities such as paresis and speech defects.[1] Several studies have confirmed that the inflammatory response that follows cerebral ischemia is a critical factor in secondary brain damage.[2–4] Neuroinflammation affects important processes in the brain, such as adult neurogenesis and neurodegenerative diseases.[5,6] Thus, modulating neuroinflammation by targeting the relevant cells or ameliorating its harmful effects may have implications for stroke treatment.

The morphologic and functional plasticity that is associated with microglial activation varies with the nature, strength, and duration of the activating stimulus and depends on intercellular interactions, including cell-surface molecules and soluble mediators.[7] Microglia may achieve

What We Already Know about This Topic

- Neuroinflammation after cerebral ischemia is a critical factor in secondary brain damage
- Brain microglia are resident macrophage cells and mediate immunity in the brain
- Triggering receptor expressed on myeloid cells 2 regulates the phenotype of microglial cells

What This Article Tells Us That Is New

- In a mouse model of middle cerebral artery occlusion, activation and up-regulation of triggering receptor expressed on myeloid cells 2 (TREM2) promoted microglial switching from the detrimental M1 phenotype to the beneficial M2 phenotype
- Administering a TREM2 agonist systemically or delivering TREM2 lentivirus directly into the cerebral ventricle caused neuroprotection in mice
- TREM2 regulates microglial phenotype after stroke and may affect short-term outcome after stroke in mice

Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site (www.anesthesiology.org). Q.Z., F.L., and X.C. contributed equally to this article.

Submitted for publication October 20, 2016. Accepted for publication March 7, 2017. From the Departments of Anesthesiology (Q.Z., J.J., S.S., D.Z., L.M., T.J., F.B., L.X.), Orthopedic Surgery (F.L.), and Geriatrics (X.C.), Xijing Hospital, Fourth Military Medical University, Xi'an, China and Department of Anesthesiology, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China (Q.Z., Q.W.).

 Anesthesiology 2017; 127:98-110

a spectrum of functional phenotypes, including the classical activated phenotype (M1) and the alternative activated phenotype (M2).[8,9] M1 microglia acquire a ramified morphology in response to invading pathogens and/or central nervous system damage and also release a wide array of inflammatory cytokines, oxygen-free radicals, and other harmful substances during early inflammation. In contrast, M2 microglia are characterized by an activated, amoeboid morphology and high phagocytic activity. M2 microglia secrete neurotrophic substances, remove necrotic or apoptotic neuronal debris, make dynamic contacts with neurons, and promote the formation of glial scar tissue during late inflammation.

Triggering receptor expressed on myeloid cells 2 (TREM2) is an immunoglobulin-like receptor of the TREM family and is expressed on activated macrophages, immature dendritic cells, osteoclasts, and microglia.[10,11] Currently, cells expressing high levels of TREM2 are thought to fulfill important functions in immune surveillance, cell–cell interactions, tissue debris clearance, and the resolution of latent inflammatory reactions.[12] By comparison, the absence of TREM2 expression on these cells not only impairs their capacity to phagocytose cellular debris but also increases their production of proinflammatory cytokines.[13] TREM2 is emerging as an important negative regulator of autoimmunity and associates with an adaptor molecule, DNAX activation protein 12 kDa (DAP12), which contains an immunoreceptor tyrosine-based activation motif that is phosphorylated upon activation of DAP12-linked receptors.[14,15] The brain pathology observed in Nasu–Hakola disease patients suggested that disruption of the TREM2/DAP12 pathway leads to neurodegeneration with demyelination and axonal loss.[16] Moreover, it has been reported that TREM2 variants strongly increase the risk of developing Alzheimer disease and are involved in the microglial response to Aβ plaque deposition.[17,18] Because the role of TREM2 after stroke had not been determined, we hypothesized that modulation of TREM2 by administration of heat shock protein (HSP) 60 or injection of a recombinant TREM2 virus might control poststroke microglial activity and phenotypes.

The present study focused on the role of TREM2 in the phenotypic conversion of microglia after stroke. Here, we report that during ischemia–reperfusion injury, microglia underwent a rapid shift in their effector program, involving morphologic transformation, proliferation, and cytokine release.

Materials and Methods

Animals and Surgical Procedures

All animal-related procedures were approved by the Ethics Committee for Animal Experimentation of Fourth Military Medical University (Xi'an, Shaanxi, China) and proceeded in accordance with the guidelines for Animal Experimentation of the University. Male wild-type C57BL/6 mice were obtained from the Experimental Animal Center of the Fourth Military Medical University. Mice (8 to 12 weeks old) were housed under controlled conditions with a 12-h light/dark cycle, a temperature of 21 ± 2°C, and 60 to 70% humidity for at least 1 week before drug treatment or surgery.

Focal cerebral ischemia was induced by middle cerebral artery occlusion (MCAO) using an intraluminal filament technique as described in our previous studies.[19] Randomization methods were used to assign individual mice to experimental conditions. All mice were anesthetized with 10% chloral hydrate (350 mg/kg) *via* intraperitoneal injection. To control MCAO severity, regional cerebral blood flow was monitored using transcranial laser Doppler flowmetry (PeriFlux 5000, Perimed AB, Sweden). The MCAO was considered effective if the regional cerebral blood flow showed a sharp drop to less than 30%, and animals that did not meet this requirement were excluded (see Supplemental Digital Content 1, http://links.lww.com/ALN/B413, which is an analysis report of regional cerebral blood flow in this study). During the surgical procedures, the rectal temperature of the mice was monitored and maintained at approximately 37 ± 0.5°C. After the suture was withdrawn and the mice had recovered from the anesthesia, they were returned to their cages with free access to food and water. For data missing or excluded from the experiments, please see supplemental tables 1 and 2 (see Supplemental Digital Content 1, http://links.lww.com/ALN/B413, which is a table showing missing or excluded data in this study).

Ischemic Penumbra

The ischemic penumbras were microdissected according to established protocols in rodent models of unilateral proximal MCAO. Briefly, a 4-mm-thick coronal brain slice was cut, beginning 6 mm from the anterior tip of the frontal lobe. Next, a longitudinal cut (from top to bottom) was made approximately 1 mm from the midline through the ischemic hemisphere to remove medial tissue. Then a transverse diagonal cut was made at approximately the 2 o'clock position to separate the wedge-shaped penumbra (see Supplemental Digital Content 1 figure 1, http://links.lww.com/ALN/B413), which is a schematic showing of the ischemic penumbra during MCAO).

Intraperitoneal Injection of HSP60

Active mouse HSP60 full-length protein (Abcam, Cambridge, MA) was intraperitoneally administered 1 h before the onset of ischemia. After ischemia, the mice were kept alive for 3 days. The animals were then sacrificed, and their brains were extracted, sectioned, and stained using 2,3,5-triphenyltetrazolium chloride to analyze infarct volume. The mice were divided into four groups as follows: (1) MCAO group treated with intraperitoneal injection of bovine serum albumin (0.2 μg/g) in saline solution; (2) MCAO group treated with a single intraperitoneal injection of HSP60 at 2.5 μg/mouse; (3) MCAO group treated with HSP60 at 3.75 μg/mouse; and (4) MCAO group treated with HSP60 at 5 μg/mouse.

N9 Cell Culture and Treatment

The murine N9 microglial cell line was cultured in Iscove's modified Dulbecco's medium (IMDM; HyClone, Logan, UT) containing 4 mM glutamine, HEPES, 5% heat-inactivated fetal bovine serum (FBS; Gibco, Rockville, MD), and 1% penicillin/streptomycin (Gibco) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The medium was changed every 3 days. Cells were grown to 90% confluence in 6-well plates or 100-mm dishes for experiments involving insult with various cytokines, including lipopolysaccharide (100 ng/ml; Sigma, St. Louis, MO), recombinant murine interferon-γ (10 ng/ml; Peprotech, Rocky Hill, NJ), interleukin-10 (10 ng/ml; Peprotech), interleukin-4 (10 ng/ml; Peprotech), and interleukin-13 (10 ng/ml; Peprotech) for 24 h.

Oxygen–glucose Deprivation

Oxygen–glucose deprivation (OGD) was performed as described previously.[20] In brief, the murine N9 microglial cell line and the HT22 hippocampal cell line were subjected to OGD in experiments 3 and 4. N9 microglia cultured in IMDM without glucose (1% F-12 with 10% FBS; HyClone) were pretreated in a humidified hypoxic chamber (1% O_2, 5% CO_2, 94% N_2) at 37°C for 4 h. Then we replaced the medium with IMDM containing 5% FBS and returned the cells to a 37°C incubator with atmospheric oxygen. The HT22 cells were cultured in Dulbecco's modified Eagle's medium with 10% FBS (v/v), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Media and cells were collected at 24 h after OGD for protein analysis.

Lentivirus Transfection

A recombinant lentivirus containing the gene encoding full-length mouse TREM2 was produced using the GV341 Vector Expression System (GeneChem, Shanghai, China), the Ubi-MCS-3FLAG-SV40-puromycin component sequence, and *Age*I and *Nhe*I cloning sites. A recombinant lentivirus containing the gene encoding TREM2-RNA interference was produced using the GV112 Vector Expression System (GeneChem), the hU6-MCS-CMV-Puromycin component sequence, and the *Age*I and *Eco*RI cloning sites according to the manufacturer's instructions. A control vector containing the *EGFP* gene and no transgene was constructed in the same manner. The cells were cultured in IMDM supplemented with 5% FBS. Microglia were infected with the recombinant TREM2 virus or the control virus (multiplicity of infection, 10–20) with 5 mg/ml Polybrene (Santa Cruz Biotechnology, Santa Cruz, CA).

Western Blot Analysis

See Supplemental Digital Content 2 (http://links.lww.com/ALN/B414), which presents the methods used in this study.

Immunohistochemistry

See Supplemental Digital Content 2 (http://links.lww.com/ALN/B414), which presents the necessary methods used in this study.

Transfection of Small Interfering RNA into the Mouse Brain

We performed *in vivo* transfection of small interfering RNA (siRNA) in C57BL/6 mice according to the method described by Nakajima *et al.*[21] See Supplemental Digital Content 2 (http://links.lww.com/ALN/B414), which presents the necessary methods used in this study.

Neurobehavioral Evaluation and Infarct Measurement

See Supplemental Digital Content 2 (http://links.lww.com/ALN/B414), which presents the necessary methods used in this study.

Terminal Transferase-mediated dUTP Nick-end Labeling Staining

See Supplemental Digital Content 2 (http://links.lww.com/ALN/B414), which presents the necessary methods used in this study.

Primary Microglia Culture

See Supplemental Digital Content 2 (http://links.lww.com/ALN/B414), which presents the necessary methods used in this study.

Co-culturing HT22 Cells and N9 Cells Using the Transwell System

See Supplemental Digital Content 2 (http://links.lww.com/ALN/B414), which presents the necessary methods used in this study.

Determination of Apoptotic Rate by Flow Cytometry

See Supplemental Digital Content 2 (http://links.lww.com/ALN/B414), which presents the necessary methods used in this study.

Cell Proliferation and Cytotoxicity Assay

See Supplemental Digital Content 2 (http://links.lww.com/ALN/B414), which presents the necessary methods used in this study.

Lactate Dehydrogenase Release

See Supplemental Digital Content 2 (http://links.lww.com/ALN/B414), which presents the necessary methods used in this study.

Cytokine Analysis

Enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN) was used to measure mouse interleukin-1β, interleukin-4, tumor necrosis factor-α, interleukin-6, interleukin-10, and transforming growth factor-β in cell culture supernatant samples according to the manufacturer's instructions.

Statistical Analysis

Brain sections were examined by two independent and blinded investigators (T.J. and F.B.). SPSS 13.0 for Windows (SPSS Inc., Chicago, IL) was used to conduct statistical analyses. All values, except for neurologic scores, are presented as mean ± SD and were analyzed by one-way ANOVA. Between-group differences were detected with Tukey *post hoc* tests. The neurologic deficit scores are expressed as the median (interquartile range) and were analyzed using the Kruskal–Wallis test followed by the Mann–Whitney U test with Bonferroni correction. Sample size was estimated based on our previous experience.[22,23] $P < 0.05$ was considered statistically significant.

Results

Phenotypic Conversion of Immunocytes after Stroke Was Accompanied by TREM2 Up-regulation

As shown in figure 1, A and B, compared with the sham group, the expression levels of induced nitric-oxide synthase (iNOS, 2.8 ± 0.2-fold, $P = 0.0015$) and interleukin-6 (2.9 ± 0.1-fold, $P = 0.0004$) were highest at 6 h in the ischemic group. In contrast, brain-derived neurotrophic factor (5.8 ± 0.2-fold, $P = 0.0002$) and arginase-1 (3.3 ± 0.1-fold, $P = 0.0005$) expression peaked at 7 days. The immunofluorescence assay revealed iNOS and arginase-1 staining in the region of the ischemic penumbra (fig. 1, C and D, respectively), consistent with the model that immunocytes act as a double-edged sword in the process of stroke. Western blotting revealed that the level of TREM2 protein (4.4 ± 0.1-fold, $P = 0.0004$) in the ischemic penumbra increased over time and was significantly greater than that in the sham group at 3 days postreperfusion (fig. 1E). This observation was further confirmed by immunostaining for TREM2 at 3 days after reperfusion. A marked increase in TREM2 staining was observed in the MCAO group (fig. 1F). Taken together, these results indicated that TREM2 might be involved in the dynamic changes to immunocytes after ischemia–reperfusion injury.

Level of TREM2 Expression in Different Microglia Phenotypes Was Altered In Vitro

To verify whether TREM2 expression was related to the detrimental M1 and beneficial M2 phenotypes, we used lipopolysaccharide and interferon-γ as M1 triggers and interleukin-4 and interleukin-13 as M2 triggers to stimulate primary microglia. Lipopolysaccharide/interferon-γ treatment enhanced the protein expression of iNOS (1.8 ± 0.1-fold, $P = 0.0092$, control *vs.* lipopolysaccharide/interferon-γ) in microglia, which displayed the ramified morphology characteristic of the M1 phenotype (fig. 2, A and B). In contrast, the expression of arginase-1 was dramatically increased in interleukin-4/interleukin-13–treated microglia, which showed the activated, amoeboid morphology characteristic of the M2 phenotype (1.6 ± 0.2-fold, $P = 0.0214$, control *vs.* interleukin-4/interleukin-13; fig. 2, C and D). We also observed that TREM2 protein expression was induced by interleukin-4/interleukin-13 treatment (1.7 ± 0.2-fold, $P = 0.0119$, control *vs.* interleukin-4/interleukin-13) and inhibited by lipopolysaccharide/interferon-γ treatment (0.4 ± 0.1-fold, $P = 0.0226$, control *vs.* lipopolysaccharide/interferon-γ; fig. 2, E and F). These data indicated that a decrease in the level of TREM2 expression could induce microglia to adopt the detrimental phenotype, whereas an increase in TREM2 expression could lead to the beneficial microglial phenotype.

TREM2 Was Involved in the Modulation of the Microglial Phenotype after OGD In Vitro

To further investigate the role of TREM2 in the regulation of microglial activation states, we transfected N9 microglial cells with recombinant lentiviral vectors containing the gene encoding full-length mouse TREM2 or with TREM2-short hairpin (sh)RNA vectors (transfection efficiency greater than 80%; fig. 3A). In the TREM2-shRNA group, the expression levels of iNOS (2.0 ± 0.3-fold, $P = 0.0007$) and interleukin-6 (1.8 ± 0.1-fold, $P = 0.0001$) were greatly enhanced by the suppression of TREM2 (fig. 3B). In contrast, in the TREM2-vector group, the expression of arginase-1 (1.5 ± 0.1-fold, $P = 0.0056$) and brain-derived neurotrophic factor (6.0 ± 0.1-fold, $P = 0.0003$) was increased by the up-regulation of TREM2 (fig. 3C). iNOS and arginase-1 immunofluorescence in the three groups revealed that the microglia displayed a more ramified morphology when TREM2 was up-regulated, whereas microglial morphology was amoeboid when TREM2 was down-regulated (fig. 3, D and E). Taken together, these results suggested that TREM2 might contribute to the switch in microglial phenotypes.

TREM2 Contributed to Neuronal Injury after OGD by Regulating Inflammatory Cytokines In Vitro

We used the Transwell culture system to examine the effects of the factors secreted by N9 microglia on cultured HT22 neurons after OGD (fig. 4A). At 4 h after OGD, the HT22 cells were damaged, and neuronal injury was assessed at 24 h after reoxygenation. We found that TREM2 down-regulation in the microglia in the TREM2-shRNA group increased apoptosis in HT22 cells (fig. 4B) by reducing cell vitality (0.6 ± 0.1, $P = 0.0101$) and enhancing lactate dehydrogenase release (343.7 ± 18.0, $P = 0.0118$; fig. 4C). We next investigated whether the mechanism underlying the role of TREM2 in post-OGD neuronal injury involved the inflammatory response. The enzyme-linked immunosorbent assay results demonstrated that interleukin-6 (13.0 ± 2.2, $P = 0.0163$) and interleukin-1β levels (7.6 ± 1.7, $P = 0.0017$) in the TREM2-shRNA group were significantly higher than those in the control-vector group, whereas the levels of interleukin-4 (3.7 ± 1.1, $P = 0.0353$) and interleukin-10 (7.0 ± 1.9, $P = 0.0019$) were increased in the TREM2-vector group (fig. 4D). In summary, these results indicate that TREM2 protected neurons from post-OGD injury.

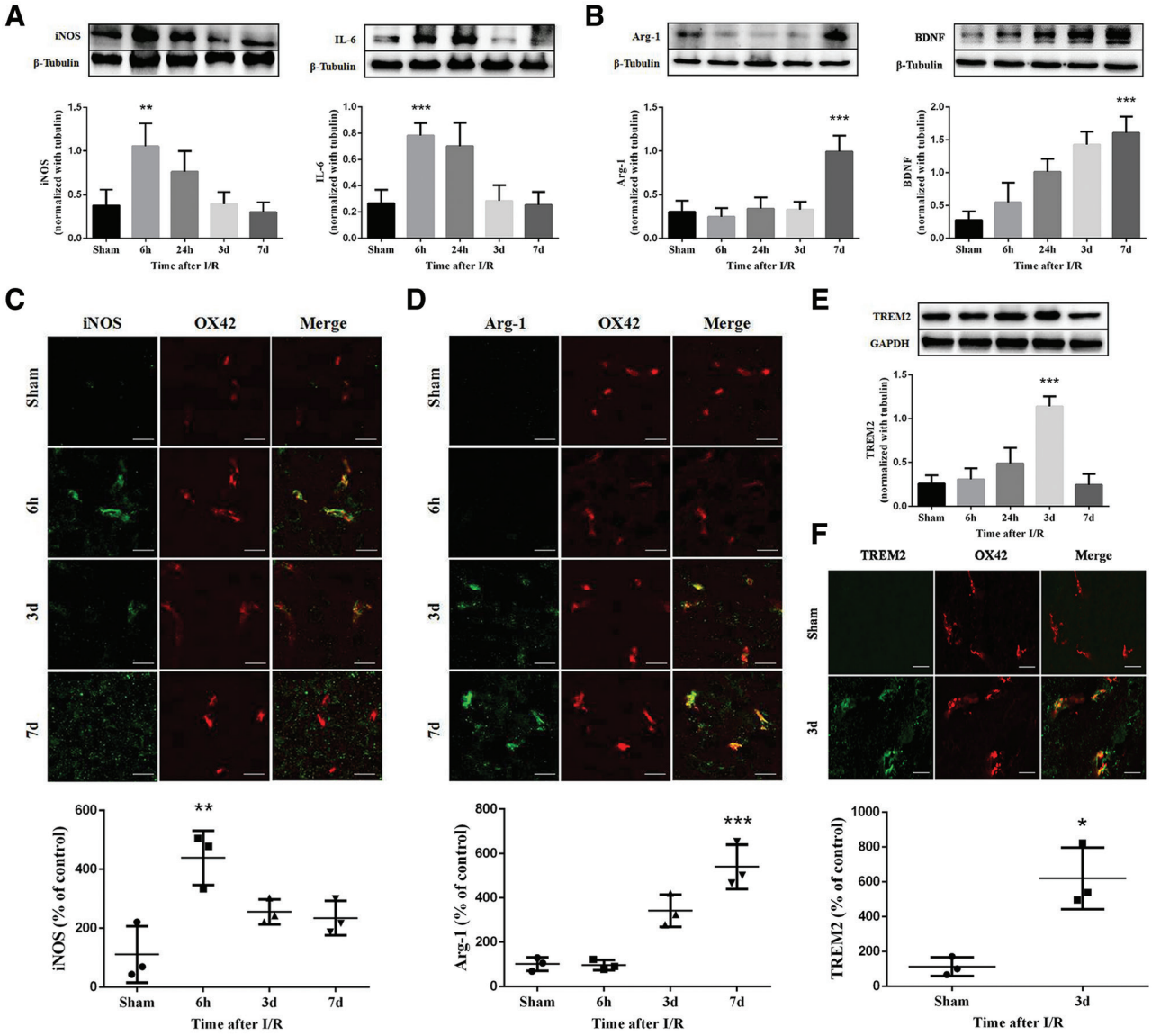

Fig. 1. The phenotypic conversion of immunocytes and the expression of triggering receptor expressed on myeloid cells 2 (TREM2) after cerebral ischemia–reperfusion (I/R) injury. Photomicrographs show levels of induced nitric-oxide synthase (iNOS), interleukin-6 (IL-6), brain-derived neurotrophic factor (BDNF), and arginase-1 (Arg-1) at 6 h, 24 h, 3 days, and 7 days after ischemia–reperfusion injury (n = 5). β-Tubulin was used as a loading control. (*A*) Quantitative analysis of the expression of iNOS and interleukin-6 after ischemia–reperfusion injury. (*B*) Expression of arginase-1 and BDNF after ischemia–reperfusion injury. (*C*) iNOS staining was observed in the wedge-shaped penumbra at 6 h, 3 days, and 7 days after ischemia–reperfusion injury in mice (n = 3). (*D*) Arginase-1 staining was observed in the wedge-shaped penumbra at 6 h, 3 days, and 7 days after ischemia–reperfusion injury after in mice (n = 3). (*E*) Quantitative analysis of the expression of TREM2 after ischemia–reperfusion injury. (*F*) TREM2 staining was observed in the wedge-shaped penumbra at 3 days after ischemia–reperfusion injury in mice (n = 3). *Bars* represent the mean ± SD of samples from three brains per group. OX42 (anti-CD11b equivalent antibody) was used as a microglia marker. **P* < 0.05 *versus* sham group, ***P* < 0.01 *versus* sham group, ****P* < 0.001 *versus* sham group. GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

TREM2 Suppression Enhanced M1 Immunocyte Polarization and Aggravated Neuronal Apoptosis after Stroke

We knocked down TREM2 by using siRNA and found that the protein level of TREM2 (0.4 ± 0.1-fold, $P = 0.0015$) in the TREM2-siRNA group was decreased by almost 70% compared with that in the sham group (fig. 5A). However, whether TREM2 suppression could exacerbate postischemic brain damage remained an important question. As expected, the expression of iNOS (1.6 ± 0.1-fold, $P = 0.0050$, TREM2-siRNA *vs.* MCAO) was markedly enhanced by TREM2 suppression. In contrast, arginase-1 expression was reduced (0.6 ± 0.1-fold, $P = 0.0385$) when TREM2 was suppressed (fig. 5B). Neuronal apoptosis in the ischemic penumbra was also more frequent in the TREM2-siRNA group (43.6 ± 13.6%, $P = 0.0462$, TREM2-siRNA *vs.* MCAO;

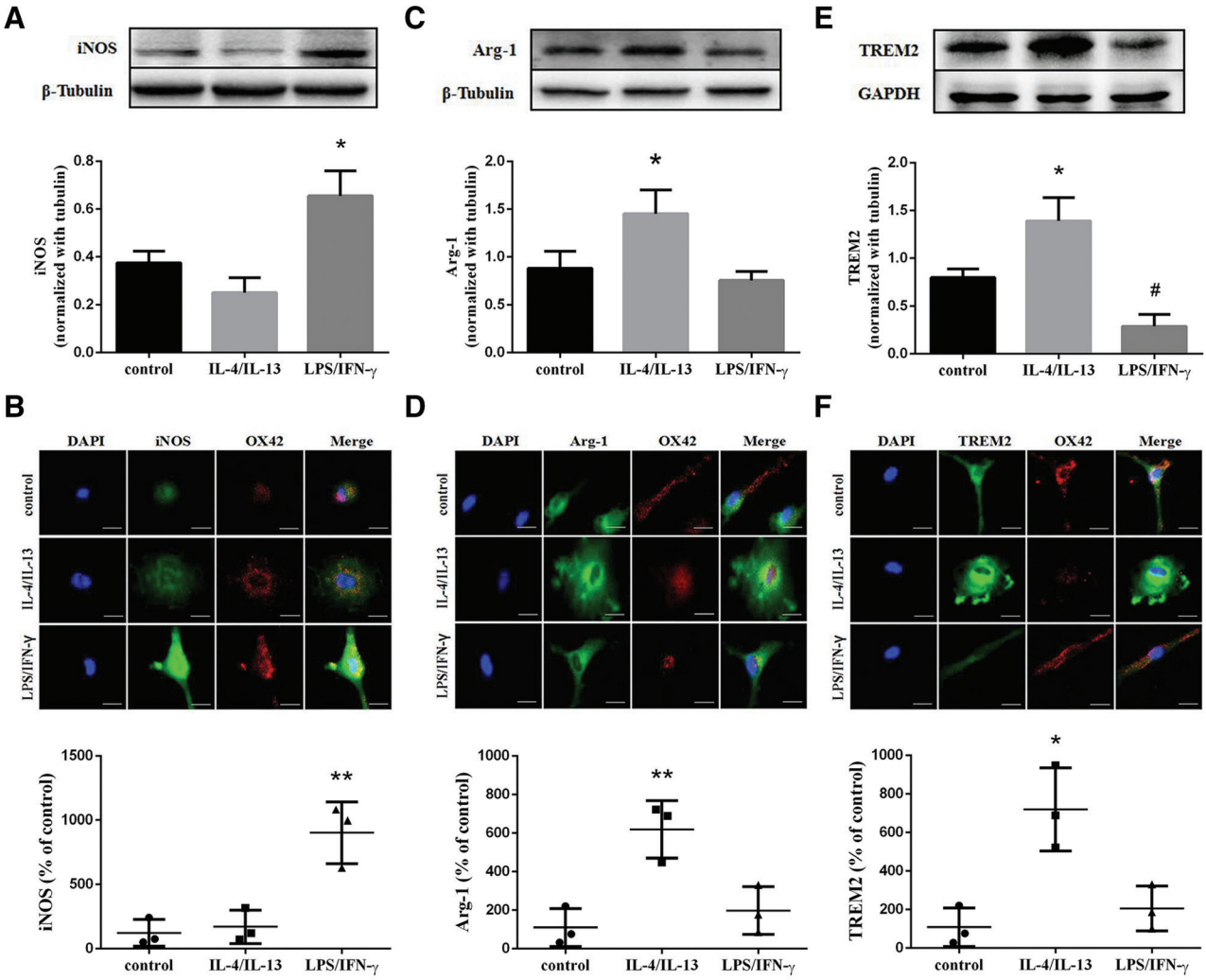

Fig. 2. The expression of triggering receptor expressed on myeloid cells 2 (TREM2) in primary microglia was accompanied by conversion into the classical activated phenotype (M1) and the alternative activated phenotype (M2). Immunofluorescent staining of primary microglia at 24 h after treatment also revealed the expression levels of induced nitric-oxide synthase (iNOS), arginase-1 (Arg-1), and TREM2 in each group (n = 5). β-Tubulin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as loading controls. (*A*, *B*) Quantitative analysis confirmed that lipopolysaccharide (LPS)/recombinant murine interferon-γ (IFN-γ) treatment increased iNOS expression (*A*), which is represented by *green fluorescence* in (*B*). (*C*, *D*) Interleukin-4 (IL-4)/interleukin-13 (IL-13) treatment increased arginase-1 expression (*C*), which is represented by *green fluorescence* in (*D*). (*E*, *F*) Interleukin-4/interleukin-13 treatment increased TREM2 expression (*E*), which is represented by *green fluorescence* in (*F*). *Bars* represent the mean ± SD of samples from three brains per group. OX42 (anti-CD11b equivalent antibody) was used as a microglia marker. **P* < 0.05 *versus* control group, #*P* < 0.05 *versus* control group, ***P* < 0.01 *versus* control group.

fig. 5C). The infarct volume and neurologic deficiency of the mice in the TREM2-siRNA group (55.2 ± 11.2%) did not differ from those of the mice in the MCAO group (46.2 ± 8.4%; fig. 5D). These results showed that blockade of TREM2 aggravated neural damage *in vivo* but did not increase the infarct volume, further demonstrating the protective effects of TREM2 against neuronal apoptosis induced by ischemia–reperfusion injury.

Up-regulation of TREM2 or TREM2 Activation Alleviated Cerebral Ischemic Injury after Stroke

The protein level of TREM2 (2.5 ± 0.1-fold, *P* = 0.0158, HSP60 + OGD *vs.* control) was increased in the HSP60 + OGD group at 24 h after OGD/reoxygenation injury, and the opposite phenomenon was observed in the shRNA + OGD group (fig. 6A). When HSP60 was administered at a dose of 2.5 μg, we did not observe any change in the volume of the infarct (46.2 ± 8.4%), but a dose of 3.75 μg induced a statistically significant reduction of the ischemic area (38.5 ± 6.0%, *P* = 0.0013, 3.75 μg of HSP60 *vs.* MCAO). Further increasing the dose to 5 μg had a considerable neuroprotective effect (27.8 ± 2.9%, *P* = 0.0003, 5 μg of HSP60 *vs.* MCAO; fig. 6B). Intriguingly, the number of apoptotic neurons was reduced in the group with 5 μg HSP60 (28.6 ± 4.2%, *P* = 0.0005, 5 μg HSP60 *vs.* MCAO; fig. 6C). For these assays, we unilaterally injected the recombinant TREM2 virus into

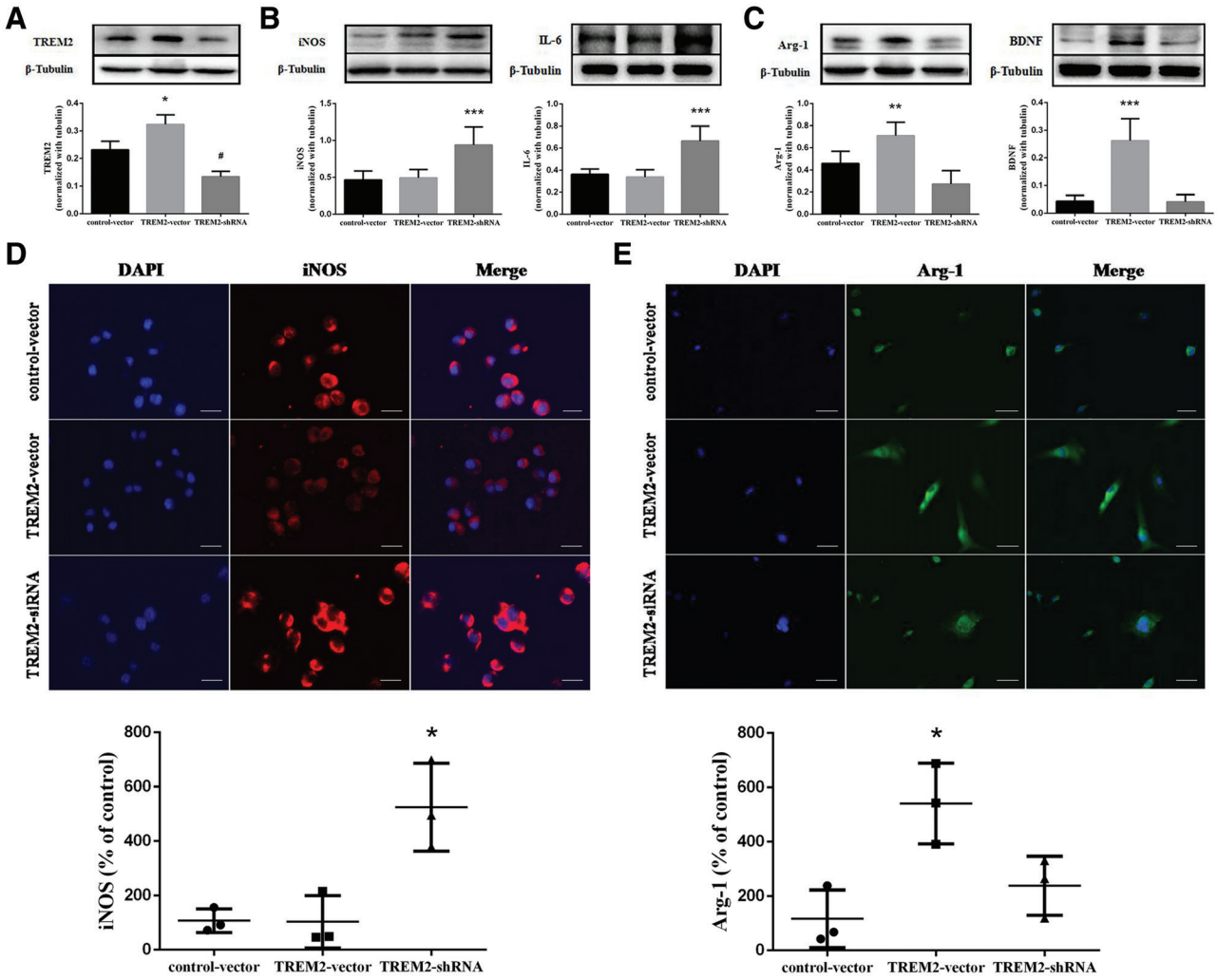

Fig. 3. Effect of triggering receptor expressed on myeloid cells 2 (TREM2) on the phenotypic conversion of microglia after oxygen–glucose deprivation *in vitro*. TREM2 vector indicates the recombinant TREM2 lentivirus, and TREM2-short hairpin RNA (shRNA) indicates the TREM2-RNA interference lentivirus. (*A*) Lentivirus transfection efficiency. **P* < 0.05 *versus* control-vector group. #*P* < 0.05 *versus* control-vector group. (*B*) Western blot analysis showed that expression of induced nitric oxide synthase (iNOS) and interleukin-6 (IL-6) was increased when TREM2 was down-regulated (n = 5). ****P* < 0.001 *versus* the control-vector group. (*C*) Expression of arginase-1 (Arg-1) and brain-derived neurotrophic factor (BDNF) was increased when TREM2 was up-regulated (n = 5). ***P* < 0.01 *versus* the control-vector group. (*D*) *Red fluorescence* represents iNOS expression (n = 3). (*E*) *Green fluorescence* represents arginase-1 expression (n = 3).

the cerebral ventricle, measured the expression of TREM2 (1.7 ± 0.2-fold, $P = 0.0377$) 10 days after the injection (fig. 6D), and assessed infarct volume and neurologic deficiency at 3 days after reperfusion. The results indicated that TREM2 up-regulation had a neuroprotective effect, as demonstrated by enhanced M2 microglial polarization (iNOS 0.2 ± 0.1-fold, $P = 0.0011$; arginase-1 0.5 ± 0.1-fold, $P = 0.0006$), decreased infarct volume (44.9 ± 5.3%), and reduced neuronal apoptosis (31.3 ± 7.6%; fig 6, E–G).

Discussion

Although substantial advances have been made in the treatment of stroke and in understanding the diverse mechanisms of neuronal death induced by ischemic stroke, the currently available neuroprotective therapies for acute ischemic stroke are limited and only moderately effective. Thus, improving neurologic outcomes and searching for reliable predictors within the pathophysiologic mechanisms of stroke is a major societal priority. In this study, we provide several pieces of evidence to support the hypothesis that microglial TREM2 is highly involved in the modulation of microglial activation states after experimental stroke (fig. 7).

Neuroinflammation, a specialized immune response that occurs in the central nervous system, is recognized as a pivotal hallmark of many pathologic conditions.[6,24] The process of neuroinflammation after ischemic stroke is characterized by the activation of microglia and astrocytes, increased concentrations of various cytokines and chemokines, disruption

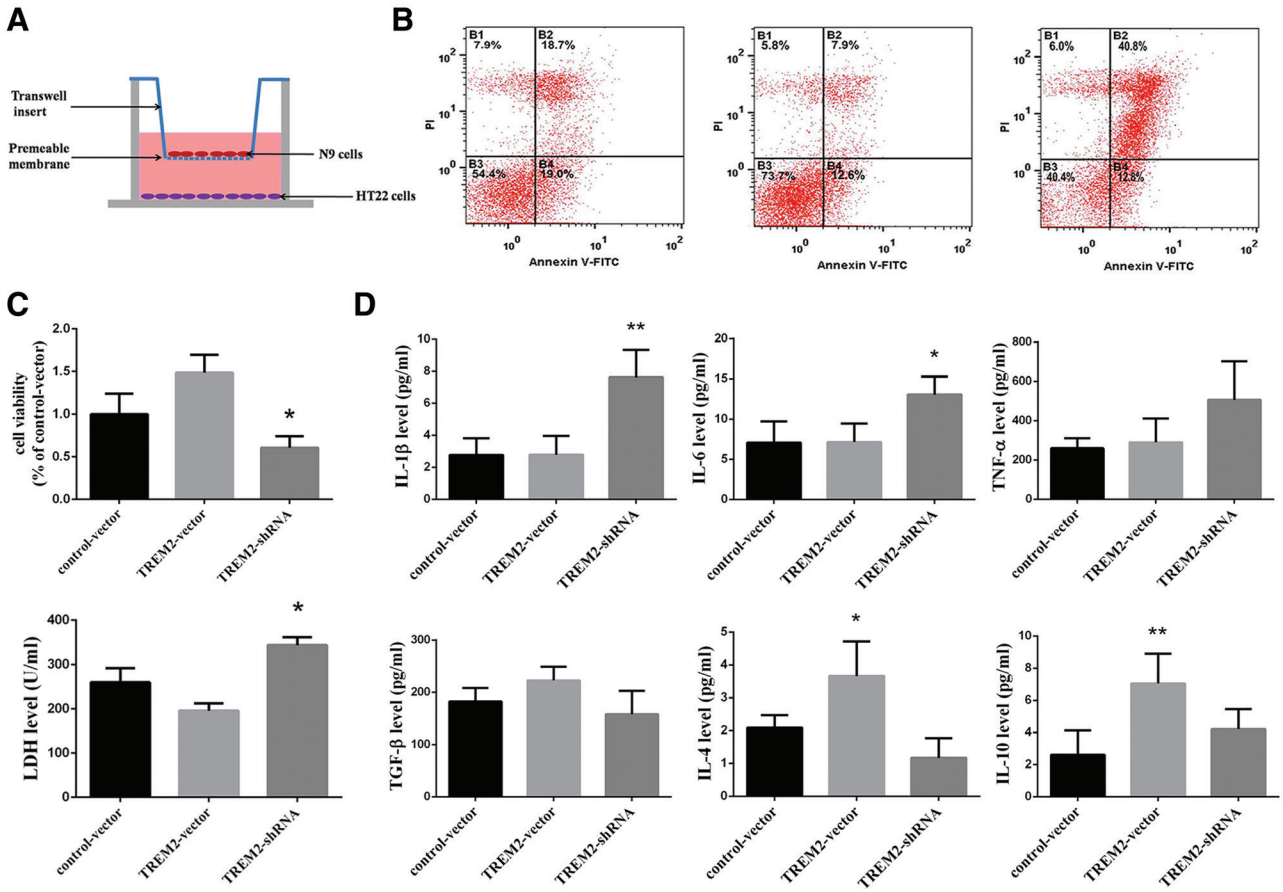

Fig. 4. Effect of triggering receptor expressed on myeloid cells 2 (TREM2) on neuronal damage and inflammatory cytokine release after oxygen–glucose deprivation in Transwell system. (*A*) Mimetic diagram of the Transwell co-culture system. (*B*) Neuronal apoptosis was reduced in the TREM2-vector group, as shown by flow cytometry analysis (n = 5). (*C*) The TREM2-short hairpin RNA (shRNA) group showed reduced cell viability (n = 8) and an increase in the lactate dehydrogenase (LDH) level in the media (n = 6). **P* < 0.05 *versus* the control-vector group. (*D*) Enzyme-linked immunosorbent assay-based comparisons of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β), transforming growth factor-β (TGF-β), interleukin-4 (IL-4), and interleukin-10 (IL-10) levels in the control-vector, TREM2-vector, and TREM2-shRNA groups (n = 6). **P* < 0.05 *versus* the control-vector group. ***P* < 0.01 *versus* the control-vector group. FITC = fluorescein isothiocyanate; PI = phosphatidylinositol.

of the blood–brain barrier, and the subsequent invasion of cells from the hematopoietic system to the site of cerebral infarction.[25,26] Studies of neuroinflammation in diverse brain diseases may contribute to the development of effective neuroprotective therapies.[27,28] One approach to such investigations is to characterize the mechanisms underlying the effects of currently established neuroprotective strategies.

Microglia, the major components of the intrinsic brain immune system and the key cellular mediators of neuroinflammatory processes, contribute to the regulation of neuronal death, neurogenesis, synapse elimination, and neuronal surveillance.[25,29] These microglia are key cellular mediators of neuroinflammatory processes, produce a variety of factors, and subserve both neurotoxic and neuroprotective functions.[9,30] As a result, microglial activation states have become a primary focus in studies of cellular neuroimmunology and neuroinflammation.[31] Specific receptors expressed on the microglial surface and the downstream signaling pathways that are involved in microglial activation have been extensively studied.[30,32] However, activated microglia and recruited macrophages (especially bone marrow-derived macrophages) present some common features and some distinct characteristics in the inflamed central nervous system, preventing the use of immunologic methods to distinguish between activated microglia and recruited macrophages *in vivo*.

Receptors belonging to the TREM family have been detected exclusively on myeloid cells and have been implicated in innate immune responses.[33] The TREM2 gene is located on chromosome 17C in mice, and in the brain, TREM2 is highly expressed by microglia.[34,35] It is well known that in humans, rare mutations causing the loss of TREM2 function lead to Nasu–Hakola disease. TREM2 is also a newly identified risk gene for Alzheimer disease, and it regulates inflammatory processes in peripheral tissues by modulating the release of inflammatory cytokines.[36] TREM2 deficiency led to uncontrolled inflammation in a model of experimental autoimmune encephalomyelitis.[13]

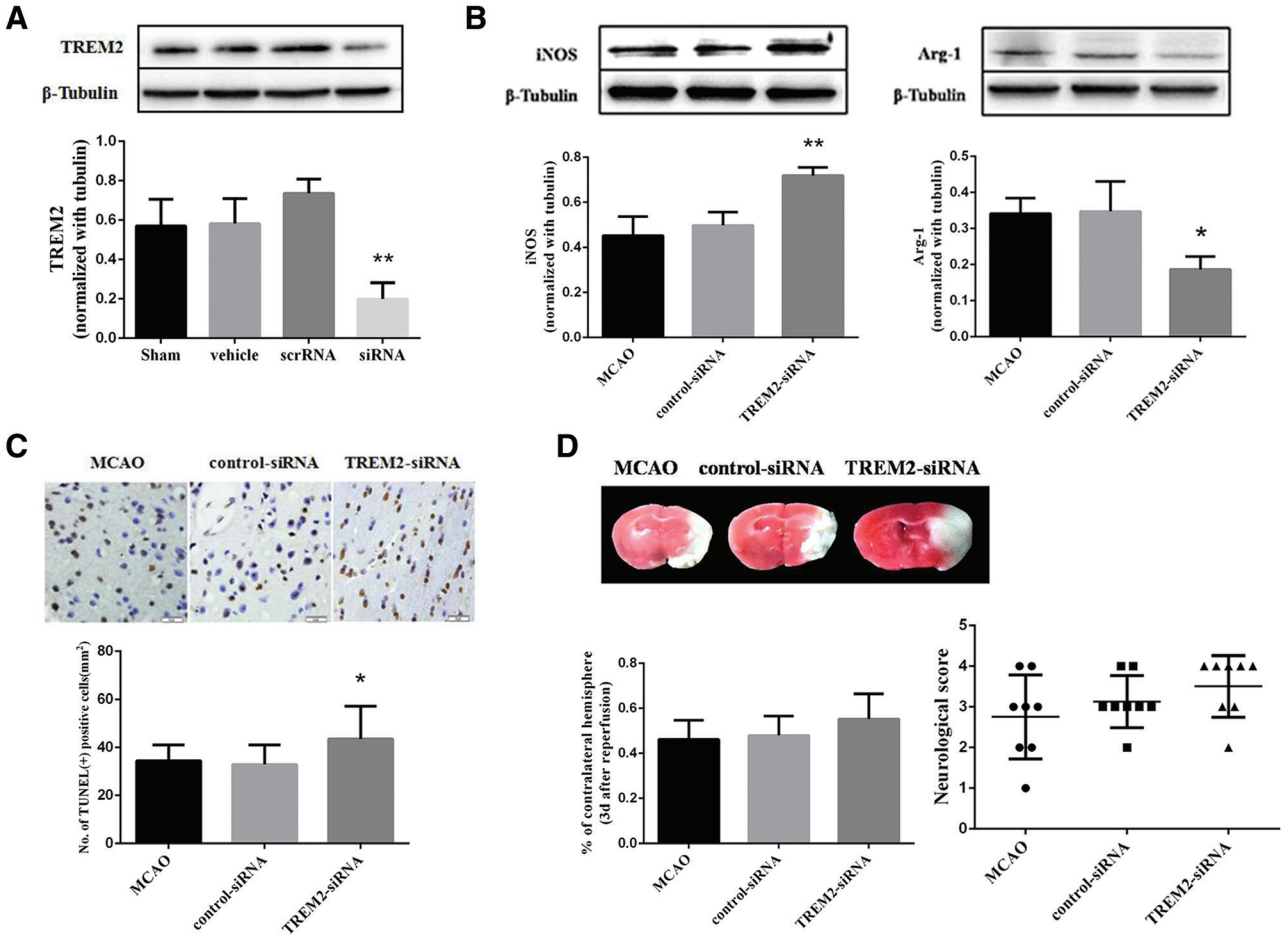

Fig. 5. Down-regulation of triggering receptor expressed on myeloid cells 2 (TREM2) induced microglia to adopt the classical activated phenotype (M1) and aggravated brain injury after stroke. (*A*) Western blotting showed the effect of TREM2-small interfering RNA (siRNA) on the expression of TREM2 in brain penumbra tissue (n = 5). $^{**}P < 0.001$ *versus* sham group. (*B*) The expression of induced nitric-oxide synthase (iNOS) and arginase-1 (Arg-1) in brain tissue at 3 days after ischemia–reperfusion injury (n = 5). $^{**}P < 0.01$ *versus* middle cerebral artery occlusion (MCAO) group. (*C*) Cellular apoptosis in the ischemic penumbra was assessed at 3 days after ischemia–reperfusion injury using terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) staining (n = 7). *Scale bar* = 20 μm. (*Lower*) Quantification and statistical analysis of the TUNEL staining in each group. $^{*}P < 0.05$ *versus* the MCAO group. (*D*) Comparison of the percentages of infarct volume among the three groups (n = 6). The effects of TREM2-siRNA on neurobehavioral measures are shown. Each *symbol* represents the score of a single mouse. The *horizontal bar* indicates the mean value of each group. scrRNA = scrambled RNA.

Therefore, TREM2 plays a key role in the negative regulation of autoimmunity and mediates microglial phagocytosis of apoptotic neurons in various neurologic diseases.[37]

Our study indicated that activation or up-regulation of TREM2 might be involved in the switch of microglial phenotypes and in promoting neurologic recovery after ischemic stroke. First, in primary microglia, the level of TREM2 expression was decreased in cells with the M1 phenotype and increased in those with the M2 phenotype. Interestingly, *in vivo*, we found that TREM2 expression increased as early as 6 h after MCAO but peaked at approximately 3 days. Nevertheless, the mechanisms underlying these changes remain incompletely understood. To further explore and understand the relationship between TREM2 regulation and the transformation of microglial activation states, we showed *in vitro* that TREM2 was involved in the modulation of microglial phenotypes and participated in neuronal injury after OGD by regulating inflammatory cytokines. We then knocked down brain TREM2 expression using RNA interference lentiviral vectors and up-regulated TREM2 expression using recombinant lentiviral vectors containing the gene encoding full-length mouse TREM2 to investigate its role in poststroke neuroinflammation and neuropathology. We confirmed that suppression of TREM2 enhanced M1 microglial polarization and increased neuronal apoptosis, whereas up-regulation of TREM2 or TREM2 activation alleviated cerebral ischemic injury after stroke. Sieber *et al.*[38] found no such effect on the lesion size and supported a contradictory model in which the subacute inflammatory reaction after stroke is attenuated in TREM2 knockout mice. Nevertheless, other groups have reported a role for TREM2 in the microglial phagocytosis in the infarcted brain and in the worsening of neurologic

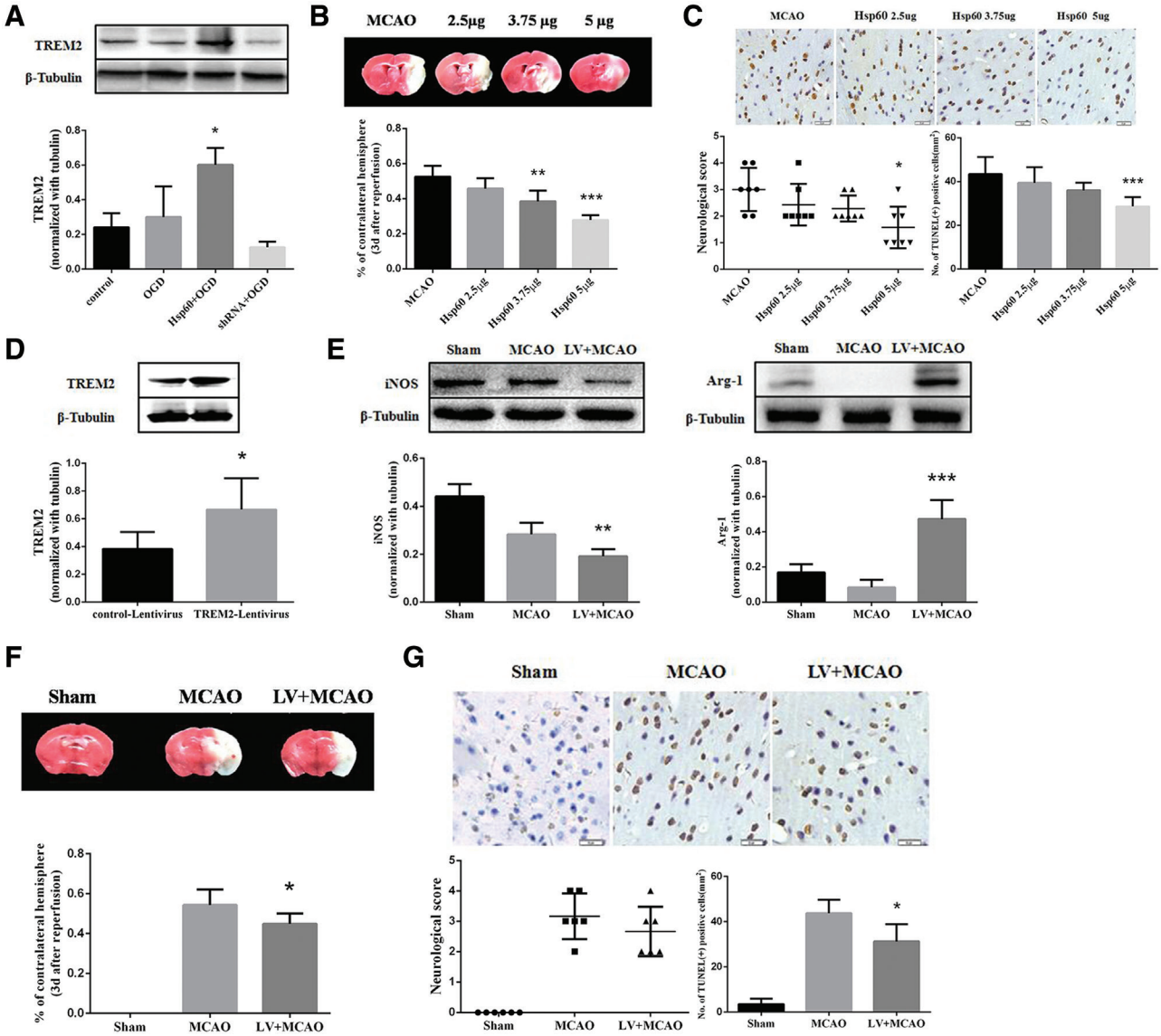

Fig. 6. Up-regulation of triggering receptor expressed on myeloid cells 2 (TREM2) induced microglia to adopt the alternative activated phenotype (M2) and conferred neuroprotection against stroke. (*A*) The heat shock protein 60 (HSP60)-induced effect on TREM2 protein expression in the oxygen–glucose deprivation model (n = 5). **P* < 0.05 *versus* control group. (*B*) Comparison of the percentage of infarct volume among the groups that received different doses (n = 7). The effects of HSP60 on neurobehavioral measures. Each *symbol* represents the score of a single mouse. The *horizontal bar* indicates the mean value of each group. ***P* < 0.01 *versus* the middle cerebral artery occlusion (MCAO) group, ****P* < 0.001 *versus* the MCAO group. (*C*) Apoptosis in the ischemic penumbra was assessed at 3 days after reperfusion using terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) staining. *Scale bar* = 20 μm. (*Lower*) quantification and statistical analysis of the TUNEL staining in each group. **P* < 0.05 *versus* MCAO group. (*D*) Unilateral transformation of the recombinant TREM2 lentivirus into the cerebral ventricle up-regulated the expression of TREM2. **P* < 0.05 *versus* control-lentivirus group. (*E*) Western blot analysis showed that TREM2 up-regulation induced microglia to adopt the M2 phenotype (n = 5). (*F*) Comparisons of percentages of infarct volume among the three groups (n = 7). **P* < 0.05 *versus* sham group. (*G*) The effect of recombinant TREM2 lentivirus on cellular apoptosis and neurobehavioral measures. Each *symbol* represents the score of a single mouse. Arg-1 = arginase-1; LV = lentivirus; OGD = oxygen–glucose deprivation; shRNA = short hairpin RNA.

outcomes after stroke.[39] Although the role of TREM2 after stroke has been controversial, our findings support the scenario that TREM2 confers neuroprotection by relieving neuroinflammation. Many signaling pathways may be involved in the interactions between neurons and microglia, and many receptors expressed on microglia participate in the processes of microglial activation; therefore, suppression of TREM2 alone cannot increase the area of cerebral infarction, but it can increase the number of apoptotic neurons.

HSP60 is the only known agonist of TREM2,[40] and whether HSP60 could exert a neuroprotective effect by binding with TREM2 remained unknown. Here, we provide the first demonstration that exogenous HSP60 activates TREM2 and reduces the detrimental effects of brain injury

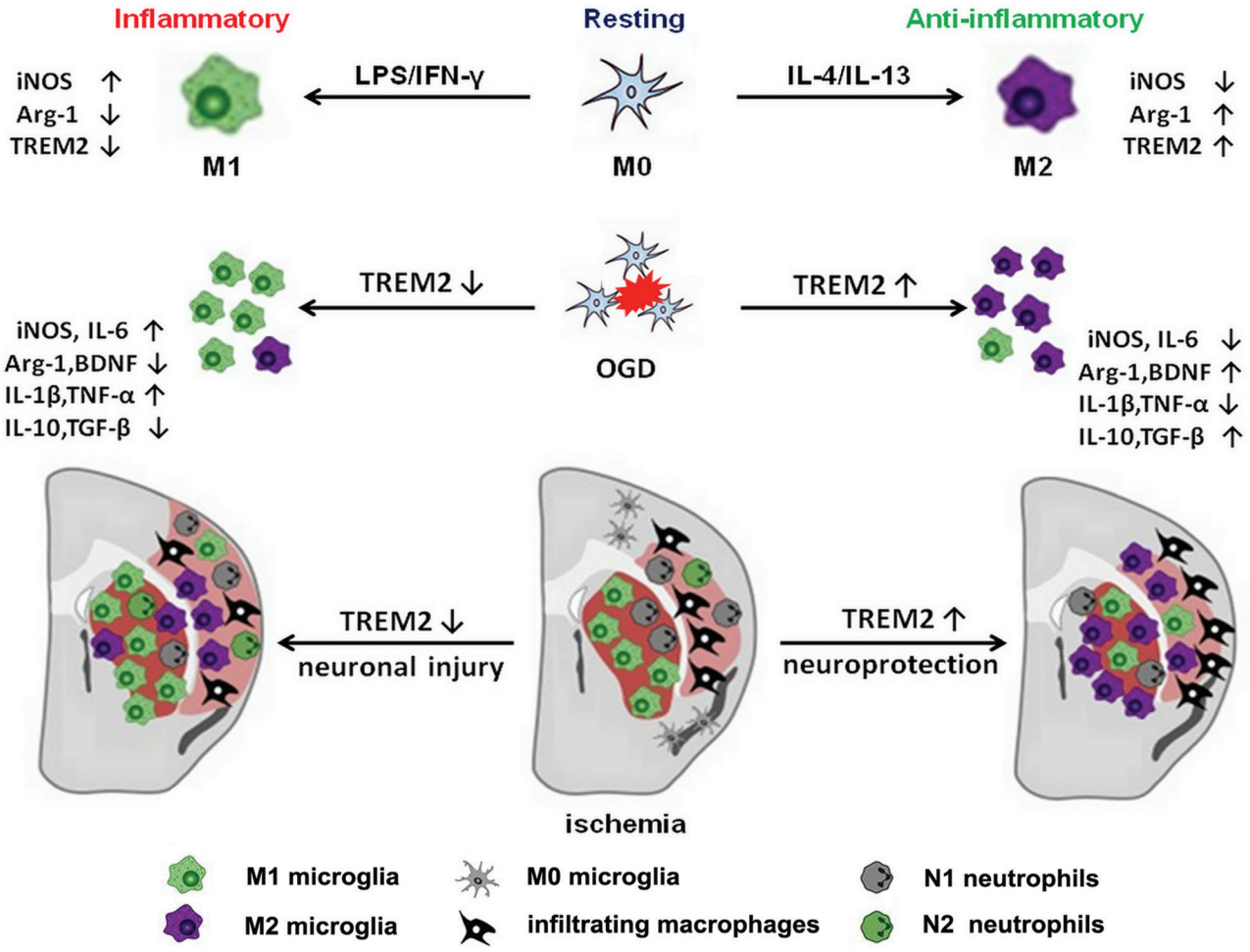

Fig. 7. Modulatory effect of triggering receptor expressed on myeloid cells 2 (TREM2) on the phenotypic conversion of microglia after stroke. Subjecting resting (M0) microglia to a proinflammatory stimulus, lipopolysaccharide (LPS), and recombinant murine interferon-γ (IFN-γ) *in vitro* drives the cells toward the classical activated (M1) phenotype and is accompanied by a decrease in TREM2 expression. The M1 microglia displayed high expression of cytokines and chemokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), as well as of induced nitric-oxide synthase (iNOS); these factors are key in cytotoxicity and tissue injury. In addition, the production of enzymes involved in tissue repair and remodeling, such as arginase-1 (Arg-1), was suppressed. In contrast, the treatment of M0 microglia with interleukin-4 and interleukin-13 (IL-4 and IL-13) drove M0 phenotypic conversion toward the augmented phagocytic M2 form and was accompanied by an increase in TREM2 expression. The M2 microglia exhibited strong expression of Arg-1 and brain-derived neurotrophic factor (BDNF) and weak expression of iNOS. Activating or up-regulating TREM2 *in vivo* had a neuroprotective effect by inducing phenotypic conversion of immunocytes and by reducing neuronal apoptosis. Down-regulation of TREM2 aggravated neuronal injury by increasing proinflammatory cytokine release, although this might be one of many pathways involved in stroke. OGD = oxygen–glucose deprivation; TGF-β = transforming growth factor-β.

in a dose-dependent manner. This result appears inconsistent with the findings of Kawabori *et al.*,[39] who did not observe changes in HSP60 expression after MCAO or hypothermia. Another previous study showed that HSP60 may be associated with the delayed death of CA1 pyramidal neurons after transient ischemia and that the induction of HSP60 protected the neurons from ischemic damage.[41] The contradictory results can be explained as follows: Recombinant 70-kDa HSP70 is an antiapoptotic protein that protects cells against stress and thus may be a useful therapeutic agent in the management of patients with acute ischemic stroke.[42] To date, several *in vitro* studies have clearly demonstrated the feasibility and efficacy of HSP70-mediated neuroprotection.[43] Emerging evidence indicates that HSPs are critical regulators of normal neural physiologic functions, as well as of cell stress responses.[44] HSP60 is a member of multiple HSP superfamilies, and its role in ischemic stroke remains unclear. In the brain, HSPD1/HSP60 is endogenously expressed in astrocytes, neurons, microglia, oligodendrocytes, and ependymal cells.[45] We did not observe endogenous HSP60 expression after MCAO. Interestingly, the recombinant 60-kDa HSP used in our study had a neuroprotective effect against MCAO in mice. The binding of HSP60 with TREM2 is one of the possible neuroprotective mechanisms. Moreover, the specificity of the binding between TREM2 and

HSP60 was confirmed in an experiment using mouse thymoma BWZ.36 cells, which are negative for surface HSP60 but became positive after transfection with TREM2 followed by incubation with the chaperone protein.[38] Nonetheless, the specificity of HSP60 and how these mechanisms function in a variety of conditions remain incompletely understood, thus requiring further study. The limitation in our experiments was that most of the mice after MCAO did not survive more than 2 weeks. Therefore, the function of microglia in the chronic phase of ischemic stroke deserves further exploration. As noted above, the main role of microglia in the chronic phase of ischemic stroke is still ambiguous. It is essential to further examine the long-term dynamics of M1/M2 polarization in a chronic inflammatory animal model and to monitor the balance of microglial polarization under microglia-specific conditions.

Conclusions

The current work provides the first demonstration of the importance of TREM2 as a novel regulator of microglia phenotypes that may hold great clinical promise as a therapeutic for stroke by altering the microglial response from one of tissue injury to one of repair. The data we have presented provide new information and tools that can be used in further studies focused on the roles of TREM2 under physiologic conditions and in the pathogenesis of stroke.

Research Support

Supported by grant No. 81473488 from the National Natural Science Foundation of China, Beijing, China; grant No. 81529004 from the Overseas Hong Kong and Macao Scholars Collaborated Researching Fund, Beijing, China; and grant No. 2014 CB543202 from the National Key Basic Research and Development Program, Beijing, China.

Competing Interests

The authors declare no competing interests.

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Management of Severe Bleeding in Patients Treated with Direct Oral Anticoagulants

An Observational Registry Analysis

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CME

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ABSTRACT

Background: The use of prothrombin complex concentrates and the role of plasma concentration of anticoagulants in the management of bleeding in patients treated with direct oral anticoagulants are still debated. Our aim was to describe management strategies and outcomes of severe bleeding events in patients treated with direct oral anticoagulants.

Methods: We performed a prospective cohort study of 732 patients treated with dabigatran, rivaroxaban, or apixaban hospitalized for severe bleeding, included prospectively in the registry from June 2013 to November 2015.

Results: Bleeding was gastrointestinal or intracranial in 37% (212 of 732) and 24% (141 of 732) of the cases, respectively. Creatinine clearance was lower than 60 ml/min in 61% (449 of 732) of the cases. The plasma concentration of direct oral anticoagulants was determined in 62% (452 of 732) of the cases and was lower than 50 ng/ml or higher than 400 ng/ml in 9.2% (41 of 452) and in 6.6% (30 of 452) of the cases, respectively. Activated or nonactivated prothrombin complex concentrates were administered in 38% of the cases (281 of 732). Mortality by day 30 was 14% (95% CI, 11 to 16).

Conclusions: Management of severe bleeding in patients treated with direct oral anticoagulants appears to be complex. The use of prothrombin complex concentrates differs depending on bleeding sites and direct oral anticoagulant plasma concentrations. Mortality differs according to bleeding sites and was similar to previous estimates. **(ANESTHESIOLOGY 2017; 127:111-20)**

DIRECT oral anticoagulants (DOACs), anti-IIa (dabigatran) or anti-Xa (rivaroxaban, apixaban, and edoxaban), are now recognized as a major step forward for patients with nonvalvular atrial fibrillation or recurrent venous thromboembolism requiring long-term anticoagulation for the prevention of thromboembolic events. The efficacy–safety ratio of DOACs is similar or even better in comparison with vitamin K antagonists (VKA) in clinical trials,[1,2] and this benefit appears to be reproduced in the real world,[3] especially with the reduction of approximately one half of intracranial hemorrhages compared with VKA. However, spontaneous severe bleeding still occurs at significant rates during DOAC treatment. Based on the reported experience of severe bleeding in clinical trials,[4–6] a limited number of case reports, and conflicting results of prothrombin complex concentrates (PCCs) and activated PCCs in animal studies or *in vitro* or *ex vivo* studies in humans,[7–10]

What We Already Know about This Topic

- Specific reversal strategies are currently available for dabigatran and in development for the anti-Xa agents. Other strategies used to manage bleeding in patients treated with these direct oral anticoagulants include measuring plasma levels of anticoagulants and therapy with prothrombin complex concentrates. However, there is little information currently available regarding these management modalities.

What This Article Tells Us That Is New

- In a prospective cohort registry study of 732 patients treated with direct oral anticoagulants and hospitalized for severe bleeding, bleeding sites were gastrointestinal in 37% and intracranial in 24% of the cases. Activated or nonactivated prothrombin complex concentrates were administered in 38% of the cases with a day 30 mortality of 13.5% and varied according to bleeding sites but was similar to previous reports. This report provides a detailed assessment of direct oral anticoagulant-treated patients managed in clinical settings.

This is a Coagulation 2016 article.

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 Anesthesiology 2017; 127:111-20

guidelines to manage bleeding in patients treated with DOACs have been published.[11,12] Owing to the lack of clinical trials until the recent introduction of specific antidotes of dabigatran (idarucizumab[13]) or xabans (andexanet alfa[14]), these guidelines mainly reflect expert opinions and have a low evidence level.

To obtain more data on the current management of these patients, we set up a large multicenter prospective observational study on a European binational basis. The present article reports the results obtained in 732 patients with major bleeding requiring hospitalization when specific antidotes were not available. The primary objective of the study was to describe the management of bleeding episodes and subsequent outcomes until day 30. The secondary objectives were to describe the typology of bleeding, to describe the clinical and biologic characteristics of the patients on admission, and to analyze how these data have influenced the choice of treatment, in particular the use of reversal agents (four-factor PCC or activated PCC).

Materials and Methods

The GIHP-NACO registry (NCT02185027) (Gestes Invasifs et Hémorragies chez les Patients Traités par les Nouveaux Anticoagulants Oraux) is a large, prospective, multicenter registry, set up by the Groupe d´Intérêt en Hémostase Périopératoire (GIHP), enrolling patients treated with a DOAC and hospitalized for spontaneous or posttraumatic bleeding or who needed urgent invasive procedures (for a list of participating centers, see the appendix) in 36 centers in university, general, and private hospitals in France and Belgium (www.ClinicalTrials.gov Identifier NCT02185027). The registry opened in June 2013 and closed in November 2015. A subsample of these data was presented at the American Society of Hematology Meeting in San Francisco, California, in 2014 and published as an abstract (*Blood* 2014; 124:2877). This report presents the data from patients with severe bleeding under treatment with DOACs (dabigatran, rivaroxaban, or apixaban) for indications including atrial fibrillation and treatment of deep venous thrombosis or pulmonary embolism. Patients treated with DOACs for prevention of venous thromboembolism after major orthopedic surgery were not included. The institutional review board (Comité d'Ethique des Centres d'Investigation Clinique de l'Inter-région Rhône-Alpes-Auvergne, France; institutional review board number 5891) approved the study (reference 2013-02). Oral consent was obtained from all patients or proxies. Written informed consent of the patients was not necessary according to French law regarding observational studies.

Local investigators screened hospitalized patients presenting with bleeding events. Data including demographic, clinical, laboratory tests, and treatment were documented using an electronic case report form providing immediate and continuous monitoring for completeness and accuracy (ClinInfo S.A., France).

Bleeding management was assessed by collecting the following: rates and counts of erythrocyte, plasma or platelet transfusions, the use of agents such as PCC, factor VIII inhibitor bypass activator (FEIBA®, France), recombinant activated factor VII concentrate, fibrinogen concentrates, or tranexamic acid. Three-factor PCCs were not available in the participating centers. In France, three four-factor PCCs with similar contents in factors II, VII, IX, and X and proteins C and S were used: KANOKAD® (LFB, Les Ulis, France), OCTAPLEX® (Octapharma, Boulogne-Billancourt, France), and CONFIDEX® (CSL-Behring, Marburg, Germany).[15,16]

Clinical Outcome Definitions

All clinical outcomes were assessed 30 days after admission. All bleeding events were classified as major or nonmajor clinically relevant.

Major bleeding was defined as overt bleeding with any of the following: fatal bleeding and/or symptomatic bleeding in a critical area or organ such as intracranial, intraspinal, intraocular, retroperitoneal, intraarticular or pericardial, or intramuscular with compartment syndrome and/or bleeding causing a fall in hemoglobin level of 20 g/l or more or leading to transfusion of two or more units of whole blood or red cells.[17]

Since all patients included in this cohort were admitted to a hospital, nonmajor bleeding fulfilled nonmajor clinically relevant criteria defined as any sign or symptom of hemorrhage that did not fit the criteria for the International Society of Thrombosis and Hemostasis (ISTH) definition of major bleeding but did meet at least one of the following criteria: (1) requiring medical intervention by a healthcare professional; (2) leading to hospitalization or increased level of care; or (3) prompting a face-to-face (*i.e.,* not just a telephone or electronic communication) evaluation.[18]

On day 30 postbleeding, patients were also evaluated for suspected major cerebral and cardiovascular events (MACCEs) after the acute bleeding event. MACCEs were defined as fatal or nonfatal cardiovascular complication events as follows: acute coronary syndrome, stroke or transient ischemic attack or systemic embolism, deep venous thrombosis or pulmonary embolism, pulmonary edema, cardiogenic shock, or any other fatal cardiovascular event as assessed by investigators. It excluded rebleeding or any new bleeding episode. The fatality rate was assessed at day 30 postbleeding.

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Statistical Analysis

Descriptive results are expressed as frequency and percentage for categoric variables and were compared using the chi-square test (or Fisher exact test for small samples). $P < 0.05$ was considered significant. For continuous variables, statistics are expressed as median, range, interquartile range, and 95% CIs (V.13.0; Stata Corp, USA).

Results

From June 2013 to November 2015, 732 patients treated with DOACs and admitted to 36 hospitals were consecutively included in the registry. Eligible patients were identified from different departments: emergency rooms, intensive care units, and the departments of neurology, cardiology, gastroenterology, and surgery. There were 535 patients (73%) admitted to the hospital from the emergency room, and 156 (21%) and 39 (5.3%) were admitted directly to intensive care units or to other medical or surgical departments, respectively.

The demographic and clinical characteristics of the patients are presented in table 1. Among the patients included in this analysis, 207 (28%) were treated with dabigatran, 472 (64%) were treated with rivaroxaban, and 53 (7.2%) were treated with apixaban. When patients were treated with rivaroxaban (10 mg) or dabigatran (75 mg twice daily; unlicensed therapeutic protocols in Europe for atrial fibrillation or venous thromboembolism treatment), the indication was confirmed with the local investigator. As a result, 85% of the patients included in the registry were treated for atrial fibrillation, and 34% of all patients had been treated for fewer than 6 months.

Among the patients, 42% were older than 80 yr, and 69% of the patients had moderate to severe renal dysfunction. There were 26% of the patients who were also receiving other medications with antithrombotic properties such as nonsteroidal antiinflammatory drugs, aspirin, VKA, or heparin, concomitantly or within days before admission for bleeding. In 64% of the cases, patients were also treated with medications interacting with P-glycoprotein or CYP450.

The sites, types, and severity of bleeding events are summarized in table 2. Severe bleeding events were mainly major according to ISTH criteria[19] (74%) and spontaneous (79%).

In 94% of the cases, conventional routine hemostasis lab tests were performed. In 62% of the cases, a plasma concentration of dabigatran, rivaroxaban, or apixaban was determined on admission (table 3). The median concentrations of dabigatran, rivaroxaban, and apixaban were 162, 124, and 111 ng/ml, respectively. Among these patients, 9.2% had a plasma concentration that was lower than 50 ng/ml at the time of blood sampling. On the other hand, 6.6% of these patients had a DOAC concentration higher than 400 ng/ml, up to 3,500, 1,245, and 537 ng/ml for dabigatran, rivaroxaban, and apixaban, respectively. The median time between the last intake of DOACs and blood sampling was 12 h. In patients with a plasma concentration of less than 50 ng/ml, intracranial hemorrhage was observed in 42%, gastrointestinal bleeding was observed in 27%, and bleeding at other sites was observed in 31% of the cases. Plasma concentrations were not significantly higher in patients treated with medications interacting with P-glycoprotein or CYP450, compared to patients who were not (table 4).

Bleeding management is described in table 4. PCCs (activated or nonactivated) were more frequently administered when bleeding occurred in a critical organ (intracranial or spinal; 57%) than in other sites (28%). PCCs were used in 28% of the patients for whom DOAC plasma concentrations were not determined. PCCs were used in 39% of cases where plasma concentrations were less than 50 ng/ml, 44% with plasma concentrations between 50 and 400 ng/ml, and 50% with plasma concentrations higher than 400 ng/ml ($P = 0.001$). This study was not designed to address the hemostatic efficacy of PCC in specific bleeding sites. The adequacy of hemostasis provided by the PCC treatment was therefore assessed subjectively by local investigators with an ordinal scale: totally, partially, or not at all in 44, 37, and 19% of the cases, respectively.

Thirty days after bleeding, MACCEs occurred in 7.4% of the cases. On day 30, case fatality was 13.5% (95% CI, 11.0 to 16.2) with central nervous system causes in 42% of the cases (table 5).

Patients with gastrointestinal bleeding events were elderly (mean age, 78.6 ± 10). Of these patients, 76% had a CHA2DS2Vasc score higher than 2, 24% had a history of heart failure, 25% had a history of stroke, and 20% had previous bleeding. The mortality in patients presenting with gastrointestinal bleeding was high (11.9% 30-day mortality).

Discussion

Patients with bleeding during dabigatran, rivaroxaban, or apixaban treatment have been analyzed in several substudies of pivotal trials evaluating the efficacy and safety of dabigatran, rivaroxaban, and apixaban.[4,6,20–23] These studies aimed to compare the management and prognosis of major bleeding in patients treated with dabigatran or rivaroxaban *versus* warfarin. However, bleeding management during these treatments was not formally analyzed. Our registry aimed to describe the actual management of patients regarding the use of PCC and plasma concentration determination in a real world cohort and association with the outcome. This registry was not designed to obtain an estimation of incidence or the relative importance of bleeding in patients treated with dabigatran, rivaroxaban, or apixaban.

As observed in patients with bleeding events in phase III trials, the patients included in our registry were mainly elderly. Creatinine clearance was lower than 60 ml/min in 61% of the patients. Similar results have been previously reported.[13,14] Indeed, renal function may have been altered in different ways: (1) slow deterioration of renal function before the bleeding events due to comorbidities, treatment, or an acute illness (infection or dehydration) owing to accumulation of DOACs; (2) acute renal failure

Table 1. Demographic and Clinical Characteristics

	Dabigatran	Rivaroxaban	Apixaban	All
n	207	472	53	732
Age, yr, median [25th–75th]	81 [75–86]	77 [69–83]	78 [70–83]	78 [70–84]
Sex, % female	86 (42)	195 (41)	24 (45)	305 (42)
BMI	25 [23–28]	26 [24–29]	25 [23-28]	26 [23–29]
History (%)				
Hypertension	132 (65)	311 (67)	34 (65)	477 (65)
Heart failure or reduced LVEF	39 (19)	84 (18)	10 (19)	133 (18)
Coronary artery disease	44 (22)	91 (20)	13 (25)	148 (21)
Stroke	51(25)	101 (22)	11 (21)	163 (23)
Diabetes mellitus	39 (19)	100 (22)	13 (25)	152 (21)
Peripheral vascular disease	52 (26)	100 (22)	13 (25)	165 (23)
Liver disease	9 (4.5)	14 (3.0)	2 (3.5)	25 (3.5)
Alcohol abuse	23 (11)	28 (6.0)	1 (1.9)	52 (7.2)
Bleeding	31 (15)	60 (13)	10 (19)	101 (14)
Creatinine clearance (Cockcroft and Gault), ml/min (%)				
> 60	41 (20)	148 (31)	12 (23)	201 (27)
30–59	102 (49)	214 (45)	25 (47)	341 (47)
15–29	38 (18)	43 (9.1)	10 (19)	91 (12)
< 15	9 (4.3)	6 (1.3)	2 (3.8)	17 (2.3)
Patients with AF				
n (%)	197 (95)	376 (80)	51 (96)	624 (85)
CHA2DS2-VASC score, median [25th–75th]	4 [3–4]	3 [3–4]	3 [3–4]	3 [3–4]
CHA2DS2-VASC > 2 (%)	156 (79)	292 (78)	40 (78)	488 (78)
Treatment regimen (%)				
	150 mg BID: 39 (20) 110 mg BID: 146 (74) 75 mg BID: 6 (3.0) Other and unknown: 6 (3.0)	20 mg OD: 187 (50) 15 mg OD: 163 (43) 10 mg OD: 12 (3.2) Other and unknown: 14 (3.7)	2.5 mg BID: 23 (45) 5 mg BID: 27 (53) — Other and unknown: 1 (2.0)	— — — —
Time between first treatment and bleeding (%)				
< 1 week	5 (2.5)	12 (3.2)	4 (7.8)	21 (3.4)
1 week to 1 month	11 (5.6)	37 (9.8)	12 (23)	60 (9.6)
1 month to 6 months	31 (15)	65 (17)	17 (33)	113 (18)
> 6 months	92 (47)	150 (40)	9 (18)	251 (40)
Unknown	58 (29)	112 (30)	9 (18)	179 (29)
Patients with VTE, n (%)	1 (0.5)	73 (16)	1 (1.9)	75 (10)
Treatment regimen (%)				
	150 mg OD: 1 (100) — — —	15 mg BID: 10 (14) 20 mg OD: 52 (71) 15 mg OD: 7 (9.6) Other and unknown: 4 (5.5)	Unknown: 1 (100) — — —	— — — —
Time between first treatment and bleeding (%)				
< 1 week		5 (17)		5 (6.7)
1 week to 1 month	1 (100)	14 (19)	1 (100)	16 (21)
1 month to 6 months	—	32 (44)	—	32 (43)
> 6 months	—	20 (27)	—	20 (27)
Unknown	—	2 (2.4)	—	2 (2.7)
Indication unknown (%)	6 (2.9)	9 (1.9)	1 (1.9)	16 (2.2)
Off-label indication (%)	3 (1.4)	14 (3.0)	—	17 (2.3)
Medication taken within 3 days of admission				
n (%)	48 (23)	104 (22)	14 (26)	166 (23)
NSAIDs	6 (2.9)	10 (2.1)	—	16 (2.9)
Aspirin	28 (14)	72 (15)	11 (21)	111 (15)
Clopidogrel	6 (2.9)	14 (3.0)	1 (1.9)	21 (2.9)
VKA	3 (1.4)	2 (0.4)	1 (1.9)	6 (0.8)
LMWH	2 (1.0)	3 (0.6)	2 (3.8)	7 (0.9)
UFH	1 (0.5)	6 (0.3)	—	7 (0.9)
Medication interacting with PgP or CYP450 (n = 730) (%)	139 (67)	291 (62)	39 (74)	469 (64)

AF = atrial fibrillation; BID = twice a day; BMI = body mass index; CYP450 = cytochrome P450; LMWH = low molecular weight heparin; LVEF = left ventricular ejection fraction; NSAID = nonsteroidal antiinflammatory drugs; OD = once a day; PgP = P-glycoprotein; UFH = unfractionated heparin; VKA = vitamin K antagonist; VTE = venous thromboembolism; UFH = unfractionated heparin.

Table 2. Site Type and Severity of Bleeding

	Dabigatran (n = 207)	Rivaroxaban (n = 472)	Apixaban (n = 53)	All (n = 732)
Spontaneous bleeding, n (%)	156 (75)	384 (81)	40 (76)	580 (79)
Site n (%)				
Gastrointestinal	82 (52)	114 (30)	17 (43)	212 (37)
Intracranial	25 (16)	104 (27)	12 (30)	141 (24)
Intramuscular/skin	10 (6.4)	48 (13)	1 (2.5)	59 (10)
Epistaxis	9 (5.8)	42 (11)	2 (5.0)	53 (9.1)
Hematuria	16 (10)	22 (5.7)	2 (5.0)	40 (6.9)
Hemoptysis	4 (2.6)	16 (4.2)	3 (7.5)	23 (4.0)
Retroperitoneal	3 (1.9)	6 (1.6)	2 (5.0)	11 (1.9)
Pericardial	2 (1.3)	8 (2.1)	—	10 (1.7)
Intraperitoneal	2 (1.1)	7 (1.8)	—	9 (1.6)
Vaginal	—	5 (1.3)	1 (2.5)	6 (1.0)
Hemothorax	2 (1.3)	4 (1.3)	—	6 (1.0)
Intraarticular	1 (0.6)	3 (0.8)	—	4 (0.7)
Intraspinal	—	3 (0.8)	—	3 (0.5)
Other	1 (0.6)	2 (0.5)	—	3 (1.9)
Bleeding related to trauma (%)	51 (25)	86 (18)	13 (25)	150 (21)
Multiple trauma (%)	18 (35)	22 (26)	2 (15)	42 (28)
Head trauma (%)	33 (65)	59 (69)	10 (77)	102 (69)
Unknown (%)	—	5 (5.8)	1 (7.7)	5 (3.4)
Other (%)	—	2 (0.4)	—	2 (0.3)
Major bleeding (according to ISTH criteria) (%)	157 (76)	342 (73)	39 (74)	538 (74)

ISTH = International Society of Thrombosis and Hemostasis.

Table 3. Laboratory Results on Admission

	Dabigatran (n = 207)	Rivaroxaban (n = 472)	Apixaban (n = 53)	All (n = 732)
Hemoglobin				
n (%)	204 (99)	467 (99)	53 (100)	724 (99)
Median [25th–75th] (g/dl)	11.7 [8.6–13.3]	12.3 [9.6–13.8]	12.2 [10–14]	12.1 [9.3–13.7]
Platelet count				
n (%)	198 (96)	451 (96)	53 (100)	702 (96)
Median [25th–75th], g/l	204 [178–275]	226 [180–286]	214 [167–287]	225 [179–284]
Fibrinogen				
n (%)	147 (72)	294 (62)	18 (34)	479 (65)
Median [25th–75th], g/l	3.7 [3–4.3]	3.8 [3.1–4.5]	3.9 [3.3–4.5]	3.8 [3.1–4.4]
aPTT ratio				
n (%)	193 (93)	440 (93)	45 (85)	678 (93)
Median [25th–75th]	1.7 [1.4–2.2]	1.2 [1.0–1.3]	1.1 [1.1–1.3]	1.2 [1.1–1.5]
Prothrombin ratio				
n (%)	159 (77)	371 (79)	39 (74)	569 (78)
Median [25th–75th]	1.4 [1.2–1.7]	1.4 [1.2–1.7]	1.2 [1.1–1.3]	1.4 [1.2–1.7]
Plasma concentration of DOACs				
n (%)	123 (59)	285 (60)	34 (64)	452 (62)
Median (range), ng/ml	162 (3–3,500)	124 (0–1,245)	111 (18–537)	128 (0–3,500)
In patients with drugs interacting with PgP and/or CYP450				
n (%)	82 (40)	183 (39)	25 (47)	290 (40)
Median (range), ng/ml	165 (8–3,474)	120 (0–1,245)	109 (18–334)	125 (0–3,474)
Time between last intake and plasma concentration sampling				
n (%)	76 (37)	186 (39)	19 (36)	281 (38)
Median (range), h	8.5 (0.8–29)	14 (0.2–62)	11 (2.6–87)	12 (0.2–87)

aPTT = activated partial thromboplastin time; CYP450 = cytochrome P450; DOAC = direct oral anticoagulant; PgP = P-glycoprotein.

Table 4. Bleeding Management

	Dabigatran (n = 207)	Rivaroxaban (n = 472)	Apixaban (n = 53)	All (n = 732)
Transfusion, n (%)	94 (45)	150 (32)	17 (32)	261 (36)
Packed erythrocyte, n (%)	86 (42)	143 (30)	14 (26)	243 (33)
Platelets, n (%)	12 (5.8)	16 (3.4)	4 (7.5)	32 (4.4)
Fresh frozen plasma, n (%)	32 (16)	32 (6.8)	6 (11)	70 (9.6)
Fibrinogen concentrate, n (%)	5 (2.4)	6 (1.3)	—	11 (1.5)
PCC, n (%)	60 (29)	129 (27)	19 (36)	208 (28)
Total dose				
Median [25th–75th], U	3,000 [1,700–4,000]	3,000 [2,000–4,000]	3,000 [2,000–3,500]	3,000 [2,000–4,000]
Median [25th–75th], U/kg	40 [24–50]	44 [25–50]	42 [29–49]	43 [25–50]
Second dose, n (%)	5 (8.3)	20 (16)	2 (11)	27 (13)
aPCC, n (%)	26 (13)	41 (8.7)	6 (11)	73 (10)
Total dose				
Median [25th–75th], U	3,500 [2,500–4,000]	3,000 [2,500–3,575]	3,500 [3,000–4,000]	3,000 [2,500–4,000]
Median [25th–75th], U/kg	46 [40–52]	44 [37–50]	48 [46–48]	46 [38–50]
Second dose, n (%)	2 (7.7)	3 (7.3)	—	5 (6.8)
Recombinant factor VIIa	—	—	—	—
Tranexamic acid (%)	13 (6.3)	18 (3.8)	3 (5.8)	34 (4.7)
Hemodialysis (%)	7 (3.4)	2 (0.4)	—	9 (1.2)
Mechanical means (%)*	60 (29)	151 (32)	13 (25)	224 (31)
Intervention for hemostasis control (%)	48 (23)	119 (25)	8 (15)	175 (24)
Endoscopy (%)	26 (13)	64 (14)	7 (13)	97 (13)
Surgery (%)	4 (1.9)	17 (3.6)	1 (1.9)	22 (3)
Embolization (%)	18 (8.7)	38 (8.1)	—	56 (7.7)

*Compression, gauze packing.
aPCC = activated PCC; PCC = prothrombin complex concentrate.

Table 5. 30-day Outcome

	Dabigatran (n = 207)	Rivaroxaban (n = 472)	Apixaban (n = 53)	All (n = 732)
MACCE, n (%)	21 (10)	30 (6.4)	3 (5.7)	54 (7.4)
Venous thromboembolism, n (%)	1 (0.5)	6 (1.3)	—	7 (1.0)
Ischemic stroke, n (%)	2 (1.0)	7 (1.5)	1 (1.9)	10 (1.4)
Systemic emboli, n (%)	—	2 (0.4)	—	2 (0.3)
Acute coronary syndrome, n (%)	5 (2.4)	4 (0.8)	1 (1.9)	10 (1.4)
Pulmonary edema, n (%)	8 (3.9)	10 (2.1)	—	18 (2.5)
Cardiogenic shock, n (%)	5 (2.4)	6 (1.3)	1 (1.9)	12 (1.6)
All causes of mortality, n	41	53	5	99
% [95% CI]	20 [15–26]	11 [7.6–15]	10 [3.3–21]	14 [11–16]
Mortality among patients with the following, % [95% CI]				
Intracranial hemorrhage (spontaneous)	36 [18–58]	28 [20–38]	17 [2.1–48]	28 [21–37]
Head trauma	24 [11–42]	14 [6–25]	10 [0.2–45]	17 [10–25]
Gastrointestinal bleeding	16 [8.8–27]	10 [5.1–17]	5.9 [0.1–29]	12 [7.8–17]
Cause of death (%)				
Neurologic/CNS	14 (6.8)	26 (5.5)	2 (3.8)	42 (5.7)
Bleeding	9 (4.3)	14 (3.0)	1 (1.9)	24 (3.3)
Cardiac	6 (2.9)	5 (1.1)	2 (3.8)	13 (1.8)
Sepsis	3 (1.4)	—	—	3 (0.4)
Other or undetermined	9 (4.3)	8 (1.7)	—	17 (2.3)

CNS = central nervous system; MACCE = major cerebral and cardiovascular events.

associated with the bleeding event; or (3) off-label use of DOACs (not recommended when creatinine clearance is lower than 15 ml/mn for rivaroxaban, apixaban, or edoxaban and lower than 30 ml/mn for dabigatran). Monitoring renal function is recommended, at least yearly, to detect changes in renal function and adapt treatment doses, particularly in patients with previously altered renal function and elderly or frail patients.[12]

A major consideration of DOACs is that no specific reversal strategy was proposed before the manufacturers made them available for clinical use. Antidotes were developed several years after the first approval of DOACs.[24] To date, except for dabigatran, reversal strategies rely on nonspecific measures and on the administration of hemostatic agents, mainly activated or nonactivated PCC.[12] Idarucizumab, a humanized monoclonal antibody that selectively binds dabigatran, has been recently approved.[13] Andexanet alfa is a recombinant modified human factor Xa decoy protein that has been shown to reverse the inhibition of factor Xa in healthy volunteers[25] and patients.[14] Ciraparantag (PER977) is a small cationic and water-soluble molecule designed to bind with high affinity to oral FXa inhibitors (edoxaban, rivaroxaban, and apixaban) and to dabigatran.[26]

Little information has been made available on the use and timing of specific reversal strategies in pivotal studies on DOACs.[4,6,23] In these studies, few patients with major bleeding have been treated with PCCs. In the Dresden registry, among 1,082 bleeding events observed during rivaroxaban exposure, 59% were classified as ISTH minor bleeding, 35% were classified as ISTH nonmajor clinically relevant bleeding, and 66 (6.1%) patients had major bleeding treated with PCC in 9.1% of the cases.[27] Our registry includes a population principally with major bleeding events that is large enough to obtain a reasonable estimate for specific management strategies and outcome.

The use of PCCs to reverse the anticoagulation action of DOACs has not yet been assessed in clinical practice but is still suggested in international and national guidelines.[11,12] Only animal[8,28] or healthy volunteer studies[7,9,29] have assessed the efficacy of PCCs (activated or not) on different endpoints. A study of healthy volunteers compared the effect of a three-factor with a four-factor PCC on reversal of the anticoagulant effects of rivaroxaban.[30] Both four-factor PCCs and three-factor PCCs partially reversed the anticoagulant effects of rivaroxaban with good tolerance and no signs of thromboembolic events. In our registry, only four-factor PCCs or FEIBA were used. The present study shows that 38.4% of the patients were treated with PCCs with a distribution between activated or nonactivated PCC that is similar in patients treated with each DOAC. The use of PCC was related to the anatomic site of the bleeding. A majority of investigators consider that the use of PCCs partially or totally contributed to cessation of bleeding.

The use of coagulation testing during the clinical development of DOACs was not used in clinical trials based on considerations they would not require monitoring because of their highly predictable pharmacokinetics and therapeutic effects. Although this position has been challenged in subsequent publications regarding dabigatran,[31] specific plasma concentration techniques have been developed[32] for each DOAC, in theory either to answer specific questions (*i.e.*, compliance to treatment) or to guide periprocedural or bleeding management.[11] Indeed, numerous case reports and series have shown that bleeding events could be related to the high plasma concentrations of these new agents,[31,33] as already observed with other anticoagulant agents.

In the present study, the plasma concentration of DOACs was determined on admission in 62% of the patients. Among these patients, 9.2% had a plasma concentration that was lower than 50 ng/ml at the time of blood sampling. These results could in part be explained by the time between the last dose and sampling. On the other hand, 6.6% of these patients had a DOAC concentration higher than 400 ng/ml, suggesting that in several patients, DOACs could accumulate owing to renal failure or major drug interaction. Very high DOAC plasma concentrations (higher than 400 ng/ml) have been observed in previous studies, either in cases of intentional overdose cohorts[34] and particularly in the context of bleeding.[13,14]

In our registry, 63% of the patients were also treated with medication interacting with the pharmacokinetics of DOACs. However, DOAC plasma concentration was not significantly higher than in patients not treated with these medications. The importance of these potential interactions in explaining high DOAC plasma concentration was not explored in the present study.

In addition to these observations, the usefulness of DOAC plasma concentration determination to guide major bleeding management has never been evaluated. The present study shows that plasma concentration was positively related to the use of PCCs. Hence, plasma concentration could be of major importance in identifying patients in whom the use of an antidote or, if not available, PCC could be useful in reversing the anticoagulant effects of DOACs. However, our study does not show that this strategy lowers mortality.

Several analyses of mortality related to major bleeds have been performed from pivotal studies of DOACs. Thirty-day mortality after the first major bleed in phase III trials comparing dabigatran with warfarin was 9.1% in the dabigatran group and 13.0% in the warfarin group.[23] Using data from the ROCKET AF study, the outcomes after major bleeding, including all causes of death, were similar in patients treated with rivaroxaban and warfarin (20.4 *vs.* 25.6% in the rivaroxaban and warfarin group, respectively).[4] In the ARISTOTLE study, 8.9% with major nonintracranial bleeding died in the apixaban arm, and 9.5% died in the warfarin arm. Of those with intracranial hemorrhage, 45.3% died in the apixaban arm, and 42.3% died in the warfarin arm.[6]

The outcomes of intracranial hemorrhage in the ROCKET AF and RE-LY studies have been analyzed more specifically.[21,22] In the RE-LY study, 154 intracranial hemorrhages occurred in 153 subjects.[22] The mortality rate ranged from 24 to 49% depending on the intracranial location of the bleeding in both the dabigatran and the warfarin groups. During ROCKET AF follow-up, 172 patients experienced 174 intracranial bleeding episodes.[21] Mortality ranged from 20 to 69% depending on the intracranial location of the bleeding in both the rivaroxaban and the warfarin groups.[4]

In the present study, the mortality for spontaneous intracranial hemorrhage (n = 141) was 36, 28, and 17% in patients treated with dabigatran, rivaroxaban, and apixaban, respectively, with large confidence intervals.

Several factors impair the comparability with different studies on bleeding complications: history, severity, cause, and sites of bleeding. Depending on the cause of bleeding, mortality can differ greatly owing to the rate of intracranial hemorrhage, whose mortality rate is high,[35] and to other anatomic sites with lower mortality.

Our study has several strengths. The majority of the bleeding events described are classified based on major ISTH criteria. A detailed clinical and biologic assessment was available for most of the patients in this real world cohort. This included the plasma concentrations of dabigatran, rivaroxaban, or apixaban in a large subset of patients. Step-by-step management of these bleeding episodes is described including the use of hemostatic agents as suggested in most guidelines.

Our study has the limitations inherent to the observational nature of the study. This impacts the distribution of the bleeding sites and the severity of the cases. Moreover, the rate of inclusion among the 36 participating centers ranged from 1 to 67 depending on the investigator's department.

Conclusions

The data available in the GIHP-NACO registry provide an instant picture of the complex management of DOAC-induced major bleeding events in two European countries. Despite compliance to specific management, including the use of hemostatic agents or plasma concentration determination, mortality remains high. Specific analysis of bleeding sites is needed to assess the role of present or future (antidote) reversal strategies to improve the outcome of major bleeding in patients treated with DOACs.

Research Support

This study was supported by an unrestricted grant from Octapharma (Boulogne-Billancourt, France), CSL-Behring (Marburg, Germany), and LFB (Les Ulis, France).

Competing Interests

Dr. Albaladejo and Dr. Samama report personal fees and nonfinancial support from Bayer (Lyon, France), Boehringer Ingelheim (Paris, France), Daiichi Sankyo (Rueil-Malmaison, France), Bristol-Myers Squibb (Rueil-Malmaison, France), and Pfizer (Paris, France) outside the submitted work; Dr. Gruel reports personal fees from Boehringer Ingelheim (Paris, France) and Bristol-Myers Squibb (Rueil-Malmaison, France) and personal fees and nonfinancial support from Bayer (Lyon, France) outside the submitted work. The other authors declare no competing interests.

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Appendix: GIHP-NACO Study Group: Severe Bleedings

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Iron Loading Exaggerates the Inflammatory Response to the Toll-like Receptor 4 Ligand Lipopolysaccharide by Altering Mitochondrial Homeostasis

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ABSTRACT

Background: Perioperative and critically ill patients are often exposed to iron (in the form of parenteral-iron administration or blood transfusion) and inflammatory stimuli, but the effects of iron loading on the inflammatory response are unclear. Recent data suggest that mitochondrial reactive oxygen species have an important role in the innate immune response and that increased mitochondrial reactive oxygen species production is a result of dysfunctional mitochondria. We tested the hypothesis that increased intracellular iron potentiates lipopolysaccharide-induced inflammation by increasing mitochondrial reactive oxygen species levels.

Methods: Murine macrophage cells were incubated with iron and then stimulated with lipopolysaccharide. C57BL/6 wild-type mice were intraperitoneally injected with iron and then with lipopolysaccharide. Markers of inflammation and mitochondrial superoxide production were examined. Mitochondrial homeostasis (the balance between mitochondrial biogenesis and destruction) was assessed, as were mitochondrial mass and the proportion of nonfunctional to total mitochondria.

Results: Iron loading of mice and cells potentiated the inflammatory response to lipopolysaccharide. Iron loading increased mitochondrial superoxide production. Treatment with MitoTEMPO, a mitochondria-specific antioxidant, blunted the proinflammatory effects of iron loading. Iron loading increased mitochondrial mass in cells treated with lipopolysaccharide and increased the proportion of nonfunctional mitochondria. Iron loading also altered mitochondrial homeostasis to favor increased production of mitochondria.

Conclusions: Acute iron loading potentiates the inflammatory response to lipopolysaccharide, at least in part by disrupting mitochondrial homeostasis and increasing the production of mitochondrial superoxide. Improved understanding of iron homeostasis in the context of acute inflammation may yield innovative therapeutic approaches in perioperative and critically ill patients. **(Anesthesiology 2017; 127:121-35)**

IRON is an essential trace element.[1] Increased appreciation of the adverse effects of anemia in the perioperative period together with awareness of the risks of blood transfusion have led to the preoperative use of intravenous iron preparations as part of patient blood management protocols.[2–4] Critically ill patients in intensive care units are also exposed to acute iron loading (defined here as an increase in intracellular iron concentration in response to iron administration) in the form of blood transfusions, as well as oral and parenteral iron treatment.[5,6] Although patients are often exposed to both iron loading and inflammatory stimuli such as major surgery or critical illness, the effects of iron loading on the inflammatory response are incompletely understood.

Because of the ability of iron to generate reactive oxygen species (ROS) by the Fenton reaction,[7] iron administration might be expected to potentiate the response to subsequent inflammatory stimuli. Studies supporting a proinflammatory role for iron include those by Zager *et al.*,[8] who showed that

What We Already Know about This Topic

- Inflammation may play a role in critical illness
- Iron can increase the formation of reactive oxygen species, potentially affecting inflammation
- Critically ill patients may be exposed to iron through transfusion

What This Article Tells Us That Is New

- In rodent and cellular models, iron loading potentiated inflammation caused by lipopolysaccharide
- Iron loading in this model increased the production of mitochondrial superoxide and disrupted mitochondrial homeostasis

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Submitted for publication February 21, 2016. Accepted for publication March 14, 2017. From the Anesthesia Center for Critical Care Research, Department of Anesthesia, Critical Care and Pain Medicine (K.H., D.B.B., J.A.G., F.I., A.B.), Division of Rheumatology, Allergy and Immunology, Department of Medicine (D.B.B.), and Division of Cardiovascular Medicine, Department of Medicine (R.M.), Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts; and Department of Anesthesiology, University Hospital of the RWTH Aachen, Aachen, Germany (K.H.).

 Anesthesiology 2017; 127:121-35

iron administration worsened *Escherichia coli*–induced sepsis, and Wang *et al.*,[9] who reported that the iron chelator deferoxamine blunted lipopolysaccharide-induced inflammation. However, Pagani *et al.*[10] found that iron-deficient mice had a more robust inflammatory response to lipopolysaccharide than iron-replete mice, and De Domenico *et al.*[11] demonstrated that iron administration diminished the inflammatory response to lipopolysaccharide. The reason for the lack of agreement between these studies is unclear but may be related to differing routes and timing of iron administration.

Iron homeostasis is tightly controlled to minimize the risk of toxicity. Hepcidin is a key regulator of iron homeostasis.[12,13] Hepcidin binds to and down-regulates ferroportin, the only known iron exporter in mammals. Acute inflammation has been shown to increase hepcidin levels,[14] inducing systemic hypoferremia with an increase in intracellular iron.

Mitochondria have an important role in the acute inflammatory response. Pathogen-associated molecular patterns such as lipopolysaccharide, a Toll-like receptor 4 (TLR4) agonist, induce inflammation in part by increasing the production of mitochondrial reactive oxygen species (mtROS).[15] Mitochondria are a major site of iron utilization within the cell[16] and exist in a dynamic equilibrium between biogenesis and mitophagy (removal of dysfunctional mitochondria by autophagy).[17] Perturbations in mitochondrial homeostasis (defined here as the balance between mitochondrial biogenesis and mitophagy) may increase the proportion of damaged or nonfunctional mitochondria, increasing mtROS production.[18]

In this study, we examined the effect of acute iron loading on the inflammatory response using *in vivo* and *in vitro* models of inflammation. We hypothesized that iron loading would exaggerate the proinflammatory effect of lipopolysaccharide by increasing mtROS production.

Materials and Methods

Reagents and Chemicals

Escherichia coli lipopolysaccharide (O55:B5), a TLR4 agonist, was purchased from List Biologicals (USA). The TLR2 and TLR3 agonists Pam-3-Cys (P3C) and poly(I:C) (PIC), respectively, were obtained from Invivogen (USA). Formyl peptide *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLF), iron dextran, 20% dextran, ferric ammonium citrate, deferoxamine, antimycin, and 3-methyladenine were purchased from Sigma-Aldrich (USA). Calcein-acetoxymethyl (AM), MitoSOX, MitoTracker Deep Red, MitoTracker Green, and SYTOX Blue were purchased from Molecular Probes (USA). Antibodies, isotype controls, and reagents for flow cytometry were obtained from BD Biosciences (USA). MitoTEMPO, a mitochondria-specific antioxidant,[19] was purchased from Enzo Life Sciences (USA).

Animals

The Institutional Animal Care and Use Committee at the Massachusetts General Hospital approved the animal studies. Male C57BL/6 mice (6 to 8 weeks old) were purchased from Jackson Laboratories (USA). The mice were fed a standard, iron-replete diet and were injected intraperitoneally with one dose of iron dextran (1 g/kg in a volume of 10 μl/g) or normal saline (control) for iron loading experiments. Pilot experiments were performed with a range of iron doses (0.5 to 2.0 g/kg), and serum iron levels and liver hepcidin messenger RNA (mRNA) were measured at various time points (1, 3, 4, and 7 days; data not shown). Mice injected with 1 g/kg iron dextran demonstrated both a sustained increase in serum iron levels and a strong induction of hepcidin after 3 days, leading us to choose this 72-h time point for further investigation. A separate group of wild-type mice was injected with normal saline or 7.5% dextran (10 μl/g, the same volume as iron dextran). Three days later the mice were injected intraperitoneally with lipopolysaccharide (5 mg/kg) or an equal volume of normal saline (control). The mice were sacrificed 6 h later, and blood and organs (lungs and liver) were collected. Serum and organs were stored at –80°C until use.

Cell Culture

The murine macrophage cell line RAW 264.7 was obtained from American Type Cell Collection (USA). RAW 264.7 (hereafter referred to as "RAW") cells were cultured in 6-well (at 8×10^5 cells/well) or 96-well tissue culture plates (at 1.28×10^5 cells/well) in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum with glutamine, penicillin, and streptomycin. For iron supplementation experiments, the cells were incubated with ferric ammonium citrate (final concentration of elemental iron, 200 μM) overnight and then treated with lipopolysaccharide (150 ng/ml) or medium alone for 6 h. The concentration of iron was chosen so as to approximate the serum iron concentrations used in pilot, *in vivo* dose-response experiments.

Human Monocyte Isolation

The Institutional Review Board at the Massachusetts General Hospital approved the collection of whole blood from volunteers for the purpose of monocyte isolation (Institutional Review Board protocol No. 2014P001656). Mononuclear cells were isolated from human whole blood collected in EDTA using Polymorphprep density gradient (Axis-Shield, Norway) according to the manufacturer's instructions. Mononuclear cells were incubated (at 8×10^5 cells/well) in 6-well tissue culture plates in serum-free DMEM for 4 h. The cells were then washed with serum-free DMEM to remove nonadherent cells and incubated overnight in DMEM with 10% fetal calf serum. The cells were incubated with iron and/or lipopolysaccharide as in the RAW cell experiments.

Mouse Serum Studies

Serum interleukin-6 and tumor necrosis factor (TNF) levels were measured using mouse interleukin-6 and TNF Quantikine enzyme-linked immunosorbent assay kits (R&D Systems, USA). Serum iron levels were measured using an Iron-SL assay (Japan).

Quantitative Reverse Transcription-Polymerase Chain Reaction

TaqMan primers for quantitative reverse transcription (RT)-polymerase chain reaction (PCR) were purchased from ThermoFisher Scientific (USA). SYBR Green primers were synthesized by the Massachusetts General Hospital DNA core facility. The sequences of SYBR Green and TaqMan primers used in this study are listed in supplemental table 1 (Supplemental Digital Content 1, http://links.lww.com/ALN/B431). Total RNA was extracted from mouse liver and lung tissues or RAW cells using TRIzol (Invitrogen, ThermoFisher Scientific, USA). Reverse RNA transcription was accomplished using Moloney murine leukemia virus RT (Promega, USA). Quantitative RT-PCR was performed using Applied Biosystems SYBR Green or TaqMan master mix (ThermoFisher Scientific) and an Eppendorf MasterCycler RealPlex2 (ThermoFisher Scientific). The level of target transcripts was normalized to the level of 18S rRNA using the relative CT method.

Intracellular Labile Iron Measurement

Intracellular labile iron was measured as described previously.[20] Briefly, cells were incubated with calcein-AM, which was transported across the cell membrane by viable cells and deesterified, producing intracellular, fluorescent, free calcein. Calcein binds with intracellular labile (or free) iron, a reaction that quenches calcein fluorescence. The concentration of labile iron in a cell is inversely proportional to the intensity of calcein fluorescence. SYTOX Blue was used to identify and exclude nonviable cells, which do not take up calcein-AM, and may thereby confound results.

Flow Cytometry

Mouse whole blood was incubated in erythrocyte lysis buffer for 3 min and washed twice in flow cytometry buffer (phosphate-buffered saline with 2% fetal calf serum). The cells were then incubated with PerCP-Cy5.5-conjugated mouse monoclonal anti-CD11b antibody, allophycocyanin-conjugated mouse monoclonal anti-Ly6G antibody, or isotype controls. The cells were then incubated for 30 min with calcein-AM (0.125 μM). RAW cells incubated with iron and/or lipopolysaccharide were treated with calcein-AM (0.125 μM), MitoSOX (2.5 μM), MitoTracker Deep Red (50 nM), or MitoTracker Green (50 nM) for 30 min at 37°C in DMEM. Flow cytometry was performed using a FACS Aria III machine (BD Biosciences, USA), and the results were analyzed using FlowJo software (TreeStar, USA). In all cases, the gating parameters were set to exclude doublets and nonviable cells.

Determination of the Ratio of Mitochondrial to Nuclear DNA

Total genomic DNA was isolated from RAW cells with a DNeasy blood and tissue kit (Qiagen, USA). Quantitative PCR was used to measure the amounts of cytochrome c oxidase I (CO1, a mitochondrial gene) and 18S ribosomal DNA (18S, a nuclear gene), as previously described.[21] The ratio of CO1 to 18S was used as a measure of the relative proportions of mitochondrial DNA (mtDNA) and nuclear DNA.

Statistics

For *in vitro* studies, the data are expressed as mean and SD of individual experiments replicated thrice. The data were tested for a normal distribution by the Shapiro–Wilk test and analyzed using Student's *t* test (or Mann–Whitney U test if the data were not normally distributed) or two-way ANOVA (iron × lipopolysaccharide). If the iron × lipopolysaccharide interaction was statistically significant, we applied all possible pairwise comparisons (Bonferroni *post hoc* tests). If the interaction was not statistically significant, we interpreted the main effects only and refrained from *post hoc* testing. For data that were not normally distributed, we used the Kruskal–Wallis test (with Dunn *post hoc* tests for all possible pairwise comparisons). For the sake of clarity, not all pairwise comparisons have been reported in the figures. Hypothesis testing was two-tailed. Values of $P < 0.05$ were considered statistically significant. Statistical analyses were performed using GraphPad Prism 7.0 (USA). Sample sizes for *in vivo* experiments were based on our prior experience with lipopolysaccharide injection without *a priori* power calculations. Conditions in the *in vivo* experiments were nonsequential, and processing of samples for the *in vivo* experiments was performed by investigators who were blinded to the experimental conditions.

Results

Iron Dextran Administration Increases Serum Iron and Intracellular Iron in Circulating Neutrophils and Monocytes

Injection of mice with iron dextran increased serum iron levels 10-fold (fig. 1A). Lipopolysaccharide injection alone reduced serum iron levels by more than 50%, consistent with previous reports.[22] Mice that were treated with both iron and lipopolysaccharide had iron levels similar to those of mice injected with iron alone. Iron loading or lipopolysaccharide administration each increased hepatic hepcidin gene expression, as has been described by others[23] (fig. 1B). Intracellular labile iron levels in circulating neutrophils (Ly6G-positive cells) and circulating monocytes (CD11b-positive cells) were elevated as shown by decreased calcein fluorescence in iron-treated mice (fig. 1, C and D). These observations demonstrate that parenteral administration of iron dextran induces hepcidin production and increases intracellular iron levels in circulating neutrophils and monocytes.

Iron Administration Potentiates the Inflammatory Effects of Lipopolysaccharide

Iron administration alone did not induce inflammation in mice, as determined by the absence of increase in either

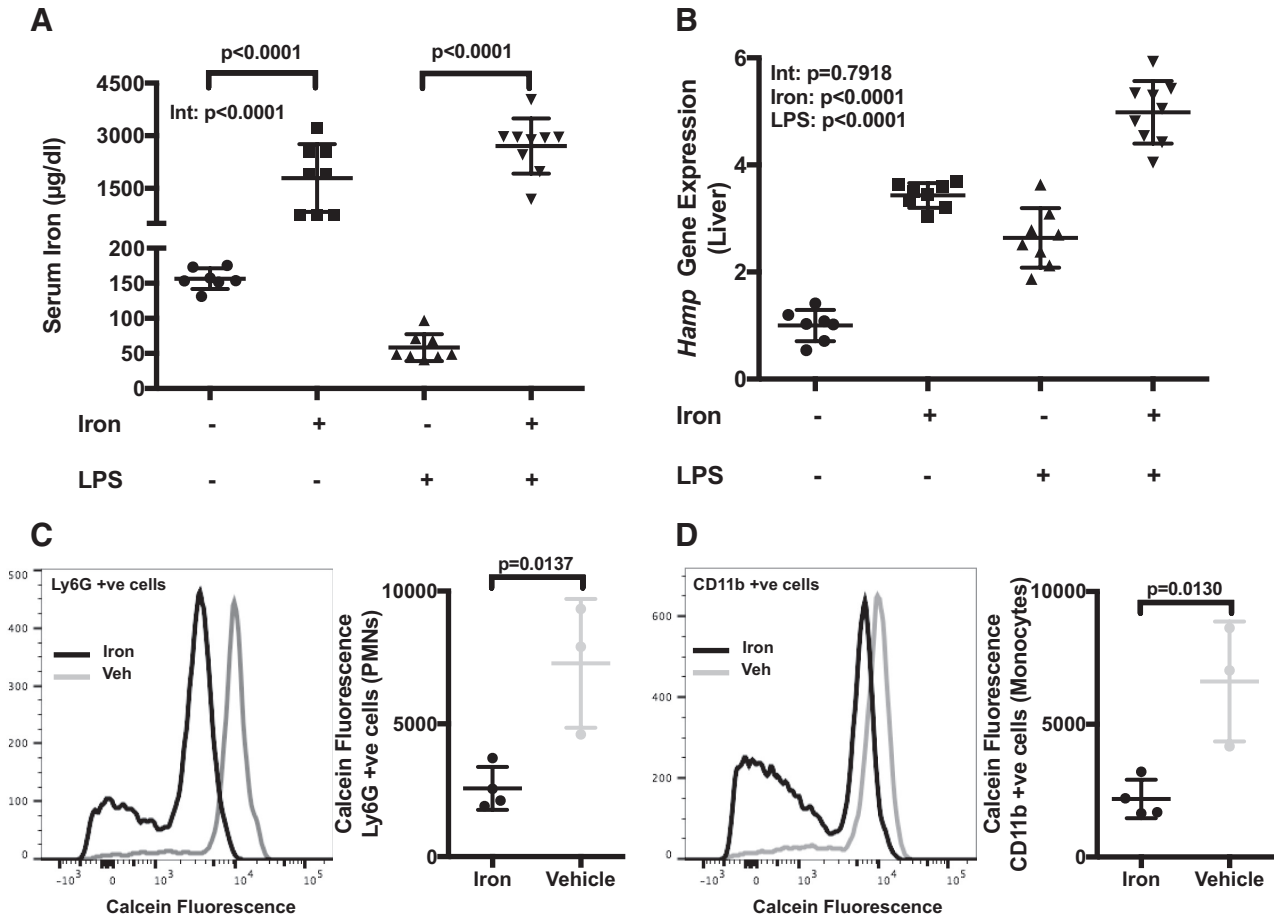

Fig. 1. Effects of parenteral iron overload in mice. (*A*) Mice treated with iron had significantly higher serum iron levels, while mice treated with lipopolysaccharide (LPS) alone became hypoferremic (two-way ANOVA with Bonferroni *post hoc* tests performed on $\log_{10}$-transformed values; *P* values adjusted for all possible comparisons). (*B*) Iron and lipopolysaccharide independently induce hepcidin (*Hamp*) mRNA levels in the liver (two-way ANOVA, interaction between iron and lipopolysaccharide not significant, therefore only main effects reported). N = 7 to 9 mice/group for (*A*, *B*). (*C*, *D*) Iron injection increases intracellular labile iron in circulating neutrophils and monocytes. Both neutrophils (Ly6G-positive cells) and monocytes (CD11b-positive cells) from the iron-treated mice had lower calcein fluorescence intensities, consistent with higher intracellular iron levels. Representative histograms are from one vehicle-treated (Veh) and one iron-treated mouse. The dot plots show mean fluorescent intensities of 3 to 4 mice/group (unpaired *t* test). Int = interaction *P* value; PMN = polymorphonuclear leukocytes.

serum protein levels or mRNA levels (in liver and lungs) of the cytokines interleukin-6 and TNFα (fig. 2, A–F). A nonlethal lipopolysaccharide challenge induced a marked increase in serum interleukin-6 and TNFα levels, as well as the corresponding mRNA levels in mouse liver and lungs. Iron-treated mice challenged with lipopolysaccharide showed more than 5-fold higher serum cytokine levels than mice challenged with lipopolysaccharide alone. Similarly, the mRNA levels of *interleukin-6* and *Tnfα* in lungs and liver of mice treated with iron and lipopolysaccharide were between 1.5- and 2.5-fold greater than in mice treated with lipopolysaccharide alone. Iron treatment and subsequent lipopolysaccharide challenge did not alter mRNA levels of the antiinflammatory cytokine interleukin-10 in the lung compared to lipopolysaccharide challenge alone (supplemental fig. 1, Supplemental Digital Content 1, http://links.lww.com/ALN/B431). In control studies, we showed that 7.5% dextran (the vehicle for iron) did not have an independent proinflammatory effect on a subsequent stimulation with 5 mg/kg lipopolysaccharide (supplemental fig. 2, A and B, Supplemental Digital Content 1, http://links.lww.com/ALN/B431). Taken together, these observations indicate that parenteral iron administration strongly augments the proinflammatory response to lipopolysaccharide in mice.

Preincubation with Iron Augments Lipopolysaccharide-induced Cytokine mRNA Induction in Human Monocytes and RAW Cells

Human monocytes were found to have a more intense response to lipopolysaccharide stimulation after being iron-loaded (fig. 3, A and B). *In vitro* incubation of RAW cells with iron increased intracellular labile iron (nonferritin-bound, catalytically active iron) concentration in RAW cells. Deferoxamine, an iron chelator, was used as an assay control, demonstrating

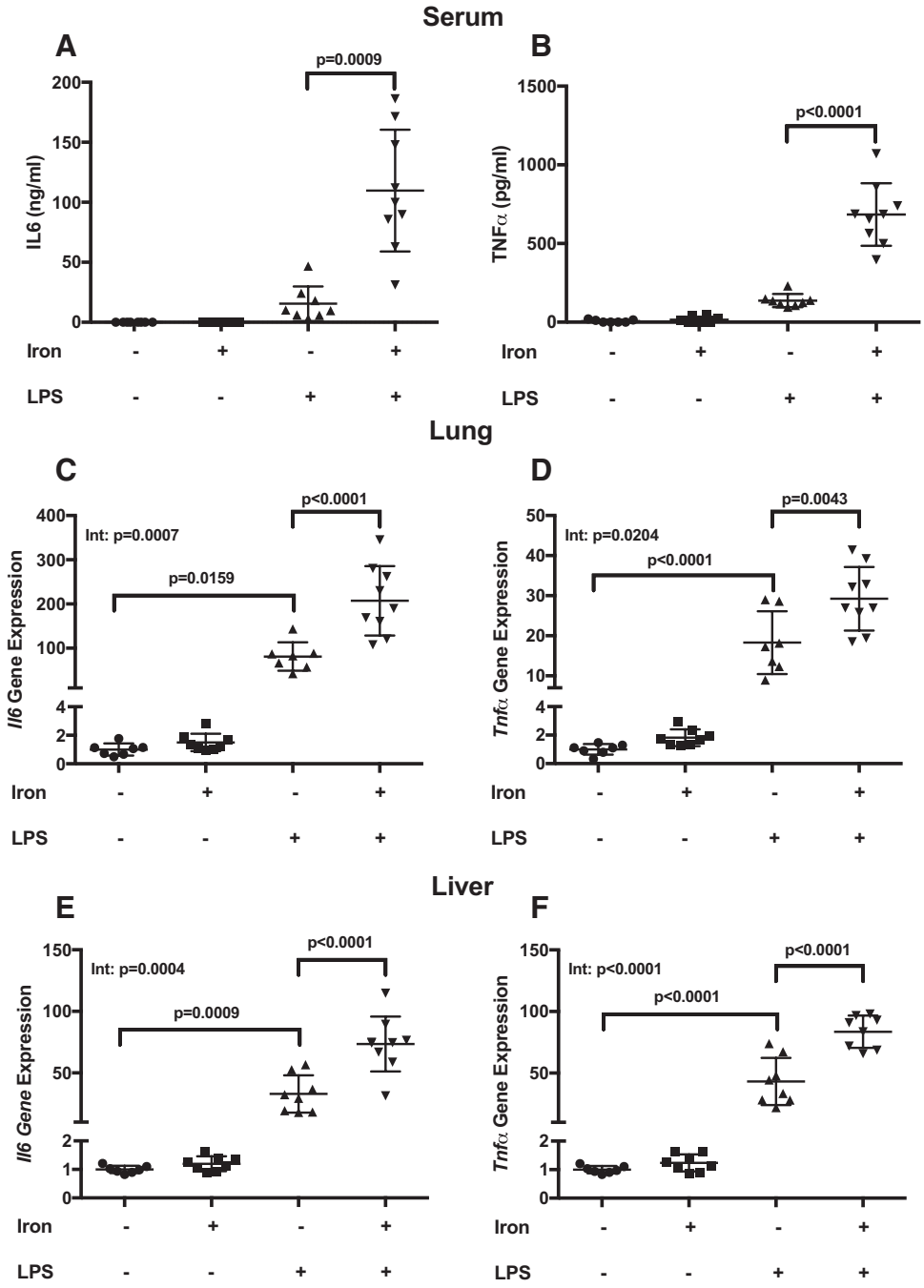

Fig. 2. Effect of parenteral iron administration on inflammatory gene expression and serum cytokine levels. Serum, lung, and liver samples were harvested from C57Bl/6 mice treated with iron and/or lipopolysaccharide (LPS) and analyzed for serum *Il6* (*A*), serum *Tnfα* (*B*), and mRNA levels of the two cytokines in the lungs (*C*, *D*) and livers (*E*, *F*). In each case, iron supplementation by itself did not induce inflammation. Lipopolysaccharide alone induced increases in cytokine protein levels, as well as mRNA levels. Serum cytokine levels in the control and iron-treated groups were below the detection level of the assay in many cases. Iron administration significantly enhanced the effect of lipopolysaccharide-induced inflammation in the mice. N = 7 to 9 mice per group (Mann–Whitney U test [*A*, *B*] and two-way ANOVA with Bonferroni *post hoc* tests [*C–F*]; *P* values adjusted for all possible comparisons). Il = interleukin; Int = interaction *P* value; TNF, tumor necrosis factor.

the inverse relationship between cellular labile iron levels and calcein fluorescence (fig. 4A). Iron-loaded RAW cells stimulated with lipopolysaccharide had significantly higher mRNA levels of interleukin-6 and TNFα than cells treated with lipopolysaccharide alone (fig. 4, B and C). Iron-loaded RAW cells stimulated with lipopolysaccharide also produced significantly more interleukin-6 protein than cells stimulated with lipopolysaccharide alone (supplemental fig. 3, Supplemental

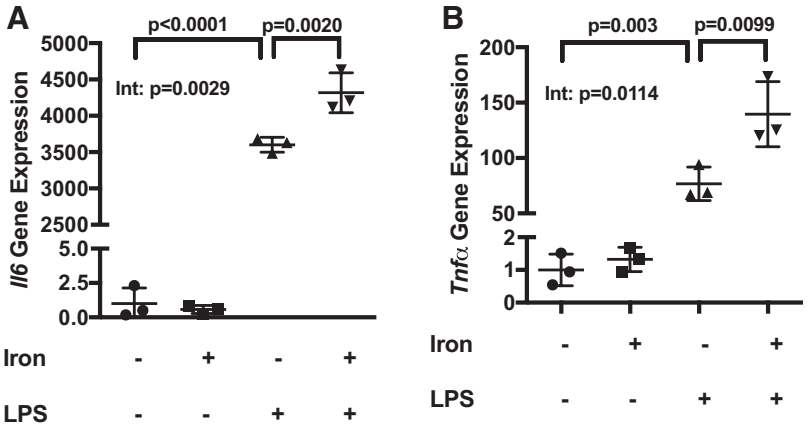

Fig. 3. Effect of iron loading on human monocytes *in vitro*. Human monocytes were isolated from peripheral blood and incubated in six-well plates. The cells were incubated with iron (200 μM) or control overnight and then stimulated for 6 h with lipopolysaccharide (LPS; 150 ng/ml) or medium. Lipopolysaccharide alone induced *Il6* mRNA (*A*) and *Tnfα* mRNA (*B*). Iron loading significantly increased mRNA levels in response to LPS (two-way ANOVA with Bonferroni *post hoc* tests; *P* values adjusted for all possible comparisons; n = 3 replicates/condition). Int = interaction *P* value; Il = interleukin.

Digital Content 1, http://links.lww.com/ALN/B431). Iron-treated RAW cells challenged with lipopolysaccharide showed increased mRNA expression of the chemokine monocyte chemotactic protein 1 (*Mcp1*) compared to cells treated with lipopolysaccharide alone (fig. 4D). Iron pretreatment with subsequent lipopolysaccharide reduced the expression of the antiinflammatory cytokine *interleukin-10* compared to cells treated with lipopolysaccharide alone (fig. 4E). Conversely, RAW cells preincubated with 30 μM deferoxamine showed a blunted response to lipopolysaccharide (fig. 5, A and B). Of note, incubation of cells with 200 μM iron had no adverse effects on cell viability, as determined by flow cytometric detection of nonviable cells (data not shown). These results demonstrate that iron loading augments the proinflammatory effect of lipopolysaccharide on RAW cells *in vitro* and that the use of an iron chelator blunts the cytokine response to lipopolysaccharide. Similarly, pretreatment of human monocytes with iron augments the proinflammatory effect of a subsequent exposure to lipopolysaccharide.

Response to Iron Loading In Vitro *Differs Depending on the Type of Inflammatory Stimulus*

We examined the effect of iron loading on the response of RAW cells to three additional proinflammatory mediators: P3C, PIC, and fMLF. P3C is a TLR2 agonist found in Gram-positive bacteria, PIC is a viral TLR3 agonist, and fMLF is a formylated peptide found in bacteria and mitochondria and an example of a damage-associated molecular pattern (DAMP). In contrast to the 2.5-fold increase in *interleukin-6* mRNA levels induced by iron and lipopolysaccharide, compared to lipopolysaccharide alone, iron-loaded cells stimulated with PIC did not show any significant increase in *interleukin-6* mRNA compared to cells treated with PIC alone. The combination of iron and P3C produced a 1.5-fold increase in *interleukin-6* mRNA compared to P3C alone. Iron together with the mitochondrial DAMP fMLF produced a 2.5-fold increase in *interleukin-6* mRNA compared to DAMP alone (fig. 5C). These results suggest that the effects of iron loading on enhancing the inflammatory response are pathway specific and depend on the type of proinflammatory stimulus.

mtROS Contribute to the Proinflammatory Effect of Iron Loading

To determine the effect of iron loading on mtROS levels, RAW cells were incubated with iron and stained with MitoSOX, a fluorescent dye that specifically detects mitochondrial superoxide.[24] Compared to untreated RAW cells, RAW cells exposed to iron had a 40% increase in fluorescence intensity, suggesting that iron loading results in an increased level of intracellular mtROS. The combination of iron and lipopolysaccharide increased RAW cell mtROS levels by 50% compared to cells treated with lipopolysaccharide alone (fig. 6, A and B).

To consider the possibility that an antioxidant might blunt the proinflammatory effect of iron, RAW cells were incubated with MitoTEMPO (100 μM), a mitochondria-specific antioxidant,[19] before stimulation with lipopolysaccharide. Preincubation with MitoTEMPO significantly blunted (but did not abolish) the inflammatory response to lipopolysaccharide compared to similarly treated cells that were not exposed to MitoTEMPO (fig. 6, C and D), showing *interleukin-6* and *Mcp1* mRNA levels, respectively). These results demonstrate that iron induces mtROS in RAW cells and that inhibiting mtROS production diminishes the inflammatory response in iron-loaded cells treated with lipopolysaccharide. However, increased mtROS alone (as caused by iron loading) is not sufficient to increase inflammatory mRNA levels, as cells treated with iron alone did not express increased cytokine mRNA levels (fig. 4, B and C).

Iron Administration Modifies mRNA Levels of Genes Involved in Mitochondrial Homeostasis

Compared to control mice, mice treated with lipopolysaccharide had lower mRNA levels of genes associated with

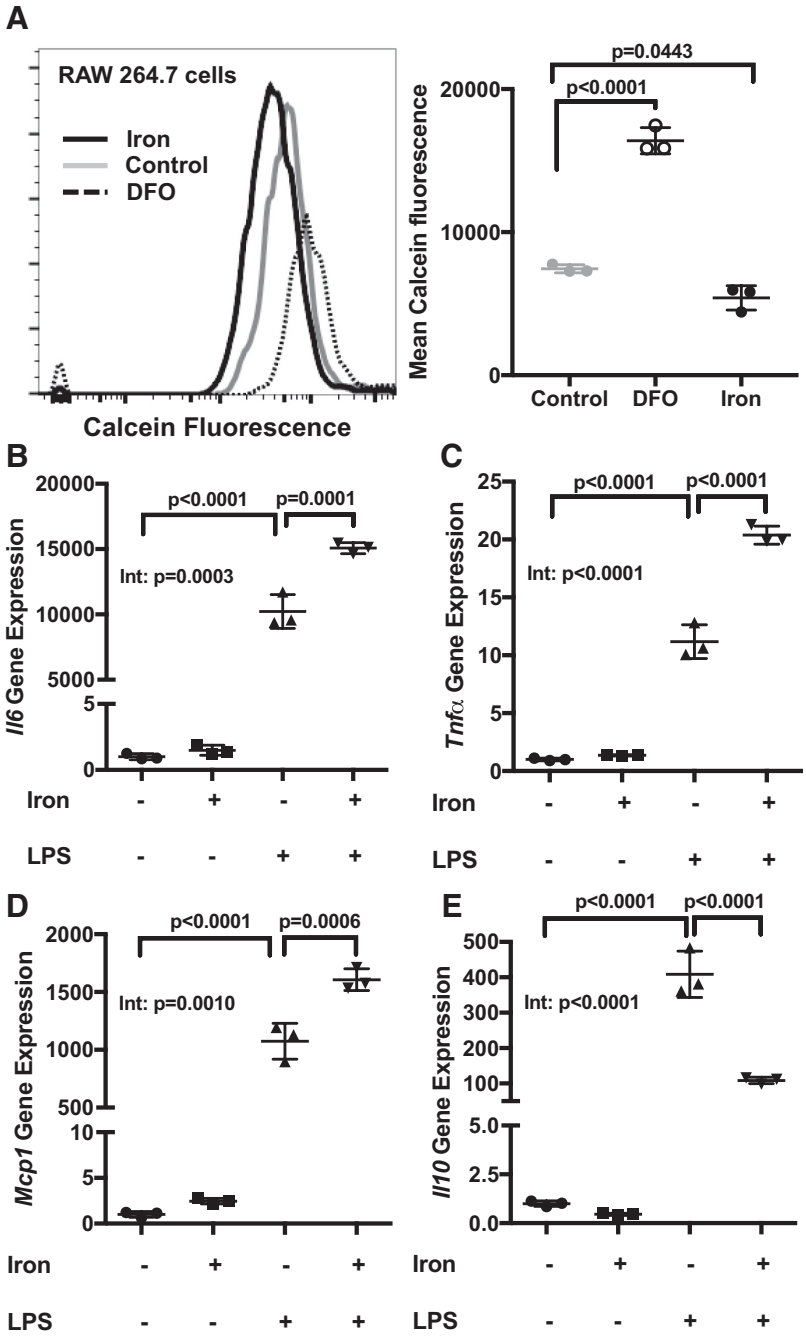

Fig. 4. Effect of iron loading on RAW 264.7 cells *in vitro*. (*A*) RAW cells treated with lipopolysaccharide (LPS), iron, or deferoxamine (DFO, an iron chelator) were incubated with calcein and subjected to flow cytometry, and calcein fluorescence was quantified. Calcein fluorescence decreased in iron-treated cells (one-way ANOVA with Bonferroni *post hoc* tests; *P* values adjusted for all possible comparisons). A representative histogram from one of three independent experiments is shown. The dot plots show mean fluorescent intensities of three replicates/condition. (*B*, *C*) RAW cells were incubated with iron (200 μM) or control overnight and then stimulated for 6 h with lipopolysaccharide (150 ng/ml). Lipopolysaccharide alone induced *Il6* and *Tnfα* mRNA. Iron loading significantly increased mRNA levels in response to lipopolysaccharide. (*D*) The combination of iron and lipopolysaccharide increased *Mcp1* mRNA levels to a greater extent than lipopolysaccharide alone. (*E*) Conversely, the combination of lipopolysaccharide and iron resulted in less *Il10* mRNA levels than lipopolysaccharide alone (two-way ANOVA with Bonferroni *post hoc* tests [*B*–*E*]; *P* values adjusted for all possible comparisons; n = 3 replicates/condition). Il = interleukin; Int = interaction *P* value.

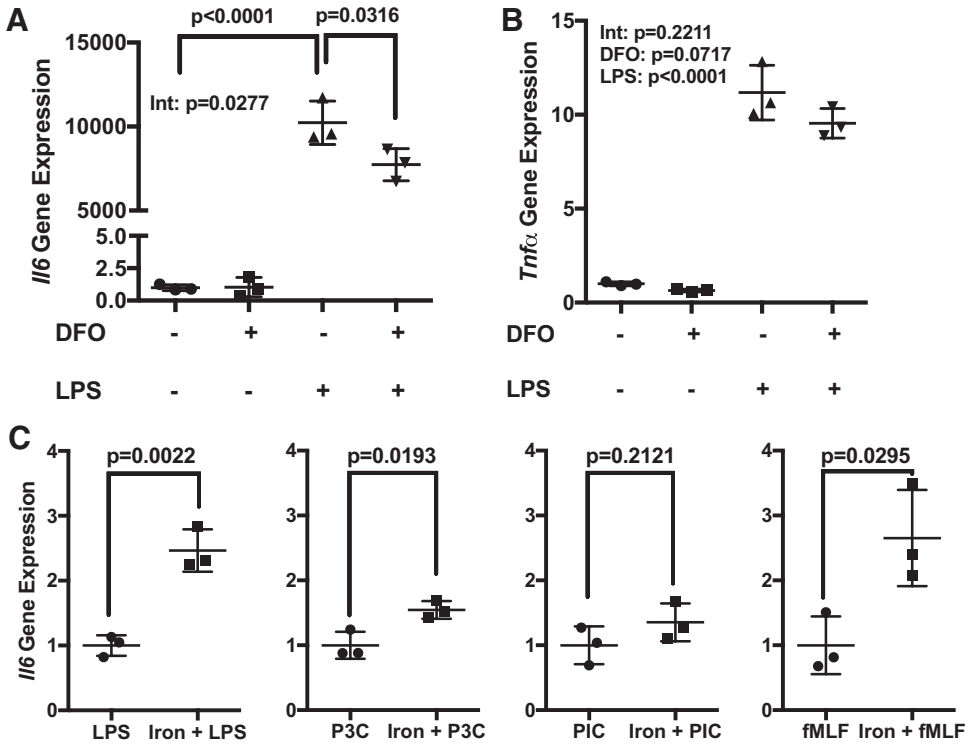

Fig. 5. (*A*, *B*) Effect of an iron chelator on RAW 264.7 cells *in vitro*. RAW cells were pretreated with 30 μM deferoxamine (DFO, an iron chelator) and subsequently stimulated with lipopolysaccharide (LPS) for 6 h. Deferoxamine pretreatment decreases *Il6* mRNA induced by lipopolysaccharide significantly. There is a trend towards decreasing TNF mRNA in deferoxamine-pretreated cells that did not reach statistical significance (two-way ANOVA with Bonferroni *post hoc* tests; *P* values adjusted for all possible comparisons; n = 3 replicates/condition). (*C*) RAW cells were incubated with iron (200 μM) or control overnight and then stimulated for 6 h with lipopolysaccharide (150 ng/ml), Pam-3-Cys (P3C; 1 μg/ml), poly(I:C) (PIC; 25 μg/ml), or *N*-formyl-L-methionyl-L-leuyl-L-phenylalanine (fMLF; 20 μM). *Il6* gene expression was normalized to the response to lipopolysaccharide alone. The response to iron loading is not uniform across different proinflammatory agents. Iron loading augments the inflammatory response to lipopolysaccharide (a TLR4 ligand) and P3C (a TLR2 ligand), but not to PIC (a TLR3 ligand). Iron loading also augments the response to *N*-formyl-L-methionyl-L-leuyl-L-phenylalanine, a formylated peptide that is considered a damage-associated molecular pattern (unpaired *t* tests; n = 3 replicates/condition). Il = interleukin; Int = interaction *P* value.

mitochondrial biogenesis (*Pgc-1α* and *Ampk*; fig. 7, A and B) in the liver, but higher expression of *Lc3b*, a gene involved in mitophagy (fig. 7C). Conversely, iron-treated mice that were challenged with lipopolysaccharide had greater expression of *Pgc-1α* and *Ampk* than mice treated with lipopolysaccharide alone (fig. 7, A–C), suggesting a shift towards either greater mitochondrial biogenesis or decreased mitophagy. We found similar changes in *Ampk* gene expression in mouse lungs and in RAW cells (supplemental fig. 4, A and B, Supplemental Digital Content 1, http://links.lww.com/ALN/B431). These results suggest that lipopolysaccharide alone induces a relative shift towards mitophagy, while iron pretreatment before lipopolysaccharide administration tilts the balance towards mitochondrial biogenesis.

Iron Loading Increases the Proportion of Nonfunctional to Total Mitochondria in RAW Cells

To examine the effect of iron treatment on the functional status of mitochondria, we stained RAW cells that were treated with iron and/or lipopolysaccharide with MitoTracker Deep Red (a dye that stains functional mitochondria only) and MitoTracker Green (a dye that stains all mitochondria, functional and otherwise).[18] The proportion of RAW cells stained with MitoTracker Green alone (*i.e.*, [MT Green – MT Deep Red]/MT Green) was significantly increased in RAW cells treated with iron compared to control cells and was also significantly higher in iron-loaded cells treated with lipopolysaccharide than in lipopolysaccharide-only cells (fig. 8, A and B). This suggests that iron loading increases the proportion of nonfunctional mitochondria in RAW cells.

Mitochondrial genomic DNA content, a measure of mitochondrial mass, was higher in iron-loaded RAW cells treated with lipopolysaccharide than in cells treated with lipopolysaccharide alone (fig. 8C). The mtDNA content in iron-loaded cells that were not treated with lipopolysaccharide was similar to lipopolysaccharide-treated cells that were not iron-loaded. Taken together, these data suggest that iron-loaded cells stimulated with lipopolysaccharide have both a higher mitochondrial mass and a greater proportion

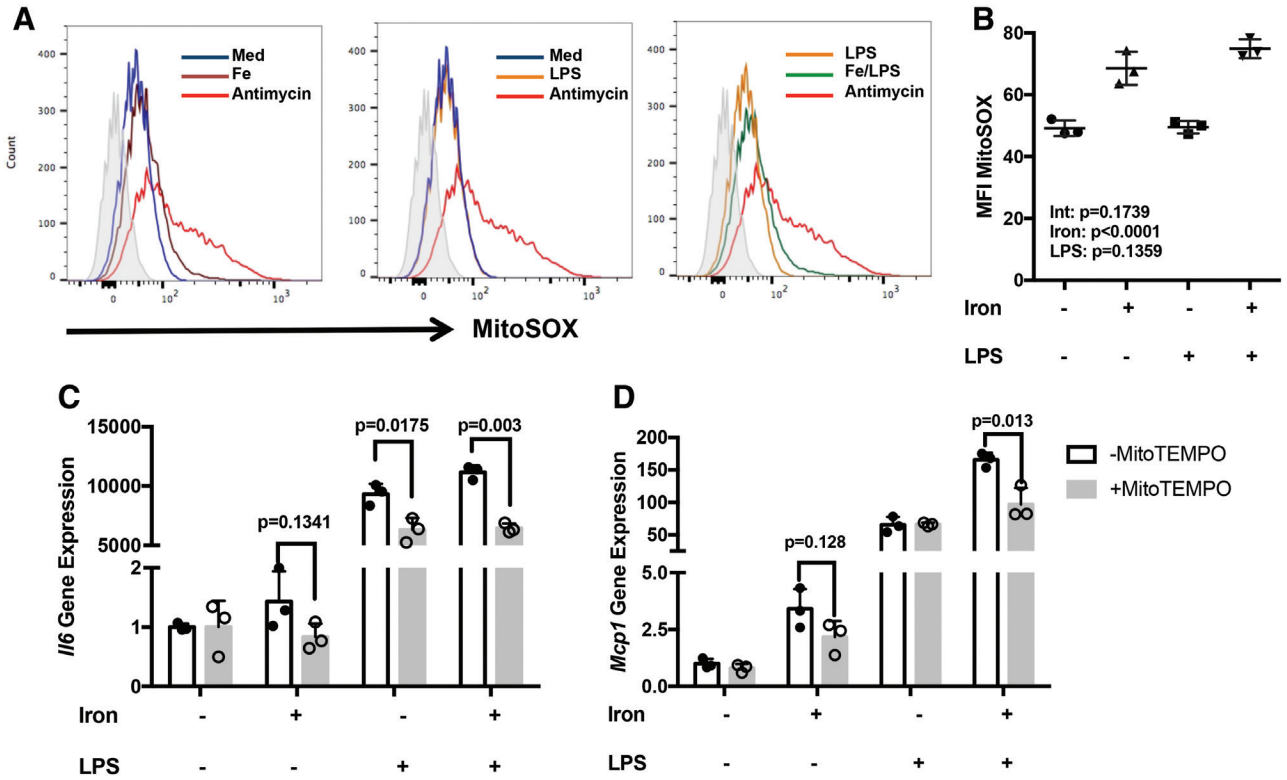

Fig. 6. The effect of iron and lipopolysaccharide (LPS) on mitochondrial reactive oxygen species production. (*A*) Effect of iron supplementation on mitochondrial reactive oxygen species production. The shaded histogram depicts unstained cells. The histogram in red shows the effects of antimycin (20 μg/ml), an inhibitor of mitochondrial complex III used as a positive control. Iron-naïve cells treated with lipopolysaccharide do not show increased MitoSOX fluorescence compared to controls (*middle*). However, iron-pretreated RAW cells exposed to lipopolysaccharide have more mitochondrial reactive oxygen species production compared to cells treated with lipopolysaccharide alone (*right*), although the increase is similar to that caused by iron incubation alone (*left*). Data are representative of three experiments. (*B*) Summary bar graphs showing relative MitoSOX fluorescence (two-way ANOVA; interaction between iron and lipopolysaccharide not significant, therefore only main effects reported; n = 3 replicates/condition). (*C*, *D*) Incubating RAW cells with 100 μM of the mitochondrion-specific antioxidant MitoTEMPO blunted the mRNA response to lipopolysaccharide (*Il6* and *Mcp1*) in iron-loaded macrophages (unpaired *t* tests; n = 3 replicates/condition). Il = interleukin; Int = interaction *P* value; Med = medium; MFI = mean fluorescent intensity.

of nonfunctional mitochondria than cells that were treated with lipopolysaccharide alone.

Discussion

In this study, we observed a strong proinflammatory effect of iron loading on a subsequent challenge with lipopolysaccharide, *in vivo* and *in vitro*. Parenteral iron loading increased intracellular labile iron in circulating neutrophils and monocytes and strongly increased the cytokine response to a subsequent lipopolysaccharide challenge. Similarly, the addition of iron to RAW cells increased intracellular labile iron and further augmented the lipopolysaccharide-induced increase in mRNA levels of proinflammatory genes. As with RAW cells, iron-loaded human peripheral blood monocytes also had higher inflammatory cytokine mRNA levels after lipopolysaccharide stimulation. Iron loading induced mtROS, and inhibition of mtROS formation blunted the augmented response to lipopolysaccharide in iron-loaded RAW cells. Iron loading in conjunction with lipopolysaccharide stimulation also increased the expression of genes associated with mitochondrial biogenesis *in vivo* (in liver and lungs) and increased mitochondrial genomic DNA *in vitro* (in RAW cells), suggesting that iron loading alters mitochondrial homeostasis. Iron loading increased the proportion of nonfunctional mitochondria in RAW cells. Taken together, the data suggest that a combination of iron loading together with an inflammatory stimulus results in an increased proportion of defective mitochondria and increased mtROS production.

Anesthesiologists often provide care for patients given parenteral iron supplements before major surgery and for critically ill patients in intensive care units, who may be iron-loaded from blood transfusion or iron therapy. Thus the effect of acute iron loading on the inflammatory response is of particular relevance in perioperative medicine, especially because there are few studies that have examined the effects of iron infusions on outcomes.[25]

Wang *et al.*[26] used a mouse model of hemochromatosis (*Hfe* knockout mice) to show that low intracellular labile iron in *Hfe*

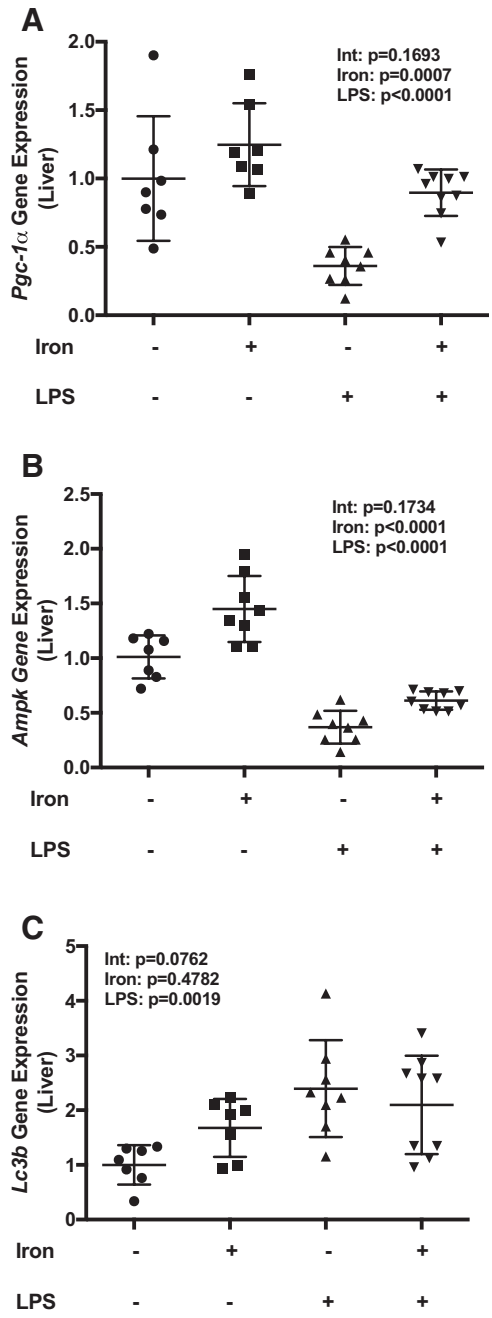

Fig. 7. Iron loading changes the balance between genes responsible for mitochondrial biogenesis and mitochondrial autophagy (mitophagy). C57Bl/6 mice were treated with iron and/or lipopolysaccharide (LPS); N = 7 to 9 mice per group. Total RNA was extracted from mouse liver, and quantitative reverse transcription-polymerase chain reaction was performed using primers for *Pgc-1*α (a mitochondrial biogenesis-associated gene [*A*]), *Ampk* (a mitochondrial biogenesis-associated gene [*B*]), and *Lc3b* (a mitophagy-associated gene [*C*]). Lipopolysaccharide alone increases *Lc3b* mRNA levels, while decreasing *Pgc-1*α and *Ampk* mRNA levels. Iron-loaded mice stimulated with lipopolysaccharide had the opposite profile, with an increase in the mRNA levels of the mitochondrial biogenesis-associated genes *Pgc-1*α and *Ampk* (two-way ANOVA; interaction between iron and lipopolysaccharide not significant, therefore only main effects reported). Int = interaction *P* value.

KO macrophages caused a diminished inflammatory response to lipopolysaccharide. In addition, pretreatment of macrophages from wild-type mice with an iron chelator reduced the inflammatory response to lipopolysaccharide.[26] Other studies showed that the iron chelator deferoxamine attenuated the inflammatory response to lipopolysaccharide in RAW cells and decreased inflammation in mouse models of endotoxemia[9] and peritonitis.[27] In contrast, Pagani *et al.*[10] found that iron-deficient mice (with low macrophage iron levels) had a greater inflammatory response to lipopolysaccharide compared to iron replete mice. De Domenico *et al.*[11] found that oral iron supplementation followed by a lipopolysaccharide challenge blunted the response of mice to lipopolysaccharide. In both of the last two studies, the authors attributed their findings to antiinflammatory effects of the iron-regulating hormone hepcidin. Iron supplementation increases hepcidin production, while low hepcidin production in iron-deficient mice induces an exaggerated response to lipopolysaccharide. However, the mechanism proposed for the presumptive antiinflammatory effect of hepcidin (activation of the janus kinase-signal transducer and activator of transcription pathway by hepcidin-ferroportin binding[11]) has since been questioned.[28] In this study, we demonstrated a robust proinflammatory response to iron loading in spite of an increase in hepcidin gene expression (fig. 1B). The mode of iron supplementation (enteral and parenteral), as well as the formulation of iron, may impact the bioavailability of iron and hence intracellular iron concentrations,[7,29] possibly accounting for differences between our study and that of De Domenico *et al.*

We found that the response to iron loading differs depending on the type of proinflammatory stimulus, suggesting the presence of specific pathways that are influenced by intracellular iron. Indeed, Wang *et al.*[26] found that intracellular iron influences lipopolysaccharide signaling specifically by modifying the MyD88-independent adaptor toll/interleukin-1 receptor domain-containing adapter inducing interferon beta-related adaptor molecule-related response to lipopolysaccharide.

To further examine the mechanisms responsible for the proinflammatory effects of iron loading, we measured mtROS production. Iron loading increased mtROS production in RAW cells, and a mitochondrial-specific antioxidant (Mito-TEMPO) blunted the proinflammatory effect of iron on RAW cells, reducing cytokine (*interleukin-6*) and chemokine (*Mcp1*) mRNA levels to those of macrophages treated with lipopolysaccharide alone. These findings suggest that iron-induced mtROS may have a "priming" effect on macrophages, augmenting the response to a subsequent lipopolysaccharide challenge. In spite of the lack of a demonstrated increase in mtROS production with lipopolysaccharide, lipopolysaccharide stimulation appears to increase mtROS production, because treatment with Mito-TEMPO blunts the inflammatory response to lipopolysaccharide alone. These results are consistent with findings reported by Bulua *et al.*,[30] who found that mouse embryonic fibroblasts have a decreased response to lipopolysaccharide when pretreated with a different mitochondrial superoxide inhibitor, MitoQ. Bulua

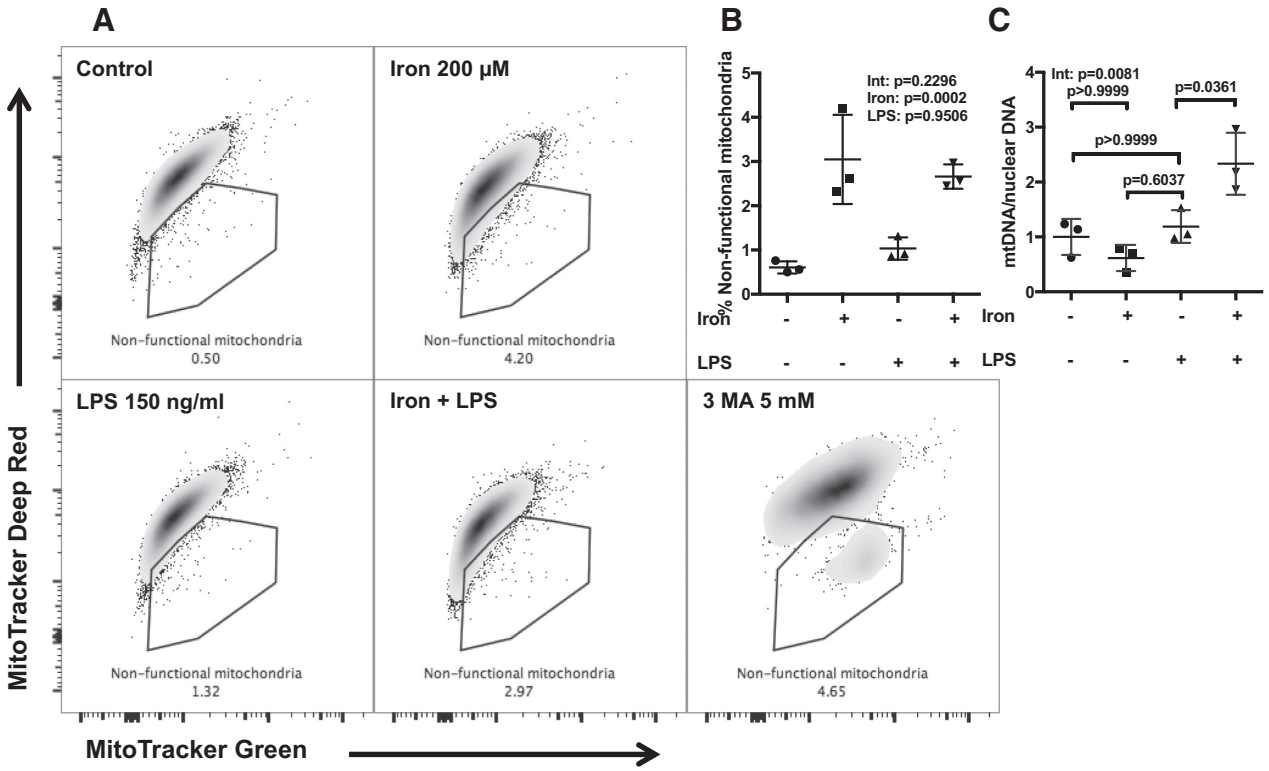

Fig. 8. Iron loading increases the proportion of nonfunctional mitochondria in RAW 26.7 cells. RAW cells were treated with iron and/or lipopolysaccharide (LPS), then incubated with MitoTracker Deep Red and MitoTracker Green, and subjected to flow cytometry. (*A*) Density plots for each condition, representative of three independent experiments. The gate shown contains the cells that are MitoTracker DeepRed–negative/MitoTracker Green–positive cells (nonfunctional mitochondria). 3-Methyladenine (3 MA), an inhibitor of autophagy, was used as a positive control. (*B*) A summary histogram of the cytometry results (two-way ANOVA; interaction between iron and lipopolysaccharide not significant, therefore only main effects reported; n = 3 replicates/condition). (*C*) Iron-loaded, lipopolysaccharide-treated RAW cells have higher mitochondrial mass defined by mitochondrial gene: nuclear gene ratio (two-way ANOVA with Bonferroni *post hoc* tests; *P* values adjusted for all possible comparisons; n = 3 replicates/condition). Int = interaction *P* value; mtDNA = mitochondrial DNA.

et al.[30] also did not find increased MitoSOX staining in the fibroblasts treated with lipopolysaccharide alone.[3] We speculate that while lipopolysaccharide does increase mtROS production, the effects of lipopolysaccharide on mtROS levels may be opposed by increased mitophagy, resulting in no change in net mtROS levels.

Other studies have found that increased intracellular iron increased mtROS production and that mtROS was associated with increased inflammation in different cell types, such as cardiomyocytes[31] and macrophages.[32] The results of this study therefore add to the existing literature by showing that iron loading potentiates inflammation by augmenting mtROS production.

Although the *in vitro* data in our study were derived from monocytes and macrophages, it is possible that iron loading may impact ROS production by neutrophils, which are a major source of ROS *in vivo*.[33] Sampaio *et al.*[34] showed that iron dextran administration in a streptozotocin-induced model of diabetes in rats was associated with a strong increase in neutrophil ROS production. Iron loading may therefore be proinflammatory in both monocytes and neutrophils.

Iron-loaded cells exposed to lipopolysaccharide had a greater mitochondrial mass, as determined by the relative abundance of mitochondrial DNA to nuclear DNA. Of note, we did not find a significant difference in the proportion of mtDNA to nuclear DNA between iron-loaded cells and lipopolysaccharide-treated cells. Iron loading RAW cells increased the proportion of nonfunctional mitochondria relative to total mitochondria. Others have shown that damaged or nonfunctional mitochondria produce more mtROS.[18,35] Mitochondrial mass can be increased by increasing mitochondrial biogenesis, decreasing mitophagy, or both. In this study, the combination of iron and lipopolysaccharide increased mRNA levels of genes involved in mitochondrial biogenesis, *Pgc-1α* and *Ampk in vivo*. Lipopolysaccharide-treated mice had higher expression of hepatic *Lc3b*, a gene involved in mitophagy, which is consistent with prior data reporting that lipopolysaccharide increases mitophagy.[36] The data therefore suggest that the combination of iron loading and lipopolysaccharide stimulation increases mitochondrial biogenesis *in vivo*. Of note, increased mitochondrial biogenesis is not necessarily deleterious; some studies have shown

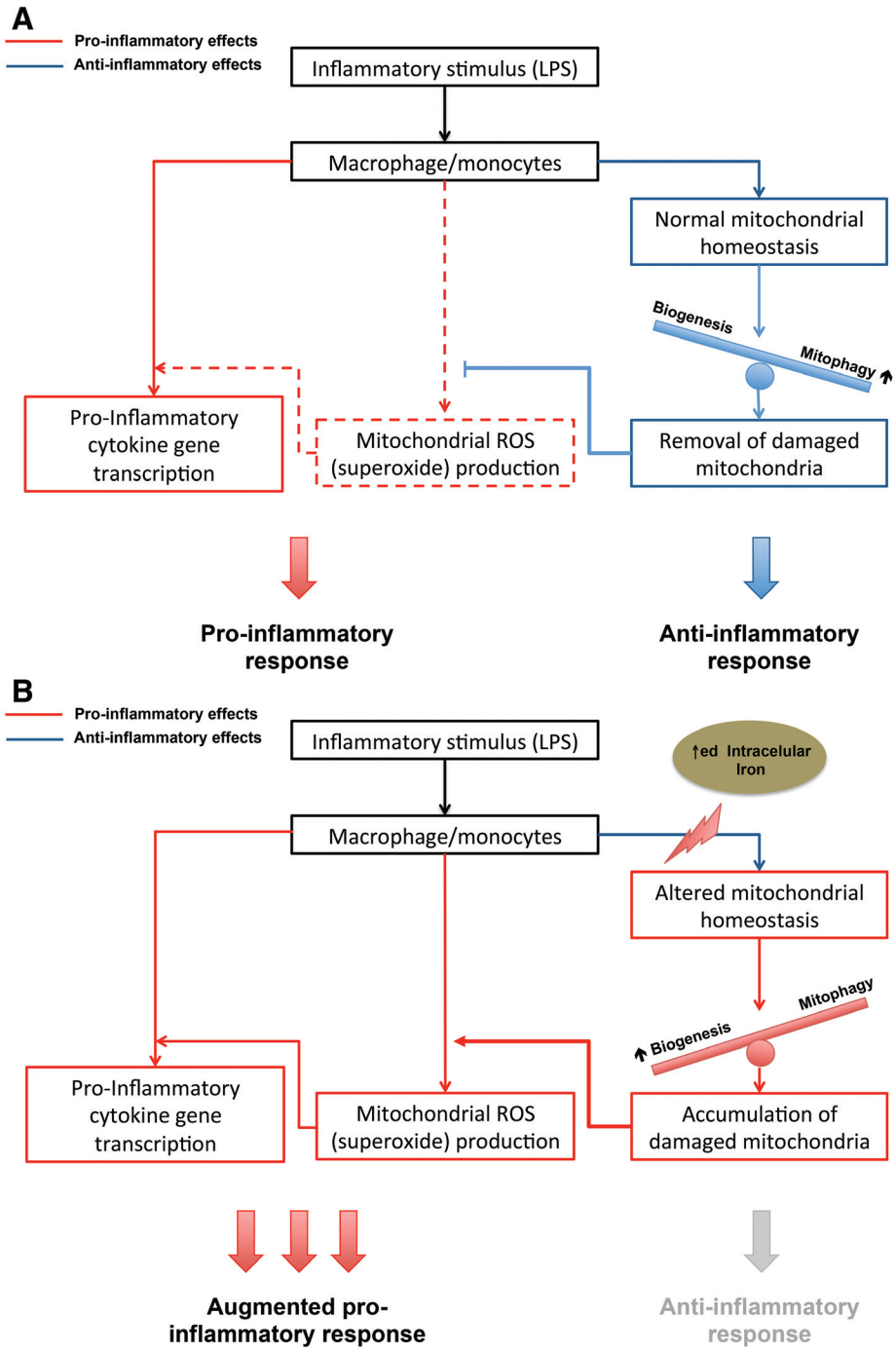

Fig. 9. A schematic view of the role of mitochondrial homeostasis (the balance between mitochondrial biogenesis and mitophagy) in the acute inflammatory response. (*A*) Lipopolysaccharide (LPS)-induced inflammation induces inflammatory gene transcription. Simultaneously, maintenance of mitochondrial homeostasis allows removal of damaged mitochondria by mitophagy (mitochondrial "quality control"), resulting in a blunted inflammatory response, which may be why mitochondrial reactive oxygen species (ROS) does not appear to increase in lipopolysaccharide-stimulated cells. (*B*) In the presence of excess intracellular iron, the balance of mitochondrial homeostasis shifts towards increased biogenesis and less effective mitophagy, resulting in an accumulation of damaged mitochondria, increased mitochondrial reactive oxygen species, and an augmented proinflammatory effect.

that mitochondrial biogenesis imparted a prosurvival phenotype in acute inflammatory states.[37]

Studies using mouse models of defective mitophagy showed that accumulation of nonfunctional mitochondria potentiated mtROS formation and induced a more potent inflammatory response to innate immune stimulants, including lipopolysaccharide.[18,21,38] Duvigneau *et al.*[39] showed that endotoxin-induced iron accumulation in cells was associated with altered mitochondrial respiration and mitochondrial dysfunction. Lowering intracellular

iron levels using iron chelators induced mitophagy in a *Caenorhabditis elegans* model of *Pseudomonas* infection,[40] while iron loading promoted mitochondrial biogenesis in osteoclasts.[41] These reports are consistent with our finding that intracellular iron levels modulate mitochondrial homeostasis.

This study has some limitations. We used iron dextran, rather than iron sucrose or iron gluconate, because iron dextran was previously shown to be the least likely to cause direct iron-induced toxicity.[42] Because we did not test other forms of iron *in vivo*, we cannot comment on the effects of other iron formulations. A second potential limitation is that we examined the effects of increased intracellular iron on the early inflammatory response and thus cannot comment on the effect of iron loading on the temporal course of inflammation. In addition, while we have found that iron loading has a proinflammatory effect on macrophages, we did not investigate the effect of iron loading on macrophage phenotype, although others have shown that iron loading induced an M1 phenotype in macrophages.[43] In RAW cells, we observed a decrease in *interleukin-10* mRNA in response to iron loading, but a similar effect was not observed in murine lungs *in vivo* (supplemental fig. 1, Supplemental Digital Content 1, http://links.lww.com/ALN/B431). Finally, our data demonstrating the effects of iron loading on mitochondria function are limited to *in vitro* assays. Further work is needed to determine whether iron loading alters mitochondrial function *in vivo*.

The results of this study suggest that increased intracellular iron leads to an increased proinflammatory response to the TLR4 ligand lipopolysaccharide, raising the possibility of targeting intracellular iron as a therapeutic modality in acute inflammatory states. However, many questions need to be addressed before intracellular iron can be considered a viable biologic target. Critically ill patients are often hypoferremic, and iron chelators will likely exacerbate hypoferremia. Therapy with iron chelators[44,45] may increase the risk of bacterial infections by organisms that can extract iron from the iron-chelator complex. Finally, we do not currently have reliable assays for monitoring intracellular iron levels to guide therapy.

Conclusions

Our results suggest that iron loading alters mitochondrial homeostasis, leading to the accumulation of defective mitochondria and increasing production of mtROS. The production of mtROS primes macrophages for a "second hit," such as exposure to lipopolysaccharide, greatly augmenting the inflammatory response to the second stimulus. Figure 9 presents a schematic of the role increased intracellular iron plays in modulating the acute inflammatory response. The data presented here highlight the proinflammatory effects of iron loading in acute inflammation and suggest that clinicians should consider the risks of treatments that result in iron loading in acutely ill patients.

Acknowledgments

We gratefully acknowledge advice on statistical methods from Timothy T. Houle, Ph.D., Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital, Boston, Massachusetts.

Research Support

Supported by a fellowship from Boehringer Ingelheim Fonds, Mainz, Germany (to Mr. Hoeft); grant No. R01DK082971 from the National Institutes of Health, Bethesda, Maryland, and the Foundation Leducq, Paris, France (to Dr. Bloch); grant No. DFG GR 4446/1-1 from Deutsche Forschungsgemeinschaft, Bonn, Germany (to Dr. Graw); grant No. 5R01HL101930 from the National Institutes of Health, Bethesda, Maryland (to Dr. Ichinose); and a mentored research training grant from the Foundation for Anesthesia Education and Research, Schaumburg, Illinois (to Dr. Bagchi).

Competing Interests

Dr. Bagchi is a consultant for Lungpacer Medical Inc., Burnaby, British Columbia, Canada. The other authors declare no competing interests.

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ANESTHESIOLOGY REFLECTIONS FROM THE WOOD LIBRARY-MUSEUM

Laughing Gas in Baltimore, Hagerstown, and Smithsburg: Maryland's Dr. D. W. Crowther

Son of an English army officer, David William Crowther, D.D.S. (1834 to 1916), was born in Devonshire but relocated as a baby with his family to Drummondville, Canada. As a young man, he subsequently moved to New York and then Alabama to serve from Mobile in the Confederacy's First Alabama Battery. Following the Civil War, he trained in dentistry, earning his D.D.S. in Maryland in 1868 from the Baltimore College of Dental Surgery. Around 1874 he moved 74 miles northwest in Maryland to Hagerstown, where he established his dental practice on North Potomac Street. According to this lovely trade card from the Ben Z. Swanson Collection of the Wood Library-Museum, Dr. Crowther extracted teeth "with [laughing] gas" just a "door above Clapp's Junior Hall Store." Never forsaking Maryland professionally, Crowther moved in 1890 about 8 miles east to Smithsburg. He retired from dentistry in 1896 and passed away a decade later. All told, during his 28 yr of practice as a degreed dentist, Crowther administered laughing gas in Baltimore, Hagerstown, and finally Smithsburg, Maryland.

George S. Bause, M.D., M.P.H., Honorary Curator and Laureate of the History of Anesthesia, Wood Library-Museum of Anesthesiology, Schaumburg, Illinois, and Clinical Associate Professor, Case Western Reserve University, Cleveland, Ohio. UJYC@aol.com.

Pain Catastrophizing Moderates Relationships between Pain Intensity and Opioid Prescription

Nonlinear Sex Differences Revealed Using a Learning Health System

Yasamin Sharifzadeh, B.S., Ming-Chih Kao, Ph.D., M.D., John A. Sturgeon, Ph.D., Thomas J. Rico, B.S., Sean Mackey, M.D., Ph.D., Beth D. Darnall, Ph.D.

ABSTRACT

Background: Pain catastrophizing is a maladaptive response to pain that amplifies chronic pain intensity and distress. Few studies have examined how pain catastrophizing relates to opioid prescription in outpatients with chronic pain.

Methods: The authors conducted a retrospective observational study of the relationships between opioid prescription, pain intensity, and pain catastrophizing in 1,794 adults (1,129 women; 63%) presenting for new evaluation at a large tertiary care pain treatment center. Data were sourced primarily from an open-source, learning health system and pain registry and secondarily from manual review of electronic medical records. A binary opioid prescription variable (yes/no) constituted the dependent variable; independent variables were age, sex, pain intensity, pain catastrophizing, depression, and anxiety.

Results: Most patients were prescribed at least one opioid medication (57%; n = 1,020). A significant interaction and main effects of pain intensity and pain catastrophizing on opioid prescription were noted ($P < 0.04$). Additive modeling revealed sex differences in the relationship between pain catastrophizing, pain intensity, and opioid prescription, such that opioid prescription became more common at lower levels of pain catastrophizing for women than for men.

Conclusions: Results supported the conclusion that pain catastrophizing and sex moderate the relationship between pain intensity and opioid prescription. Although men and women patients had similar Pain Catastrophizing Scale scores, historically "subthreshold" levels of pain catastrophizing were significantly associated with opioid prescription only for women patients. These findings suggest that pain intensity and catastrophizing contribute to different patterns of opioid prescription for men and women patients, highlighting a potential need for examination and intervention in future studies. **(Anesthesiology 2017; 127:136-46)**

With up to 40% of the global population experiencing ongoing pain,[1,2] there is a need to better understand the experience of pain and associated treatment patterns. Pain catastrophizing[3,4]—a cascade of negative thoughts and emotions in response to actual or anticipated pain—is a key factor in pain-related outcomes. In experimental and clinical settings, pain catastrophizing is associated with amplified pain processing,[5,6] greater pain intensity,[7] and greater disability.[7,8] Pain catastrophizing may explain up to 20% of the variance in chronic pain intensity[9] and thus may influence other pain treatments, including opioid medications.

Pain catastrophizing has been identified as a risk factor for prescription opioid misuse in patients with chronic pain generally[10] and among those with a history of substance

What We Already Know about This Topic

- Pain catastrophizing is a cascade of negative thoughts and emotions in response to actual or anticipated pain
- It may explain up to 20% of the variance in chronic pain intensity and may, as a result, influence pain treatment

What This Article Tells Us That Is New

- A retrospective study of 1,794 patients with chronic pain seeking initial medical evaluation found a significant relationship between pain intensity and opioid prescription that was much stronger in women, especially those with high levels of pain catastrophizing
- Although men and women had similar levels of catastrophizing and opioid prescription, opioid prescriptions were more common at lower levels of catastrophizing for women

This article is featured in "This Month in Anesthesiology," page 1A. Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site (www.anesthesiology.org).

Submitted for publication April 7, 2016. Accepted for publication March 16, 2017. From Stanford University School of Medicine, Department of Anesthesiology, Perioperative and Pain Medicine, Division of Pain Medicine, Stanford Systems Neuroscience and Pain Laboratory, Palo Alto, California.

 Anesthesiology 2017; 127:136-46

use disorder.[11] Postsurgically, opioid use is quantified commonly either by dose or by time-to-opioid cessation.[12,13] Perioperative studies have yielded mixed findings for pain catastrophizing, with some reporting a direct relationship with morphine dose delivered either by patient-controlled analgesia devices[14] or by hospital staff,[15] whereas other studies reported no association[16] or an inverse association.[17] Findings from a recent longitudinal study of 145 patients undergoing musculoskeletal trauma surgery suggested that pain catastrophizing predicted delayed opioid cessation after surgery.[18] Using multivariate analyses, the authors found that pain catastrophizing was the strongest predictor of postsurgical opioid use 1 to 2 months after surgery. After the authors controlled for anxiety, depression, posttraumatic stress disorder symptoms, and disability, pain catastrophizing accounted for 23% of the unique variance in persistent postsurgical opioid use.

In the outpatient setting, catastrophizing has been associated with opioid craving,[19] long-term opioid use in veterans,[20] and opioid misuse.[21] Given the positive associations found between catastrophizing and the aforementioned opioid responses and behaviors, it would follow that a similar association might exist for catastrophizing and receipt of an opioid prescription in a larger civilian chronic pain population. However, to our knowledge, this latter relationship is unexplored. Characterization of the relationship between catastrophizing and opioid prescription in a larger chronic pain sample could enhance understanding and potentially reveal a therapeutic target for reducing need and use of opioids in chronic pain outpatients.

Accordingly, the purpose of this study was to characterize the relationship between existing opioid prescription and pain catastrophizing in a large sample of patients presenting for new evaluation at a chronic pain clinic. It has been found that in individuals with chronic noncancer pain, the presence of comorbid mental health diagnoses, particularly mood disorders, predicts the likelihood of opioid prescription,[21] the degree of opioid use,[11] and the likelihood of aberrant opioid use (*e.g.*, opioid abuse or dependence).[22] Consequently, we sought to characterize the relationship between pain catastrophizing and opioid prescription independent of the influences of these and other factors, including age,[11] sex,[23] and pain intensity,[16,24] known to be relevant to opioid use, and pain catastrophizing, such as symptoms of anxiety and depression.[22] We aimed solely to identify any relationships between our variables of interest, in turn allowing for future investigations to further explore any clinically significant findings.

Materials and Methods

Design and Setting

The current study used a retrospective, observational method to examine a large sample of adult patients with chronic pain. Patients were seeking treatment at a large, urban, tertiary academic pain treatment center located in the San Francisco Bay Area in the United States. Data were extracted for patients with initial pain clinic visits between January 2014 and April 2015. Study procedures, which involved exclusively retrospective review of clinical data and therefore did not require informed consent from patients, were approved by the Institutional Review Board at Stanford University in Stanford, California.

Participants

All new patients who sought treatment at a tertiary academic outpatient pain management center in the San Francisco Bay Area between the aforementioned dates were eligible to be in the study. However, only those who had completed the Pain Collaborative Health Outcomes Information Registry (Pain-CHOIR) in its entirety, 1,794 patients, were included in the study.

Data Collection

Data were collected with the Pain-CHOIR[25,26] (http://snapl.stanford.edu/choir). Pain-CHOIR is a learning health system that allows for deep phenotyping of patients while also identifying their treatment needs and facilitating rapid delivery of specialized pain services. The patient-reported outcomes component of Pain-CHOIR is an electronic patient survey. For simplicity, the survey alone will be referred to as Pain-CHOIR. Pain-CHOIR, administered to all patients in the Stanford Pain Management Center, serves as a key component of the new patient evaluation procedure. Five days before their scheduled new patient medical evaluation, all patients receive an email with instructions to follow a link to register with the Pain-CHOIR system and complete their new patient survey. Patients who do not complete their Pain-CHOIR before their visit or lack the technologies (computer/smartphone, Internet, email address) needed to access the survey are asked to complete the surveys at clinic check-in using a tablet computer provided by the clinic.

Data for the following measures were extracted from the initial Pain-CHOIR: demographic variables (education, marital status, and race), the Pain Catastrophizing Scale (PCS),[27] average pain intensity, and the Depression and Anxiety item banks of the National Institutes of Health Patient-Reported Outcomes Measurement Information System (PROMIS).[28] Other demographic variables, such as date of birth (used to calculate age) and sex, were extracted from Stanford Hospitals and Clinics electronic medical record system. In addition, all patients had accessible electronic medical records with physician notes that allowed for manual retrospective chart review. Finally, all pain diagnostic information was attained by collecting the International Classification of Diseases, Ninth Revision billing codes assigned to each patient at initial clinic visit. The codes were reviewed and categorized according to diagnoses relevant to the field.

Opioid Prescription. Patients self-reported all current opioid prescription data, either electronically *via* Pain-CHOIR

or verbally to clinic staff during their medical visit. For patients who verbally provided opioid medication information (n = 711, 40%), opioid data were extracted *via* retrospective chart review in a step-wise manner. Step 1 involved recording current opioid medications for the initial clinic visit from physician documentation (the clinical note) in the electronic medical record. If data were absent in step 1, step 2 was used, in which opioid data were extracted from the electronic medical record medication list. Step 2 was used for less than 10% of the manually extracted opioid prescription data. Data collection screened for codeine, duragesic, hydrocodone, hydromorphone, levorphanol, meperidine, methadone, morphine, oxycodone, oxymorphone, tramadol, and suboxone. Active opioid prescription was recorded as a binary variable with 0 = no opioid prescriptions and 1 = any opioid prescription.

Midway through the study period, an opioid survey was included into Pain-CHOIR containing the following item: "Are you currently taking any opioid medications (such as Vicodin, Oxycontin, Oxycodone, Morphine, MS-Contin, Codeine, Actiq, Duragesic, Dilaudid, Demoral, Methadone, Percocet, Opana, Nucynta, Stadol, Ultram)?" The addition of the opioid question rapidly identified patients with current opioid prescriptions, thereby greatly facilitating data catchment. Thus, for 1,083 patients, opioid prescription data were extracted electronically directly from Pain-CHOIR.

Patient-reported Outcome Measures

Pain Catastrophizing Scale. Pain catastrophizing was measured with the PCS.[27] The PCS asks respondents to rate how frequently they respond to pain in a manner consistent with each of the 13 statements presented (*e.g.*, "It's awful and I feel that it overwhelms me."). Each item is rated on a 5-point scale ranging from 0 (not at all) to 4 (always). A total PCS score is computed by summing the 13 items (range = 0 to 52), with higher scores reflecting higher levels of catastrophizing. The PCS contains three subscales: rumination, magnification, and feelings of helplessness. The PCS has been shown to have good internal and cross-population psychometric consistency.[27,29–31] The coefficient alpha for the total PCS is 0.87.[27]

PROMIS Depression and Anxiety. Within Pain-CHOIR, PROMIS is delivered as a computer-based survey that uses a computerized adaptive testing approach based on item response theory to allow for item-level responses, greater precision achieved through lowered SE, and a smaller set of questions[32] that gauge a psychometric domain on a continuum[33] with reduced sensitivity to population variability.[34] The PROMIS Depression and Anxiety item banks have demonstrated validity and consistency.[35] PROMIS instruments quantify level of symptoms, are normed on the U.S. population, and are reported with *t* scores with a mean of 50 and a SD of 10.[28]

Average Pain Intensity. Average pain intensity was measured with the numeric rating scale, which operates on a 0 to 10 scale with "0" being no pain and "10" being the worst pain imaginable.[36] Respondents were asked to consider the previous 7 days for rating their average pain intensity. The numeric rating scale has been validated for specificity and use in chronic pain research.

Statistical Analysis

In the statistical analysis of clinical trials and observational studies, valid characterizations of effects are contingent on the accurate selection of the statistical model. In the field of pain research, linear models predominantly are used. Linear models, including linear regression, logistic regression, and semiparametric methods such as Cox proportional hazard modeling, assume that the effects of the covariates on the outcome variable are linear and equal across the entire range of observed values. In situations in which the phenomenon under question is in fact nonlinear, model misspecification can lead to inaccurate estimates that can result in erroneous statistical inferences *via* outliers or dilution of true effects. Consequently, individual investigators may be misled into costly pursuits of inaccurate conclusions. Scientifically, neglecting nonlinearity may lead to inconsistent statistical estimates and paradoxical bodies of literature. In some cases, nonlinearity may be apparent visually during data analysis and accounted for by the incorporation of polynomial covariate terms. However, this is not always true, suggesting that this approach may not be sufficient for detecting and addressing potentially nonlinear relationships. Models involving binary outcome variables may present particular difficulties in this regard.

To bypass normal distribution assumptions and account for nonlinear relationships, we used both a generalized linear model and generalized additive model. General linear modeling is a flexible, linear statistical model that allows for the analysis of variables with non-normal distributions using a link function. General linear model building was performed with a logit link function for the binary outcome of "any opioids prescribed" with covariates (x) of pain intensity, anxiety, depression, pain catastrophizing, pain intensity × pain catastrophizing (interaction), age, and sex.

$$logit\left[P\left(Opioid\right)\right] = \beta_0 + \beta_1\left(x_1\right) + \beta_2\left(x_2\right) + \beta_3\left(x_3\right)$$

General additive modeling, a flexible nonlinear model, was used to identify and characterize the effect of potential nonlinear prognostic factors on the binary outcome of opioid prescription with smoothing spline curves to fully estimate nonlinear effects. Opioid prescription was analyzed as a possible prognostic factor in the association between pain intensity and pain catastrophizing, separately characterized by sex. Interaction terms (such as pain intensity × pain catastrophizing) were used in moderation analyses, intended to determine whether the prognostic value of predictors such as pain intensity and pain catastrophizing in predicting opioid prescription were mutually dependent. In simpler terms, this interaction term was intended to reflect whether the independent prognostic value of pain intensity for opioid

prescription was dependent on co-occurring pain catastrophizing scores and *vice versa*.

We also used mediation analyses, an analytic approach designed to estimate the extent to which a third variable (the mediator) explains or accounts for the relationship between an independent and dependent variable. Given the known positive relationships between pain intensity and pain catastrophizing and the inconsistent relationship between pain catastrophizing and opioid use, one natural question that arose was whether the relationship between pain intensity and opioid use was mediated by pain catastrophizing. To test this, we used the causal mediation analysis framework from Imai *et al.*[37] for the whole sample and separately for men and women. Analogous to mediation analysis using structural equation modeling, this framework also relies on a series of regression models. It is able to estimate the average causal mediated effect and average direct effect nonparametrically.

All statistical analyses were completed in SPSS 22.0 (IBM Corporation, USA) and R (R 3.1.0, Austria) for Windows. General additive models were estimated with the R package. mgcv. Significance was set at $P < 0.05$ unless otherwise noted.

Results

Sample Demographic and Diagnostic Characteristics

Demographic characteristics for the 1,794 patients included in this study are described in table 1. The study sample was predominantly white (n = 1,144; 67%), married (n = 794; 54%), and women (n = 1,129; 63%), with at least some college education (n = 1,199; 83%). Mean age of the sample was about 50 yr (table 2) with an age range of 18 to 94 yr (table 1). In the current study, pain diagnoses were separated into a series of categories, representing the broad location and presumed etiology of pain complaints. Given that complete diagnostic information was not available for the sample used in this study, pain diagnosis information for all pain clinic patients presenting for an initial visit between January 2014 and May 2016 were analyzed to characterize the clinic overall. Although 10,707 (28%) of the total number of diagnoses were not pain related or listed, the most common diagnoses included headache (9.2%), thoracolumbar pain (8.7%), musculoskeletal pain (7.6%), cardiac pain (5.3%), and nerve pain (5.0%) (see Supplemental Digital Content 1, http://links.lww.com/ALN/B433, which lists the distribution of pain diagnoses for the clinic). The total number of diagnoses exceeded the number of patients visiting the clinic due to multiple diagnoses per patient per visit. Most patients had one major pain diagnosis (46%), whereas close to 20% had two or more diagnoses.

Clinical Measures by Sex

Clinical measures are reported by sex in table 2. Unpaired *t* test results revealed a slight age difference between men and women, with men having greater average age. As expected, women had higher average pain intensity than men ($P = 0.02$). Despite higher pain intensity in women, we found no difference in PCS scores by sex ($P = 0.12$). Although there were no significant differences in depression between men and women, there was a difference in anxiety between the sexes, with women reporting greater anxiety.

Table 1. Sample Demographic Characteristics

Variable	n (%)
Sex	
Woman	1,129 (63)
Man	665 (37)
Age, yr	
18–30	216 (12)
31–40	301 (17)
41–50	373 (21)
51–60	475 (26)
61–70	256 (14)
71–80+	173 (10)
Marital status* (19% of patients not included)	
Separated/divorced	225 (16)
Cohabitating	104 (7)
Widowed	49 (3)
Married	794 (54)
Never married	288 (20)
Education* (19% of patients not included)	
No high school diploma	111 (8)
High school diploma or GED	143 (10)
Some university/associate's degree	534 (37)
Bachelor's degree	343 (24)
Graduate degree	322 (22)
Unknown	4 (<1)
Race*† (5% of patients not included)	
American Indian or Alaska Native	7 (<1)
Asian	117 (7)
Black or African American	51 (3)
Native Hawaiian/Pacific Islander	16 (1)
White	1,144 (67)
Other	311 (18)
Patient declined to answer	34 (2)
Unknown	31 (2)

*Not all patients included due to incomplete surveys. †Due to limitations of the electronic medical records used, ethnicity data were unreliable and not reported. Hispanic is subsumed either in "white" or "other."
GED = General Educational Diploma.

Table 2. Clinical Measures by Sex

	Men	Women
Age	51 (15)	49 (15)*
Average pain intensity (NRS)	6 (2)	6 (2)*
PCS	21 (13)	20 (13)
Depression	57 (10)	58 (9)
Anxiety	58 (10)	59 (9)*

Scores are presented as mean (SD).
*Significant sex differences on a variable: $P < 0.05$.
NRS = numeric rating scale; PCS = Pain Catastrophizing Scale.

Table 3. Clinical Measures by Opioid Status

	Full Sample	No Opioids	Opioids
Age	50 (15)	49 (16)	50 (15)
Average pain intensity	6 (2)	5 (2)	6 (2)*
PCS	20 (13)	19 (13)	21 (13)*
Depression	58 (9)	57 (9)	58 (10)†
Anxiety	58 (10)	58 (10)	59 (9)

Scores are presented as mean (SD).
Significant differences between opioid and nonopioid groups: *$P < 0.001$, †$P < 0.05$.
PCS = Pain Catastrophizing Scale.

Clinical Measures by Opioid Status

Means for age and psychometric variables are reported in table 3. In the full sample, 57% (n = 1,020) had one or more opioid prescriptions. Age was unrelated to opioid prescription. A similar proportion of men (58%; n = 387) and women (56%; n = 633) had opioid prescription ($P = 0.38$). Overall, opioid prescription was associated with higher average pain intensity ($P < 0.001$), PCS scores ($P < 0.001$), and depression ($P = 0.04$).

Opioid Prescription as a Function of Pain Intensity and Pain Catastrophizing

Given the significant differences in pain intensity and pain catastrophizing observed between opioid prescription groups, we sought to attain a preliminary understanding of any underlying relationships through a visual display—a density plot of patients by opioid prescription status. Figure 1 displays a heat map of the density of patients according to opioid status as a function of pain intensity and pain catastrophizing, with red representing the greatest patient density. As seen by the concentration of yellow at the bottom left-hand corner of the left graph, the density of patients with low levels of pain and catastrophizing without opioid prescription is much higher than that of those with opioid prescription. The data display for opioid prescription reveals a more horizontal distribution of patients with a wider range of pain catastrophizing. Also, there are a greater number of high patient-density patches spread over a larger range of catastrophizing. The data display in figure 1 allowed us to visually detect emerging relationships between pain intensity and pain catastrophizing in those without opioid prescription. However, the difference between the heat maps of the two groups called for the further modeling of these variables.

Generalized Linear Modeling of the Association between Opioid Prescription, Average Pain Intensity, and Additional Variables

We further investigated the differences in pain intensity and pain catastrophizing seen between opioid users and nonusers with a more sophisticated analysis, generalized linear modeling. Table 4 shows the result of using generalized linear modeling, a more flexible linear model, to show the association between opioid prescription, average pain intensity, and additional study variables. As a base model, pain intensity showed a significant positive association with opioid prescription. For every increase of one SD away from average pain intensity, the odds of having prescription opioids increased by 41%. The use of pain intensity with other variables showed no significant effects on opioid prescription. However, on modeling opioid prescription with pain intensity, pain catastrophizing, and their interaction term, significant associations were found. Pain intensity and pain

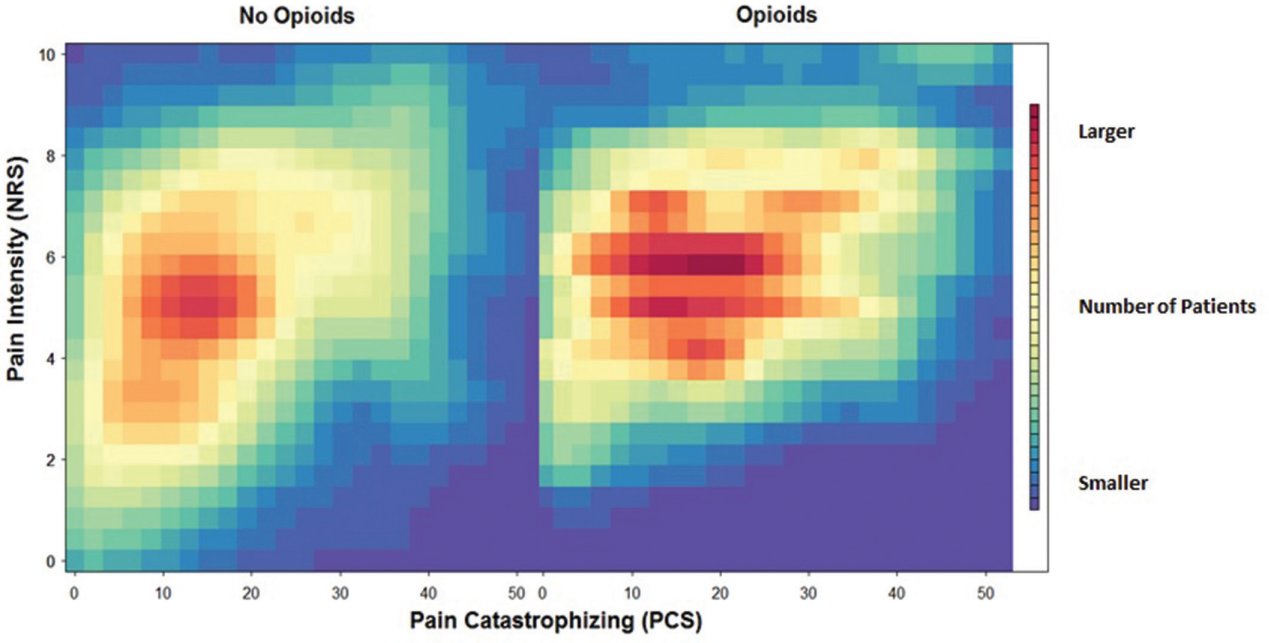

Fig. 1. Heat map of distribution of patients in terms of pain catastrophizing and pain intensity, by opioid prescription status. The color *red* represents the greatest patient density. NRS = numeric rating scale; PCS = Pain Catastrophizing Scale.

Table 4. General Linear Models for the Prediction of Opioid Prescription

	Variable	Estimate	OR (per SD)	SE	*P* Value
Base model					
1	Pain intensity	0.159	1.41	0.023	<0.001
Models with one additional predictor					
2	Anxiety	–0.001	0.99	0.006	0.834
3	Depression	0.003	1.03	0.006	0.643
4	Age	0.004	1.06	0.003	0.195
5	Woman	–0.131	0.88	0.101	0.192
6	PCS score	0.006	1.08	0.004	0.109
Model with PCS and interaction					
7	Pain intensity	0.236	1.66	0.041	<0.001
	PCS score	0.034	1.56	0.01	0.001
	PCS:pain intensity	–0.007	0.91 (PCS) 0.98 (pain intensity)	0.002	0.005
Model with PCS and interaction by sex					
8 Men	Pain intensity	0.32	2.03	0.07	<0.001
	PCS score	0.032	1.53	0.017	0.057
	PCS:pain intensity	–0.006	0.92 (PCS) 0.99 (pain intensity)	0.003	0.039
9 Women	Pain intensity	0.202	1.53	0.051	<0.001
	PCS score	0.035	1.57	0.013	0.008
	PCS:pain intensity	–0.005	0.94 (PCS) 0.99 (pain intensity)	0.002	0.032

Models were computed by using blocks of predictors in predicting opioid prescription. First, pain intensity was tested as an independent predictor. Next, psychologic and demographic factors were added. Third, pain intensity and PCS scores and an interaction between these variables were estimated for the entire sample, as well as separately in men and women.

OR = odds ratio; PCS = Pain Catastrophizing Scale.

catastrophizing independently yielded high odds ratios (1.66 and 1.56, respectively), but their interaction term, which introduced a greater degree of symmetric flexibility to the model, yielded odds ratios of about one (0.91 for PCS term, 0.98 for pain intensity term). Although the odds ratios were close to one, the *P* value of the interaction term was low ($P = 0.005$), indicating the presence of some significant variable overlap that merited more nuanced analysis.

Although we had found no sex differences in catastrophizing or opioid prescription status in our initial stages of analysis, differences in pain intensity between sexes prompted further analyses. The generalized linear models revealed pain intensity to be the best predictor for opioid prescription in both men and women. Although the overall model showed sex differences in the effect of pain catastrophizing on the prediction of opioid prescription, it also revealed the interaction between pain catastrophizing and pain intensity to have significant predictive effects in both sexes ($P = 0.039$ in men, $P = 0.032$ in women). Given that these relationships existed when we analyzed the entire sample as well as when we analyzed the sample by sex, we next aimed to characterize the potential mediators underlying these interactions.

Test of the Moderating Effect of Opioid Prescription on the Relationship between Pain Intensity and Pain Catastrophizing

Given the existence of a relationship between opioid prescription, pain intensity, and pain catastrophizing, we examined potential mediators of these linear associations. As seen in table 5, the direct effect of pain intensity on opioid prescription was greater than the mediated effect of PCS on the relationship between pain intensity and opioid prescription in both sexes. The nonsignificant *P* values indicated that PCS did not mediate, or explain, the relationship between

Table 5. Pain Intensity and Opioid Prescription by Sex and the Mediation Effect of PCS

	Total Effect	Direct Effect of Pain Intensity on Opioid Prescription	PCS Mediation of the Effect of Pain Intensity on Opioid Prescription	Proportion	*P* Value for Mediated Effect
Full sample	0.164	0.151	0.014	0.08	0.11
Men	0.218	0.217	0.001	0.005	0.95
Women	0.136	0.119	0.017	0.120	0.09

This analysis involved using pain intensity and PCS scores as predictors of opioid prescription. PCS scores were tested as a statistical mediator of the relationship between pain intensity and opioid prescription. Proportion column refers to proportion of direct effect of pain intensity on opioid prescription accounted for by PCS scores.

PCS = Pain Catastrophizing Scale.

opioid prescription and pain intensity evidenced in the linear modeling analysis. This lack of mediation suggested that pain catastrophizing had a moderating effect on the relationship between pain intensity and opioid prescription. In other words, pain catastrophizing strengthened, rather than explained, their linear relationship. Furthermore, mediation analysis also revealed that sex serves as a moderating variable.

To further elucidate potential moderators of the relationship between sex, opioid prescription, pain intensity, and pain catastrophizing, we used general additive modeling to visualize any complex nonlinear relationships. Additive modeling *P* values revealed significant effects of opioid prescription on the relationship between pain intensity and catastrophizing in the entire sample ($P < 0.001$). However, a nonlinear model as provided by general additive modeling did not fit the interactions between pain intensity, pain catastrophizing, and opioid prescription in women ($P = 0.005$) as well as it did in men ($P < 0.001$). This reveals that the relationship between these variables may be different for men and women patients, leading to the conclusion that sex moderates the relationship between pain intensity, catastrophizing, and opioid prescription.

Figure 2 (green represents lower density of patients with opioid prescription, and red represents high density of patients with opioid prescription) shows that, as expected, in both men and women, low pain intensity and pain catastrophizing were associated with patients without opioid prescription. However, moving past the lower left-hand corner of both graphs in figure 2, the graphs shows that in men, the greatest density of patients with opioid prescription is found in those with high pain intensity and low pain catastrophizing. Paradoxically, men with high pain intensities and high catastrophizing scores did *not* seem to have higher frequencies of opioid prescription. However, for women, opioid prescription was associated with both high pain intensity as well as high pain catastrophizing. Overall, there was a strong nonlinear relationship between pain intensity, pain catastrophizing, and opioid prescription in men, as seen by the horizontal gradations of increasing opioid prescription on increases in pain intensity. Given the increased density of women with opioid prescription who have high pain intensities and high levels of catastrophizing, there seems to be a more nuanced relationship between pain, pain catastrophizing, and opioid prescription in this group. Figures 3 and 4 represent these sex-dependent relationships.

Figure 3 reveals that men have a relatively flat association between opioid prescription and pain intensity, and the data for women suggest an inflection point on the numeric rating scale that emerges above pain intensity of 4 and persists until roughly pain intensity ratings of 7.

Figure 4 reveals that opioid prescription by sex diverges above scores of 10 on the PCS and similarly persists until the severe range of catastrophizing is reached, at which point the associations align for both sexes. Combined, figures 3 and 4 suggest that sensory and psychologic experience appear to associate more strongly with opioid prescription at lower levels (intensities) for women than for men.

Finally, to address concerns about model flexibility and cross-validation of our findings, we performed a bootstrapping on the general additive model fitting and the statistical inference using 500 replicates. All of the smoothed term *P* values in the bootstrap replicates were less than 0.05. In fact, the largest (least significant) *P* value was 0.0001. This suggests that the nonlinearity effect we described is highly robust.

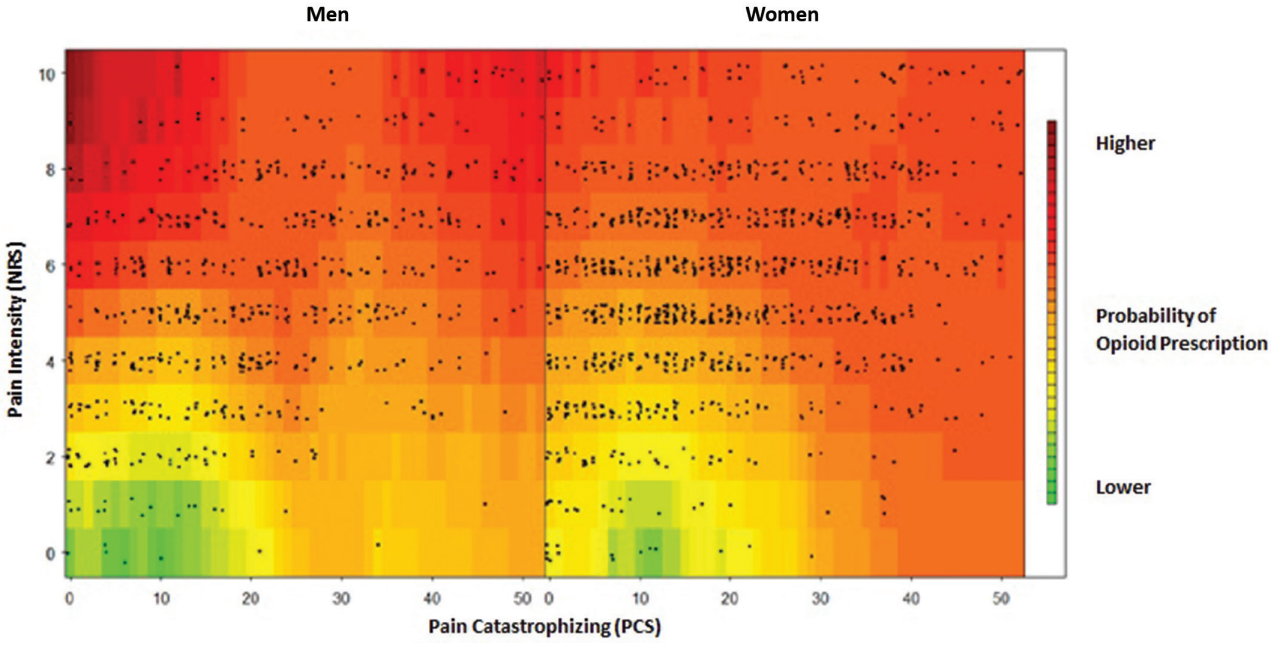

Fig. 2. Nonlinear relationship between pain intensity and pain catastrophizing, by sex. Points represent individual patients. NRS = numeric rating scale; PCS = Pain Catastrophizing Scale.

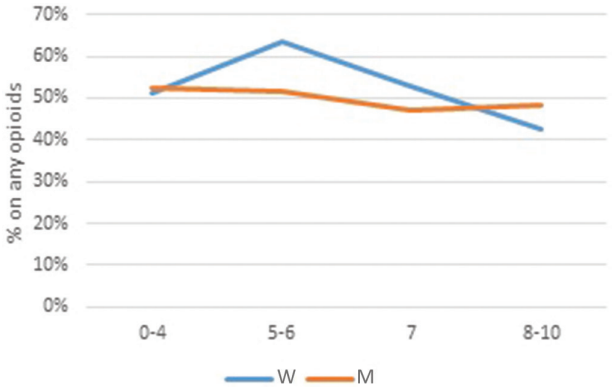

Fig. 3. Relationship between opioid prescription (*Y-axis*; % sample prescribed any opioids) and pain intensity quartile (*X-axis*; 0 to 10 pain intensity ratings) by sex. M = men; W = women.

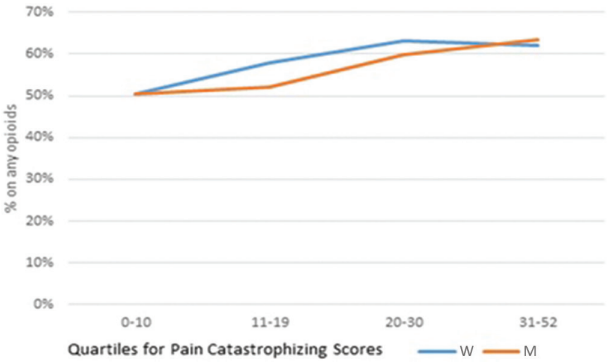

Fig. 4. Relationship between opioid prescription (*Y-axis*; % of sample prescribed any opioids) and quartiles of Pain Catastrophizing Scale scores (*X-axis*) by sex. M = men; W = women.

Discussion

In this study, we characterized the relationship between opioid prescription, pain intensity, and pain catastrophizing in 1,794 patients with chronic pain seeking initial medical evaluation at a multidisciplinary pain treatment center. Univariate analysis revealed that active opioid prescription was significantly associated with greater pain catastrophizing and higher pain intensity. Linear modeling revealed that (1) pain intensity was directly and significantly related to opioid prescription; (2) a significant interaction effect was found for pain catastrophizing and pain intensity on opioid prescription; and (3) this interaction effect remained significant yet differed by sex. Mediation analysis showed no mediating effects of pain catastrophizing or sex. In turn, catastrophizing and sex demonstrated moderating roles in the relationship between pain intensity and opioid prescription. Furthermore, additive modeling showed nuanced nonlinear relationships between the aforementioned variables in both men and women. Pain catastrophizing had greater association with opioid prescription in women. Although we could not make causal inferences due to the cross-sectional study design and opioid prescription being an outcome of previous clinic visit, nor would we be able to make these inferences had we identified any significant mediated effects in our analyses, our data reveal sex-based differences in the relationship between pain intensity, pain catastrophizing, and opioid prescription, highlighting the impact that catastrophizing may have in women with chronic pain.

Pain catastrophizing relates directly to pain intensity and serves to undermine pain treatment efficacy.[38] Similarly, pain intensity directly relates to opioid use.[39–41] Here, we show that opioid prescription is associated with greater pain catastrophizing. Notably, our univariate analysis of this large sample showed no significant sex differences in pain catastrophizing or opioid prescription. Previous studies have shown inconsistent relationships between sex and pain catastrophizing,[42–44] making it unclear whether these discrepancies are a result of smaller sample sizes.[30] Consistent with previous work, we found greater average pain intensity for women.[45]

The sex- and opioid status–based differences in pain and catastrophizing called for linear modeling to elucidate any underlying relationships between these variables. Our results showed that pain intensity was highly associated with the likelihood of having prescription opioids. However, adding pain catastrophizing and an interaction between catastrophizing and pain intensity demonstrated the strongest linear association with opioid prescription. Mediation analysis suggested that pain catastrophizing and sex served as moderating variables, strengthening the existing relationship between pain intensity and opioid prescription.

In addition to showing the moderating characteristics of pain catastrophizing and sex, we used additive modeling to show the impact of pain catastrophizing on opioid prescription by sex. For men, the greatest density of individuals with opioid prescription was colocated with lower pain catastrophizing and higher pain intensity. In women, however, the greatest density of those with opioid prescription was colocated with moderate-to-high pain intensities and pain catastrophizing levels, suggesting that pain catastrophizing has a stronger association with opioid prescription status. Given that opioids were prescribed before the study, causal inferences are impossible, but future studies may use prospective designs to confirm that these associations hold at the point of opioid prescription.

Our findings suggest that even relatively low levels of negative cognitive and emotional responses to pain may have a greater impact on opioid prescribing in women. Women may be more likely to influence provider prescribing patterns through behavioral cues during the medical visit; previous research has suggested women may engage in pain behavior for extended periods of time[46] and may appraise their pain as more threatening than men.[47] Although treatment for catastrophizing is important for both sexes, the higher additive modeling *P* values found for women suggests that risks are occurring at lower levels of catastrophizing for women than for men.

Additional studies are needed to replicate the associations we discovered. However, if confirmed, these findings would hold specific clinical relevance. Often, a pain catastrophizing scale score is considered clinically meaningful if it is near or more than 30.[27] However, given the moderating effect of pain catastrophizing and its possible predictive value for opioid prescription, treatments for pain catastrophizing may hold specific therapeutic value for women at levels considered clinically subthreshold for outpatients with chronic pain (*e.g.*, PCS less than 20). Others recently have demonstrated catastrophizing risk inflection points occurring at similarly low levels of catastrophizing for outpatient pain rehabilitation outcomes (PCS greater than 14)[48] and postsurgical pain (PCS greater than 13),[16] thereby suggesting the need for continued examination of how the field defines threshold for risk and treatment needs. We found no other studies to specifically report subthreshold associations with opioid prescription and sex differences therein.

Strengths and Limitations

Our study design involved single time-point data collection, which allowed for descriptive associations only, with no possibility for causal interpretations. Many of our variables, including about 40% of our opioid use data, were collected directly from medical records. For the 700 patients whose opioid use was collected manually from the electronic medical record, opioid dose was collected. After meticulous data cleaning and the calculation of opioid dose in morphine equivalents, opioid dosing information from the electronic medical records proved to be unreliable. Given that chart review was completed only for initial pain clinic visits, and given that not all physicians asked for nonpain-related medications, the accuracy of prescription and dosing of other medications, such as benzodiazepines, also was unreliable. We therefore structured our study to describe associations with opioid prescription and did not characterize relationships based on opioid dose or other medications.

Due to the deidentified nature of the data extracted from Pain-CHOIR, we were unable to attain the medical record numbers of all patients included in this study. For a third of the sample, we were able to locate medical record numbers by identifying exact data matches between our data set and that of Pain-CHOIR. Despite having only a small proportion of this sample's diagnostic codes, we believe that a comparison of these patients with the distribution of the entire pain clinic population is sufficient in characterizing the population, especially given that we did not use the diagnostic information for any significant analyses.

Despite the use of single time-point data, the electronic and easily accessible nature of the data allowed for the application of novel analytics on a large database, yielding more nuanced characterizations of the population. In addition to mediation analysis, we used highly flexible linear and nonlinear models, which together presented a novel battery of rigorous statistical tests that allowed for optimal characterization of the data.

Future Directions

Although our results are informative, the nature of some variables merits closer investigation. Given the limitation of using opioid prescription as a binary variable for measuring opioid use, future investigations should include opioid dose and possibly more reliable methods of opioid consumption quantification, such as a urine screen. Further investigation by pain condition or of other drugs that have shown relevance to opioid use, such as benzodiazepines, also is warranted.

Prospective, longitudinal studies also are needed to characterize patients at the point of opioid prescription. Moreover, despite our use of several important covariates, our analysis was not exhaustive. Consequently, there may be other variables (*e.g.*, pain sensitivity, pain-related disability, and pain interference) that are relevant to pain intensity, pain catastrophizing, and opioid use.

As a learning health care system,[25,26] Pain-CHOIR allowed for an inclusive range of pathology and patient characteristics that are not typical of most research studies or even registries that tend to be disease-specific. As such, it necessarily included wide ranges of patient characteristics and, more importantly, complex corelative relationships across the entire spectra of pathology. Although traditional linear model–based methods are valid and appropriate in studies with traditional data ascertainment, in all-comer learning health systems such as Pain-CHOIR, models with reduced restrictions about intervariable relationships should be more methodologically appropriate. In this work, we demonstrated that flexible models, such as additive models, can elucidate nonlinear relationships between several variables that are core to our field. In fact, these models suggest additional behavior-changing threshold effects that would be obscured in traditional methods. Although these methods are technically not new and have been in use in other fields of science, they have not seen wide use in the field of pain. Again, it is the all-encompassing nature of Pain-CHOIR that enables methods like this. Reciprocally, Pain-CHOIR and its clinical and research values are enabled by such methods.

Conclusions

This study used a large dataset of patients visiting a tertiary outpatient pain clinic. We elucidated relationships between sex, pain catastrophizing, pain intensity, and opioid prescription. Using an advanced analytic approach, we found a significant relationship between pain intensity and opioid prescription and found that this relationship was significantly stronger in women, especially those with high levels of pain catastrophizing. Despite similar levels of catastrophizing and opioid prescription among men and women, pain catastrophizing appears to have a stronger relationship with opioid status for women, calling for future studies to investigate lower pain catastrophizing thresholds for women and the potential impacts for reducing opioid prescription. These

results emphasize the importance of considering both obvious medical factors such as pain intensity and psychologic and demographic differences that may be salient predictors of the use of prescription opioids.

Research Support

The authors acknowledge funding support from the National Institutes of Health (NIH, Bethesda, Maryland), the National Center for Complementary and Integrative Health (NCCIH, Bethesda, Maryland) grant Nos. P01AT006651 (to Dr. Mackey) and P01AT006651S1 (to Drs. Mackey and Darnall); NCCIH grant No. R01 AT008561-01A1 (to Drs. Darnall and Mackey); NIH National Institute on Drug Abuse (NIDA, Bethesda, Maryland) grant Nos. K24DA029262 (to Dr. Mackey) and 3T32DA035165-02S1 (to Dr. Sturgeon); the NIH Pain Consortium grant No. HHSN271201200728P (to Dr. Mackey); and from the Chris Redlich Pain Research Endowment (to Dr. Mackey).

Competing Interests

The authors declare no competing interests.

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DNA Hydroxymethylation by Ten-eleven Translocation Methylcytosine Dioxygenase 1 and 3 Regulates Nociceptive Sensitization in a Chronic Inflammatory Pain Model

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ABSTRACT

Background: Ten-eleven translocation methylcytosine dioxygenase converts 5-methylcytosine in DNA to 5-hydroxymethylcytosine, which plays an important role in gene transcription. Although 5-hydroxymethylcytosine is enriched in mammalian neurons, its regulatory function in nociceptive information processing is unknown.

Methods: The global levels of 5-hydroxymethylcytosine and ten-eleven translocation methylcytosine dioxygenase were measured in spinal cords in mice treated with complete Freund's adjuvant. Immunoblotting, immunohistochemistry, and behavioral tests were used to explore the downstream ten-eleven translocation methylcytosine dioxygenase-dependent signaling pathway.

Results: Complete Freund's adjuvant-induced nociception increased the mean levels (± SD) of spinal 5-hydroxymethylcytosine (178 ± 34 *vs.* 100 ± 21; $P = 0.0019$), ten-eleven translocation methylcytosine dioxygenase-1 (0.52 ± 0.11 *vs.* 0.36 ± 0.064; $P = 0.0088$), and ten-eleven translocation methylcytosine dioxygenase-3 (0.61 ± 0.13 *vs.* 0.39 ± 0.08; $P = 0.0083$) compared with levels in control mice (n = 6/group). The knockdown of ten-eleven translocation methylcytosine dioxygenase-1 or ten-eleven translocation methylcytosine dioxygenase-3 alleviated thermal hyperalgesia and mechanical allodynia, whereas overexpression cytosinethem in naïve mice (n = 6/group). Down-regulation of spinal ten-eleven translocation methylcytosine dioxygenase-1 and ten-eleven translocation methylcytosine dioxygenase-3 also reversed the increases in Fos expression (123 ± 26 *vs.* 294 ± 6; $P = 0.0031$; and 140 ± 21 *vs.* 294 ± 60; $P = 0.0043$, respectively; n = 6/group), 5-hydroxymethylcytosine levels in the *Stat3* promoter (75 ± 16.1 *vs.* 156 ± 28.9; $P = 0.0043$; and 91 ± 19.1 *vs.* 156 ± 28.9; $P = 0.0066$, respectively; n = 5/group), and consequent *Stat3* expression (93 ± 19.6 *vs.* 137 ± 27.5; $P = 0.035$; and 72 ± 15.2 *vs.* 137 ± 27.5; $P = 0.0028$, respectively; n = 5/group) in complete Freund's adjuvant-treated mice.

Conclusions: This study reveals a novel epigenetic mechanism for ten-eleven translocation methylcytosine dioxygenase-1 and ten-eleven translocation methylcytosine dioxygenase-3 in the modulation of spinal nociceptive information *via* targeting of *Stat3*. **(Anesthesiology 2017; 127:147-63)**

EPIGENETIC modifications have been identified as essential mediators of chronic pain.[1] DNA methylation of cytosine (5mC) disrupts gene expression, and the 5mC-mediated dysregulation of pain-related genes occurs in different nociceptive pathways (including in the dorsal root ganglia, spinal cord, and brain) and in different types of pain models.[2–5] Inhibiting DNA methylation prevents and reverses pain behaviors, suggesting a potential role for DNA demethylation in pain.[2,3,6] A recent study found that complete Freund's adjuvant (CFA)-induced nociception decreases the 5mC level in cystathionine-β-synthetase without affecting the expressions of DNA methyltransferase 3a and 3b,

What We Already Know about This Topic

- Epigenetic changes serve to control gene expression and may contribute to the persistence of pain
- The methylation of DNA is one such epigenetic mechanism

What This Article Tells Us That Is New

- The knockdown of key DNA demethylating enzyme ten-eleven translocation methylcytosine dioxygenases (TET1, TET3) reduces nociceptive sensitization induced by inflammation
- The effects of TET1/TET3 knockdown may result from alterations in spinal signal transducer and activator of transcription 3 expression

Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site (www.anesthesiology.org). Z.P., Z.-Y.X., and G.-F.L. contributed equally to this article.

Submitted for publication August 4, 2016. Accepted for publication March 2, 2017. From Jiangsu Province Key Laboratory of Anesthesiology (Z.P., Z.-Y.X., G.-F.L., M.-L.S., M.Z., L.-Y.H., Q.-Q.T., L.-J.Z., J.-L.C.) and Jiangsu Province Key Laboratory of Anesthesia and Analgesia Application Technology (Z.P., Z.-Y.X., G.-F.L., M.-L.S., M.Z., L.-Y.H., Q.-Q.T., L.-J.Z., J.-L.C.), Xuzhou Medical University, Xuzhou; and Department of Anesthesiology, The Affiliated Hospital of Xuzhou Medical University, Xuzhou (J.-L.C.), China.

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two major enzymes for *de novo* DNA methylation,[7] suggesting an underlying active mechanism in the demethylation of aberrantly expressed genes in response to chronic pain. The functional role of DNA demethylation in nociceptive information processing, however, remains poorly understood.

Recent reports have verified that all three of the known ten-eleven translocation methylcytosine dioxygenases (TET1, TET2, and TET3) act as intermediaries between 5mC and 5-hydroxymethylcytosine (5hmC) in different mammalian cells.[8,9] Increasing numbers of studies reveal that TET and 5hmC are proficiently enriched at transcription start sites and CpG sites in gene bodies.[10] Furthermore, the distribution of 5hmC affects transcription efficiency by altering chromatin structure or recruiting/excluding DNA-binding proteins. Several studies have identified an enrichment of 5hmC within mammalian neurons. For example, the 5hmC content in brain cells is approximately 10-fold that in embryonic stem cells, suggesting 5hmC may be a stable epigenetic modification for modulating gene expression in the central nervous system (CNS).[9,11,12] Accordingly, TET participates in neuronal activity in learning and memory[13–15] and in CNS-related diseases, such as Alzheimer disease,[16] drug addiction,[17] and inflammation,[18] by altering 5hmC distribution patterns. Mice lacking *Tet1* exhibit hypermethylation of the *Npas4* promoter and reduced expression of *Npas4* and downstream genes, resulting in hippocampal long-term depression and impaired memory extinction.[15] *Tet1* overexpression or mutation in the dorsal hippocampus specifically impairs long-term memory formation.[13] Our previous findings showed that TET1- and TET3-mediated hydroxymethylation of micro(mi)RNA-365-3p regulates the nociceptive behavior induced by formalin *via Kcnh2*.[19] Despite the fact that manipulating the 5hmC content *via* TETs is a potential therapeutic tool for CNS-related diseases, the role of TET-mediated hydroxymethylation in the aberrant expression of genes has not been explored in chronic pain.

Signal transducer and activator of transcription 3 (STAT3) is a key regulator in neurophysiology and neuropathology and is implicated in pain hypersensitivity.[20–22] Spinal nerve ligation or bilateral chronic constriction increases STAT3 expression in the spinal cords of rats, and intrathecal injections of the STAT3 inhibitors AG490 and WP1066 reduced the established hyperalgesia.[20,21] Although this evidence links STAT3 with nociceptive sensitization, little is known about how the expression of *Stat3* is regulated in nociceptive information processing.

In this study, we found significant increases in the levels of 5hmC, TET1, and TET3 in mice spinal cords in a CFA-induced inflammation pain model that mimics nociceptive sensitization in patients with inflammatory pain. Moreover, the level of 5hmC in the *Stat3* promoter was up-regulated. Thus, we hypothesized that the TET-mediated increase of *Stat3* 5hmC in spinal cord contributed to nociceptive information processing in the development of chronic inflammatory pain. Here, we show that TET1 and TET3 regulate *Stat3* expression as a novel epigenetic mechanism in nociceptive information processing in a CFA-induced inflammation pain model.

Materials and Methods

Animals, Pain Model, and Behavior Testing

Adult male Shanghai populations of Kunming mice (20 to 25 g) were used in this study. For each experiment, the animals were randomized to either a control or an experimental group. Nociceptive sensitization was induced by subcutaneous administration of CFA (40 μl; F5881; Sigma-Aldrich, USA) into the plantar surface of the hind paw. A 0.9% saline solution was used as a control for CFA. Paw withdrawal latency to a thermal stimulus and paw withdrawal thresholds to a mechanical stimulus were used to measure hyperalgesia and allodynia, respectively. Before nociceptive behavior testing, mice were acclimatized to the environment for 1 h. Thermal hyperalgesia was measured by focusing a beam of light on the plantar surface of the hind paw to generate heat, and the time required for the stimulus to elicit a withdraw of the hind paw was recorded. The radiant heat intensity was adjusted to obtain basal paw withdrawal latency of 11 to 14 s. An automatic 20-s cutoff was used to prevent tissue damage. Thermal stimuli were delivered three times to each hind paw at 5-min intervals. Mechanical allodynia was assessed with the use of von Frey filaments, starting with a 0.31-g and ending with a 5.0-g filament. The filaments were presented, in ascending order of strength, perpendicular to the plantar surface with sufficient force to cause slight bending against the paw. A brisk withdrawal or flinching of the paw was considered a positive response. In climbing tests, a 0.5-mm-diameter metal wire mesh with a 5-mm-wide grid was placed vertically 30 cm above the table. Each mouse started at the bottom of the mesh with its head facing downward. After the mouse was released, the time to climb all the way to the top was recorded.[23] Behavioral testing was performed in a double-blind trial fashion. All animal procedures were approved by the animal care committee of Xuzhou Medical University (Xuzhou, China).

Sample Collection

Blood samples were collected from the facial veins of the mice. To summarize in brief, mice were picked up firmly by the scruff *via* the thumb and first finger, and the hairless freckle on the side of the jaw was pricked with a needle. A 200-μl sample of blood was collected from each mouse and placed in tubes containing EDTA anticoagulant for storage at −80°C. For harvesting tissue, mice were anesthetized with 10% chloral hydrate, and the spinal cord within the lumbar segments (L3–L5) was removed rapidly. The dorsal spinal cord ipsilateral to CFA injections was separated for subsequent analyses. Peripheral blood was obtained after the eyeballs were removed and snap-frozen in liquid nitrogen before they were stored at −80°C.

DNA Dot-Blot

Genomic DNA was extracted from tissue and blood samples with a QIAamp DNA mini kit (51306; QIAGEN,

USA) and DNA blood quick extraction kit (B5183; Shanghai Sangon Biotech, China), respectively. Global 5hmC dot-blot analysis was performed as described previously.[24] Genomic DNA was denatured in Tris-EDTA buffer for 10 min at 95°C and immediately chilled on ice for 5 min. One hundred nanograms DNA from each sample were spotted on a positively charged nylon membrane. The membrane was baked for 2 h at 80°C, ultraviolet cross-linked (254 nm) for 10 min, and then blocked with 5% nonfat milk for 1.5 h at room temperature. The membrane was incubated with a primary rabbit anti-5hmC antibody (1:10,000; 39,999; Active Motif, USA) at 4°C overnight. After it was incubated with a peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) secondary antibody at room temperature for 1 h, the signal was visualized with an enhanced chemiluminescence substrate according to the manufacturer's instructions (345818; Millipore, USA). Equal DNA loading amounts were verified by staining the membranes with 0.2% methylene blue. The reference DNA fragments containing 5hmC were used as the positive standard. The dot-blot densities were analyzed with Image J software (USA).

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed by the use of a ChIP assay kit (17 to 295; Millipore) according to the manufacturer's instructions. In brief, 1% formaldehyde was added to 40 mg spinal cord tissue for 15 min at room temperature, and the cross-linking was terminated by adding glycine (0.125 M final concentration). Samples were centrifuged, and the supernatants were removed, washed twice in cold phosphate-buffered saline (PBS) containing protease inhibitor cocktail II, and homogenized on ice. The samples were again centrifuged, and the supernatants were lysed with sodium dodecyl sulfate (SDS) lysis buffer containing protease inhibitor cocktail II for 10 min on ice, were sonicated on ice (five 10-s pulses with 30-s rest intervals) using an ultrasonic instrument in a soundproof box (Diagenode s.a., Belgium), were centrifuged once again, and the precipitate was removed. Samples were diluted 10-fold and precleared at 4°C for 60 min with protein G agarose beads. The samples immunoprecipitated for 12 h on a rotating platform at 4°C, and 10% of the supernatants containing proteins and chromatin complex were saved as the input control, whereas the remainders were immunoprecipitated at 4°C overnight with IgG (PP64; Millipore), TET1 (1:80; 61,443; Active Motif), and TET3 (sc-139186; Santa Cruz Biotechnology, USA) antibodies. Antibody-bound DNA–protein complexes were collected with protein G agarose beads and were washed and eluted from the beads according to the manufacturer's instructions. Cross-linking of DNA–protein was reversed by incubating at 65°C for 4 h. The resulting DNA was cleaned with a QIAquick PCR purification kit (28106; QIAGEN) according to the manufacturer's instructions before quantitative polymerase chain reaction (qPCR) analyses. We used SCf and SCr primers to amplify ChIP DNA and input DNA (see table S1, Supplemental Digital Content 1, http://links.lww.com/ALN/B419). ChIP PCR products were normalized to input products amplified using genomic DNA.

Sequencing of 5hmC- and 5mC-enriched Genomic DNA

The capture, sequencing, and analyses for the regions of DNA enriched in 5hmC and 5mC were carried out in accordance with the Illumina sequencing kit. Genomic DNA was sonicated to achieve a 200- to 900-base pair (bp) size with a Covaris instrument, and 800 ng of the fragmented sample was end-repaired, A-tailed, and ligated to single-end genomic adapters with a Genomic DNA sample kit (FC-102-1002; Illumina, USA) according to the manufacturer's instructions. The ligated DNA fragments of 300 to 1,000 bp in size were separated by agarose gel electrophoresis. The DNA was heat-denatured at 94°C for 10 min, rapidly cooled on ice, and immunoprecipitated with 1 μl monoclonal anti-5mC (C15200081; Diagenode) or anti-5hmC (C15200200; Diagenode) antibodies in 400-μl immunoprecipitation buffer (0.5% bovine serum albumin in PBS) overnight at 4°C with rocking agitation. To recover the immunoprecipitated DNA fragments, 20 μl magnetic beads (Life Technologies, Inc., USA) were added and incubated for an additional 2 h at 4°C with agitation.

Five washes were performed with ice-cold immunoprecipitation buffer. A nonspecific mouse IgG immunoprecipitation was performed in parallel with methylated or hydroxymethylated DNA immunoprecipitation as a negative control. Washed beads were resuspended in Tris-EDTA buffer with 0.25% SDS and 0.25 mg/ml proteinase K for 2 h at 65°C and were then allowed to cool to room temperature. Methylated DNA immunoprecipitates or hydroxymethylated DNA immunoprecipitates (hMeDIP) and supernatant DNA were purified with the use of QIAGEN MinElute columns and eluted in 16 μl elution buffer. The pull-down DNA or input DNA was used for preparing sequencing libraries. The libraries were generated in accordance with the Illumina protocol for Preparing Samples for ChIP Sequencing of DNA (111257047; Rev. A) with 25 ng 5mC- or 5hmC-captured DNA. Fourteen cycles of PCR were performed on 5 μl immunoprecipitated DNA with the single-end Illumina PCR primers. The resulting reactions were purified with QIAGEN MinElute columns, after which a final size selection (300 to 1,000 bp) was performed by electrophoresis in 2% agarose. PCR-amplified DNA libraries were quality controlled with an Agilent 2100 Bioanalyzer (Agilent, USA) and finally diluted in elution buffer to 5 ng/μl. Then, 1 μl was used in real-time PCRs to confirm enrichment for the hydroxymethylated region. The library was denatured with 0.1 M NaOH to generate single-stranded DNA molecules, was loaded onto channels of the flow cell at an 8-pM concentration, and amplified *in situ* with the TruSeq rapid PE cluster kit (PE-402-4001; Illumina) to generate the sequencing

cluster. Then, 100 sequencing cycles were carried out with the TruSeq SBS kit v5 (FC-104–5001; Illumina) protocol on the Illumina HiSeq 2000. Image analysis and base calling were performed using Off-Line Basecaller software (OLB V1.8 in HiSeq 2000 sequencer; Illumina). After passing through the Solexa CHASTITY quality filter, the clean reads were aligned to the mouse genome (UCSC MM10) using BOWTIE software (V2.1.0). A hydroxymethylation score for any region in the genome was defined as the number of fragments per kilobase.[25] In brief, the concordantly mapped 100-bp fragments were represented by extending each mapped read to 480 bp in length. We used 50-bp resolution intervals (50-bp bins) to partition the stacked fragments region and counted the number of fragments in each bin. The fragment counts in each bin determine the DNA hydroxymethylation signal of that bin. Two biologic replicates were performed for each condition.

Quantification of 5mC and 5hmC

The bisulfite conversion method for 5mC quantification was performed as described previously.[2] To summarize, genomic DNA was subjected to bisulfite conversion with an EZ DNA methylation-gold kit (D5005; Zymo, USA). Bisulfite-modified DNAs were amplified using BS primers listed in table S1. PCR products were ligated to a vector with a TA cloning kit (SK2214; Shanghai Sangon Biotech), and we quantitatively analyzed the 5mC levels after Sanger sequencing. 5hmC quantification was determined with an EpiMark 5hmC assay kit (E3317S; NEB, USA). An outline of the assay and sample calculations can be seen in the manufacturer's instructions. In brief, DNA is treated with T4 β-glucosyltransferase, which specifically glucosylates 5-hydroxymethyl cytosine to yield β-glucosyl-5hmC (5ghmC). Whereas *Msp*I cleaves recognition sequences containing 5mC ($C^{5m}CGG$) or 5hmC ($C^{5hm}CGG$), the cleavage is blocked by 5ghmC ($C^{5ghm}CGG$). qPCR is used to determine the amount of DNA template cut by *Msp*I before and after treatment with T4 β-glucosyltransferase, enabling an estimation of the 5hmC level at the *Msp*I site. The 5hmC level of the Fos promoter region was measured using 5hmC-enriched genomic DNA. The primer sequences we used are listed in table S1.

Real-time qPCR

Total RNA was isolated with a Trizol reagent (15596-026; Invitrogen, USA) to generate cDNA templates by reverse transcription reactions with oligo $(dT)_{18}$ and reverse transcriptase M-MLV (2641A; Takara Bio, Japan) at 42°C for 60 min. cDNA products were used as templates to detect *Tet1*, *Tet2*, *Tet3*, and *Stat3* expression *via* real-time qPCR (RT-qPCR) with SYBR Premix Ex *Taq*II (RR820A; Takara Bio) according to the manufacturer's instructions. RT-qPCR primers are listed in table S1. Reactions were performed in triplicate. The expression levels of the target genes were quantified relative to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) expression (cycle threshold, C_T) using the $2^{-\Delta\Delta CT}$ method, as described previously,[26] where ΔC_T was the difference in C_T values derived from the detected sample and *Gapdh* control, and $\Delta\Delta C_T$ represented the difference between paired samples calculated by subtracting the sample ΔC_T from the control ΔC_T. Any value among triplicates that had a marked difference (≥1.00) compared with the average of the other two was omitted.

Single-cell RT-PCR

Single-cell RT-PCR for spinal neurons was performed as described previously.[27] In brief, the contents of dissociated spinal neurons from CFA mice were harvested into patch pipettes with tip diameters of ~35 μm, placed gently into reaction tubes with Dnase I at 37°C for 30 min, and heated to 80°C for 5 min to remove genomic DNA. Reverse transcriptase (SuperScript III Platinum; Invitrogen) was added, the sample was incubated at 50°C for 50 min, and the reaction was terminated at 70°C for 15 min. The cDNA products were used in gene-specific nested PCR. The primers are shown in table S1. The first-round PCR was performed with the outer primer pair in the FastStart universal SYBR green master kit (Roche, Switzerland). PCR conditions were as follows: 1 cycle of 3 min at 94°C; 35 cycles of 15 s at 95°C and 15 s and 60°C, and 1 cycle of 10 min at 72°C. The second round of PCR was carried out using 0.5 μl of the first PCR product as the template and with inner PCR primers. The amplification reagents and procedure were the same as those of the first round. A negative control was obtained from pipettes that were submerged in the bath solution only. *Gapdh* was used as the reference gene.

Plasmid Construction

All constructs were produced by the use of standard molecular methods and confirmed by DNA sequencing. To construct TET1 and TET3 knockdown vectors, pLVTHM was digested with *Mlu*I (R0198S; NEB) and *Cla*I (R0197S; NEB) and then ligated with the double-strand sif1/sir1 (Lenti-T1-siRNA) and sif3/sir3 (Lenti-T3-siRNA) oligos, respectively (see table S1). To construct TET1 and TET3 overexpression vectors, Gibson DNA Assembly reactions (E5510S; NEB) were used to generate Lenti-T1 and Lenti-T3 constructs according to the manufacturer's instructions. In brief, three *Tet1* or four *Tet3* overlapping inserts were prepared by PCR with the PCR primers listed in table S1. Gibson DNA Assembly reactions containing 100 ng of each insert and 50 ng of the pWPXL vector digested with *Bam*HI (R0136L; NEB) were carried out at 50°C for 45 min.

To construct the promoter reporter vectors, a defined region of the *Stat3* promoter was amplified from mouse genomic DNA using S3-P6F1/S3-P6R1 (products with transcription start site [TSS]) and S3-P6F2/S3-P6R2 (without TSS) primer pairs. The products were then cloned into a pGL6 plasmid *via Xho*I (R0146S; NEB) and *Hind*III (R3104S; NEB) digestion to produce pGL6-Stat3-TSS and pGL6-Stat3.

Lentivirus Production and Infection

HEK 293T cells were cultured in Dulbecco modified Eagle medium with 10% fetal bovine serum (FBS). The cells were cotransfected with plasmid constructs and 16 μg of the core plasmid, 12 μg PSPAX2, and 4.8 μg PMD2G envelope plasmid in 6-well plates with Lipofectamine® 2000 (ThermoFisher Scientific, USA) according to the manufacturer's instructions (11668-027; Invitrogen). The viral supernatant was collected 48 h after transfection and concentrated with a Centricon Plus-70 filter unit (UFC910096; Millipore). Lentivirus titers were measured at $>10^8$ transduction units/ml. The assays for *in vitro* and *in vivo* lentivirus infection were performed according to the method described previously.[2] To summarize, 20 μl lentivirus and 1.5 μl polybrene (1.4 μg/μl; H9268; Sigma-Aldrich) were added in a 24-well plate containing 1×10^5 HEK 293T cells and 400 μl Dulbecco modified Eagle medium without FBS. After 24 h, the transfection medium was replaced with 500 μl fresh complete medium containing 10% FBS. Cells were collected after 48 h in culture.

Short-interfering RNA and Lenti–short-interfering RNA Delivery

Injections were performed by holding the mouse firmly by the pelvic girdle and inserting a 30-gauge needle attached to a 25-μl microsyringe between L5 and L6 vertebrae. Proper insertion of the needle into the subarachnoid space was verified by a slight flick of the tail after a sudden advancement of the needle. Injections of 5 μl of 20 μM short-interfering (si)RNAs for *Tet1*, *Tet3*, and *Stat3* or 1 μl Lenti-T1-siRNA or Lenti-T3-siRNA were performed daily for 3 days in a double-blind trial fashion. Knockdown *via Tet1*-siRNA, Lenti-T1-siRNA, *Tet3*-siRNA, Lenti-T3-siRNA, and *Stat3*-siRNA was confirmed with RT-qPCR from samples of the ipsilateral dorsal spinal cord taken 72 h after the last injection. Animals receiving intrathecal injections of scrambled siRNA or an empty vector were used as control groups. The siRNA sequences and siRNA-vector construction sequences are listed in table S1.

Methylation and Hydroxymethylation of the Promoter Reporter

CpGs (5C group) in pGL6-Stat3-TSS or pGL6-Stat3 were methylated with the use of CpG methyltransferase (M0226S; NEB) at 37°C for 1 h and then purified with a Wizard Plus SV minipreps DNA purification system (A1330; Promega, USA) according to the manufacturer's instructions. The methylated CpGs (5mC group) in reporter plasmids were hydroxymethylated (5hmC group) with recombinant TET1 protein (31363; Active Motif) in 50 mM HEPES (pH 8) with 50 μM $Fe(NH4)_2(SO4)_2$, 2 mM ascorbate, and 1 mM α-ketoglutarate for 3 h at 37°C. An empty pGL6 vector was used as the negative control, and the pRL-TK plasmid was used as an internal control (Promega).

Luciferase Reporter Assay

HEK 293T cells were seeded at 1×10^5 cells per well of a 24-well plate. Cells were transfected with 200 ng of the methylated and the hydroxymethylated pGL6-Stat3-TSS, pGL6-Stat3, empty pGL6, or control pRL-TK vectors with Lipofectamine® 2000 (11668-027; Invitrogen). Cell lysates were prepared and subjected to luciferase assays by the use of the Dual-Luciferase® reporter kit (Promega) 48 h after transfection.

Immunohistochemistry

Spinal cords were dissected rapidly from mice perfused with 4% formaldehyde and postfixed in the same solution, cryoprotected in 30% sucrose, and then sectioned into 35-μm slices. Slices were blocked with 10% bovine serum albumin. For 5hmC immunofluorescence staining, an additional process was performed: the sections were treated with 1 M HCl at 37°C for 3 min followed by blocking with 5% FBS in PBS as described previously.[28] The treated slices were incubated with anti-5hmC (1:2,000; 39999; Active Motif), anti-TET1 (1:200; 61741; Active Motif), anti-TET3 (1:200; 61743; Active Motif), anti-IBA1 (1:500: 19-19741; Wako, USA), anti-GFAP (1:300; 3670; Cell Signaling, USA), or anti-NeuN (MAB377; Millipore) antibodies at 4°C overnight. Sections were then washed twice in 0.4% Triton X-100 in PBS at room temperature for 10 min, incubated with fluorescent-conjugated secondary antibodies (AB10113, AB10081, or AB10053; Shanghai Sangon Biotech) at room temperature for 60 min, washed twice, and then sealed after drying. Fos immunohistochemistry was performed as described previously.[2] In brief, a series of 30-μm transverse sections were cut on a cryostat and stored in PBS. After they were washed in PBS, the tissue sections were incubated in PBS containing 5% normal goat serum and 0.3% Triton X-100 at room temperature for 30 min. The sections were then incubated in primary rabbit anti-Fos antibody (1:1,000; Santa Cruz Biotechnology) at 4°C for 48 h. The sections were then incubated in biotinylated goat anti-rabbit antibody (1:200) at 37°C for 1 h and with an avidin–biotin–peroxidase complex (1:100; Vector Labs, USA) at 37°C for 2 h. Finally, sections were treated with 0.05% diaminobenzidine for 5 to 10 min. Sections were rinsed in PBS to stop the reaction, mounted on gelatin-coated slides, air dried, dehydrated with 70 to 100% alcohol, cleared with xylene, and coverslipped for microscopic examination. For analyzing changes in Fos expression, we examined five L3–L5 spinal cord sections per animal, selecting the sections with the greatest number of positive neurons. For each animal, we recorded the total number of positive neurons in bilateral I–V and X laminae of the spinal cord. All positive neurons were counted without considering the intensity of the staining. Immunostaining images were acquired with a confocal microscope (FluoView FV1000; Olympus, Japan). Immunohistochemistry slides were analyzed *via* bright field on a Nikon Eclipse E600 microscope, and images were

obtained with a Nikon Digital Sight camera (DS-Fi1) and NIS Elements (Nikon Instruments, Japan).

Western Blot Analysis

Proteins (20 to 50 μg/sample) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Due to the large difference in molecular weights between the target protein and internal reference protein, they were separated from blot membranes and incubated simutaneously at 4°C overnight in the corresponding antibodies against: TET1 (1:100; 61,443; Active Motif), TET2 (1:100; sc-136926; Santa Cruz Biotechnology), TET3 (1:100; sc-139186; Santa Cruz Biotechnology), and STAT3 (1:1,000; Santa Cruz Biotechnology) or control β-actin (1:1,000; TA-09; ZSGB-Bio, China). The membranes were then washed twice in tris-buffered saline with Tween-20 at room temperature for 10 min, incubated with anti-rabbit IgG secondary antibodies (1:1000; A0208; Beyotime, China) at room temperature for 1 h, and washed twice again in tris-buffered saline with Tween-20 at room temperature for 10 min. The immune complexes were detected with an NBT/BCIP (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate) assay kit (72091; Sigma-Aldrich). Band analyses were performed in ImageJ software, with the intensities of the target signals normalized to those of β-actin for statistical analyses.

Statistical Analysis

On the basis of previous experience,[29] we used five to six mice per group for each experiment. Data are presented as mean values ± SD. The results from behavioral testing, luciferase reporter assay, 5hmC levels after TET knockdown or overexpression, 5hmC, protein, DNA dot-blot, Fos immunohistochemistry staining, qRT-PCR, and climbing test were analyzed statistically with a one-way or two-way ANOVA or paired or unpaired Student's *t* test. When ANOVA showed a significant difference, pairwise comparisons between means were tested by the *post hoc* Tukey method. Statistical analyses were performed with Prism (GraphPad 5.00, USA). $P < 0.05$ was considered statistically significant.

Results

CFA-induced Nociception Increases Spinal 5hmC Levels

5hmC has been identified as a novel epigenetic modification in mammals, and its change becomes an epigenetic feature in CNS diseases.[28] As the spinal cord plays critical roles in transducing and transmitting pain-related gene signaling, we explored the potential modulatory role of spinal 5hmC in nociceptive sensitization. We first examined the changes in total levels of spinal 5hmC in CFA-induced nociceptive sensitization. Dot-blot results revealed that the total 5hmC content increased to the highest level at 3 days (178 ± 34 *vs.* 100 ± 21; $P = 0.0019$) during the observed 14 days after CFA injection (fig. 1A), suggesting CFA-induced nociception increased the total level of spinal 5hmC, which is dynamically regulated in a time-dependent manner. The strong correlation of epigenetic marks between spinal cord and blood may be useful for diagnostic and therapeutic applications in chronic pain. Therefore, we asked whether changes in 5hmC in whole blood are similar to those in the spinal cord underlying CFA-induced nociceptive sensitization. We found that blood 5hmC was markedly increased from 1 to 5 days (all $P < 0.02$), reached a peak value at day 5 (615 ± 106 *vs.*

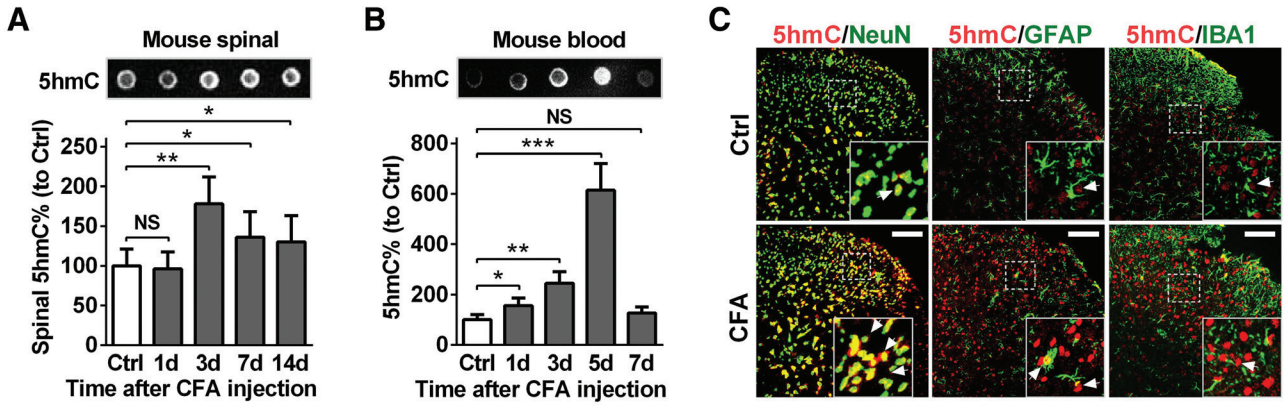

Fig. 1. Complete Freund's adjuvant (CFA)-induced nociceptive sensitization increases global 5-hydroxymethylcytosine (5hmC) levels in spinal cords. (*A*) Time course of dot-blot assay for spinal 5hmC from 1, 3, 7, and 14 days after CFA injection. Spinal DNA samples from 1, 3, 7, and 14 days after CFA injection were dot-blotted with a 5hmC-specific antibody; n = 6 mice at each time; one-way ANOVA (expression *vs.* time point) followed by *post hoc* Tukey test, F_{time} (4, 25) = 41.48, *$P < 0.05$, **$P < 0.01$. (*B*) Time course of dot-blot assay from mouse peripheral blood for 5hmC from 1, 3, 5, and 7 days after CFA injection; n = 6 mice at each time; one-way ANOVA (expression *vs.* time point) followed by *post hoc* Tukey test, F_{time} (4, 25) = 123. 3, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (*C*) Combined 5hmC (*red*) and NeuN, GFAP, or IBA1 (*green*) immunofluorescence staining in ipsilateral spinal cord 3 days after CFA or saline injection. *Scale bar*, 25 μm. *Arrows* indicate the positive 5hmC signal. Acquisition parameters of images: laser intensity, 45% for 488 (*green*) and 50% for 543 (*red*); detector voltage (high voltage), 550 for *green* and 600 for *red*; gain, 0 for *green* and 0 for *red*; offset, 53% for *green* and 72% for *red*; scanning speed, 20.0 μs/pixel. Ctrl = control; NS = not significant.

100 ± 20; $P = 0.00035$), and then returned almost to the basal level at 7 days after CFA injection (127 ± 24 *vs.* 100 ± 20; $P = 0.096$; fig. 1B), indicating the gain of 5hmC in mouse spinal cord and blood is a novel epigenetic feature in the nociceptive information processing.

To further explore the mechanism underlying the regulation of spinal 5hmC during nociceptive information processing, we investigated the genomic characteristics of changes in spinal 5hmC at 3 days after CFA injection. First, we used immunofluorescence staining to verify the increase in global spinal 5hmC in mice treated with CFA (fig. 1C) compared with that of the control group. Immunofluorescence staining showed an ~85.4% overlap between the 5hmC signal and NeuN (a neuron marker) in the control group and 90% overlap in the CFA group, whereas 5hmC signals rarely overlapped with GFAP (an astrocyte marker) and IBA1 (a microglial marker) in both groups (fig. 1C), suggesting that 5hmC may occur in the mouse spinal neurons. Then, we carried out a genome-scale evaluation of spinal 5hmC distribution at 3 days after CFA injection using a genome DNA immunoprecipitation sequencing method with an anti-5hmC antibody (hMeDIP-Seq). Before sequencing, the specificities of anti-5hmC and anti-5mC antibodies were evaluated against a negative control IgG antibody. We determined that DNAs were pulled down by anti-5hmC and anti-5mC antibodies but not by IgG *via* gel staining and NanoDrop2000 spectrophotometry, indicating that anti-5hmC and anti-5mC antibodies are specific for hMeDIP. In the subsequent sequencing, the means of 40.1 million and 35.5 million reads were obtained from the spinal cords of control and CFA groups, respectively. When the reads from the two groups were mapped to a mouse reference genome and filtered out, 20.9 million and 18.6 million uniquely mapped reads, respectively, remained that were used for subsequent analysis (data not shown). Genome-scale densities of 5hmC reads from each sample were determined in an Integrated Genomics Viewer browser (Broad Institute, USA). Global 5hmC density in CFA mice differed from that of control animals due to obvious alterations of 5hmC at numerous gene loci (fig. 2A). The normalized spinal 5hmC read densities across the transcript units of all the reference genes in the control and CFA groups showed different features and genome-wide coverages (see fig. S1A, Supplemental Digital Content 2, http://links.lww.com/ALN/B420). In addition, the clustering analysis clearly showed distinctive patterns of specific 5hmC enrichment between control and CFA-treated mice (see fig. S1B, Supplemental Digital Content 2, http://links.lww.com/ALN/B420).

Because cytosine methylation of CpG dinucleotides frequently leads to transcriptional silencing, CpG islands have been recognized as a crucial regulation region in DNA methylation.[2] To systematically identify the downstream genes

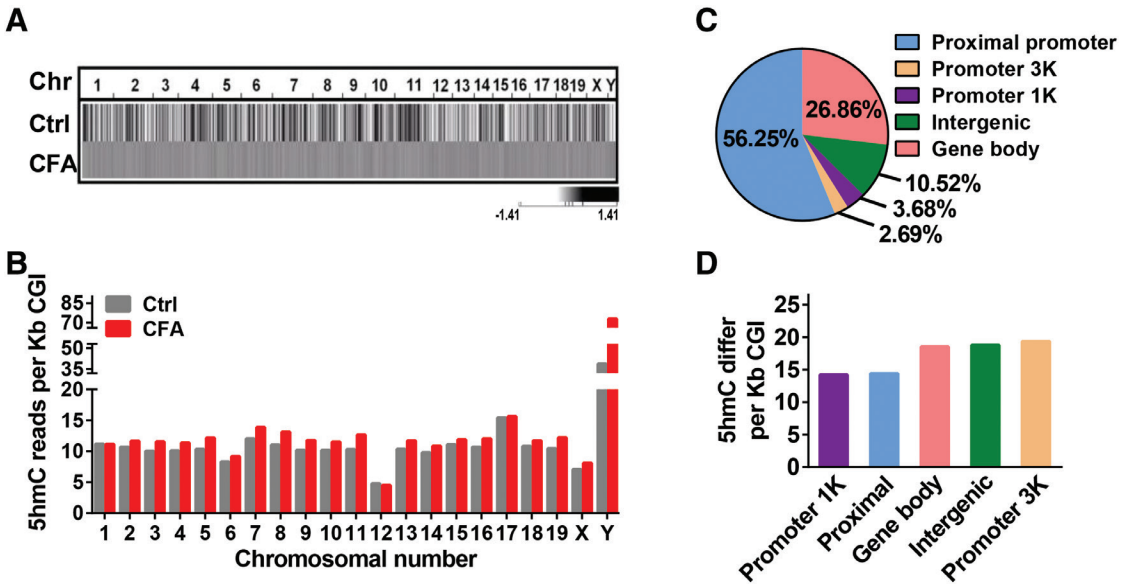

Fig. 2. Distribution of 5-hydroxymethylcytosine (5hmC)-enriched CGI in spinal cords of saline- and complete Freund's adjuvant (CFA)-treated mice. (*A*) Genome scale of 5hmC profiles and enrichment in CFA-induced nociceptive sensitization; the heatmap represents the read densities, which have been scaled equally and then normalized, based on the total numbers of mapped reads per sample. (*B*) Histogram of 5hmC peak counts (in numbers per kilobase CGI sequence) of each chromosome in control and CFA group mice. (*C*) Genomic distribution of sites that show CFA-induced changes in 5hmC enrichment in CGI regions. Regions with more than a twofold change in 5hmC. (*D*) 5hmC differential region density is presented as the number of CFA-induced differential peaks per kilobase sequence. Proximal promoter presents islands starting 250 bp upstream of RefGene transcription start site (TSS) and ends 300 bp downstream (from TSS –250 to TSS +300). Promoter 1K, from TSS –1000 to TSS –250. Promoter 3K, from TSS –3000 to TSS –1000. Gene body, from TSS +300 to transcription end site (TES) –300. Intergenic, from TES –300 bp to neighboring RefGene TSS –1000. CGI = CpG island; Chr = chromosome; Ctrl = control.

regulated by TET1 and TET3, we performed a genome-wide analysis for spinal 5hmC density of CpG island (CGI) in individual genes. In total, we identified 11,990 5hmC peak regions from spinal cords of mice in the control group and 11,844 peaks from the CFA group. Although the gross chromosomal distributions of 5hmC peak regions were equivalent between control and CFA groups, we found that 12 chromosomes had relatively low distributions, but the Y sex chromosome had extremely high 5hmC enrichment (fig. 2B). To achieve 5hmC peak regions with relatively high differentiation under saline and CFA-treated conditions, we also identified ~9,810 CGI regions with 2-fold differential spinal 5hmC densities after CFA treatment. These 5hmC differential regions were enriched heavily in promoters (~62.64%) and gene bodies (~26.94%), as determined by calculating the percentages of differential region counts across all genomic features (fig. 2C). In addition, the densities of 5hmC changes across various genomic regions were measured, and we found that the highest occurred in CGI in promoters (fig. 2D). As 5hmC in promoter CGI is associated with high levels of transcription,[30] we speculated that 5hmC might function in promoter regions during CFA-induced nociceptive sensitization. Taken together, these results suggest that the global 5hmC contents were increased in the spinal cords during nociceptive sensitization and that 5hmC modification may be involved in the process of chronic pain.

Spinal TET1 and TET3 Contribute to CFA-induced Nociception and Spinal Neuron Sensitization

TET proteins catalyze 5mC toward 5hmC; therefore, we next investigated *Tet* expression and its roles in regulating nociceptive responses using a CFA model. *Tet* expression was quantified at the messenger (m)RNA and protein levels by RT-qPCR and Western blotting, respectively. We found that protein and mRNA levels of TET1 and TET3, but not TET2, were significantly increased at 3 days after CFA injection compared with those in the control group; *Tet1* and *Tet3* mRNAs were increased by 180% (280 ± 35 *vs.* 100 ± 18; $P = 0.0026$) and 121% (219 ± 35 *vs.* 98 ± 14; $P = 0.017$), respectively (fig. 3A). Correspondingly, TET1 and TET3 proteins were increased by 47% (145 ± 28 *vs.* 98 ± 19; $P = 0.0088$) and 55% (157 ± 34 *vs.* 102 ± 22; $P = 0.0083$), respectively (fig. 3B); TET2 proteins were almost not detected (data not shown). These results suggest that increases in TET1 and TET3, but not TET2, may be involved in the nociceptive responses induced by CFA. The distribution of 5hmC in spinal neurons prompted us to determine whether TET1 and/or TET3 also are expressed in spinal neurons. Immunofluorescence double staining showed that TET1 and TET3 were highly expressed in the spinal cords of CFA-treated mice. There was 85.5% overlap between NeuN and TET1 staining and 64.3% overlap between NeuN and TET3 staining in the CFA-treated mice (fig. 3C); however, TET1 was rarely expressed in spinal glial cells, whereas TET3 was expressed in some astrocytes, but not in microglial cells (data not shown). In addition, single-cell PCR showed that five of six spinal neurons expressed TET1 and four of six neurons expressed TET3 in CFA mice (fig. 3D), further evidence that most spinal neurons express TET1 or TET3.

We explored the potential effects of manipulating spinal TET1 or TET3 expression on nociceptive responses. For this purpose, we used siRNAs (T1-siRNA or T3-siRNA for exogenous down-regulation) and lentiviruses (Lenti-T1-siRNA or Lenti-T3-siRNA for endogenous down-regulation, and Lenti-T1 or Lenti-T3 for endogenous up-regulation). The lentivirus constructs were expressed mainly in spinal neurons but also were expressed in some spinal glial cells (data not shown). The transfection efficiencies of siRNAs and lentiviruses were validated in the spinal cords of naïve and CFA mice by RT-qPCR (all $P < 0.031$; see fig. S2A-D, Supplemental Digital Content 2, http://links.lww.com/ALN/B420). Before nociceptive behaviors were tested, mice performed vertical climbing tests to verify that TET1 and TET3 manipulation did not affect motor behaviors (data not shown); however, knockdown of TET1 and TET3 *via*

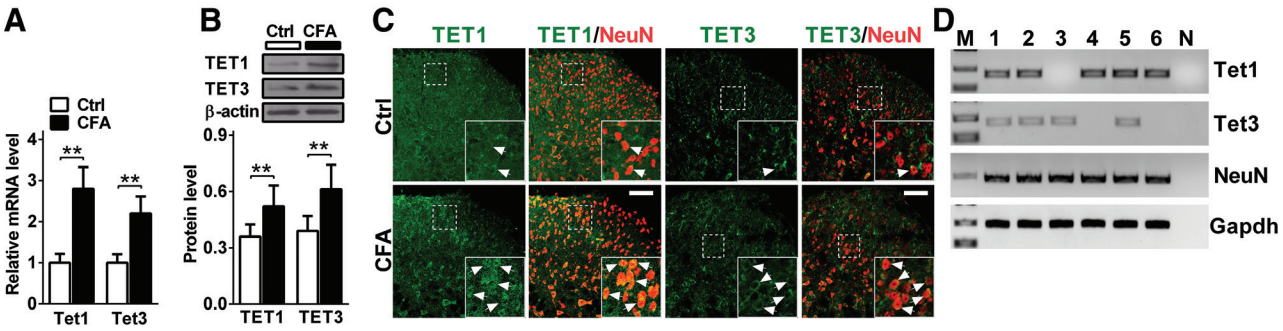

Fig. 3. Complete Freund's adjuvant (CFA)-induced nociceptive sensitization increases the expression of spinal ten-eleven translocation methylcytosine dioxygenase (TET)1 and TET3. (*A*) Messenger (m)RNA expression measured by real-time quantitative polymerase chain reaction at 3 days after CFA injection. All samples were normalized to *Gapdh*. n = 6/group; $^{**}P < 0.01$ *versus* the corresponding control group (Ctrl) by two-tailed unpaired Student's *t* test. (*B*) Protein expression by Western-blotting after normalizing to β-actin. n = 6/group; $^{**}P < 0.01$ *versus* the corresponding control group by two-tailed unpaired Student's *t* test. *(C)* Double staining of TET1 or TET3 with NeuN. *Scale bar*, 25 μm. *Arrows* indicate the positive overlayed signal. *(D)* Single-cell real-time polymerase chain reaction showing colocalization of TET1 or TET3 with NeuN. Nos. 1–6 represent six different neurons; No. 7 (N) is a negative control.

intrathecal injections for 3 consecutive days of T1-siRNA or T3-siRNA and Lenti-T1-siRNA or Lenti-T3-siRNA reversed CFA-induced thermal hyperalgesia and mechanical allodynia (all $P < 0.039$), but no effect was observed from injections of scrambled siRNA or Lenti-vector (fig. 4, A and B). To further determine the contribution of TET1 and TET3 in initiating nociceptive sensitization, we pretreated animals with Lenti-T1-siRNA or Lenti-T3-siRNA for 3 days before CFA injection and then assessed the preventive effect on nociceptive responses. We found that this pretreatment inhibited CFA-induced thermal hyperalgesia and mechanical allodynia (all $P < 0.037$; fig. 4C). Finally, we found that overexpressing TET1 and TET3 *via* intrathecal injections of Lenti-T1 and Lenti-T3, respectively, promoted thermal hyperalgesia and mechanical allodynia (all $P < 0.047$; fig. 4D). These findings suggest that spinal TET1 and TET3 contribute to the modulation of nociceptive sensitization.

Fos, the protein encoded by the protooncogene *c-fos*, has been used extensively as a marker for activity in activated nociceptive neurons in the spinal cord.[31] In our genomic 5hmC profiling, we found no change in spinal 5hmC in the promoter of *c-fos* after CFA-induced nociceptive sensitization. Moreover, the knockdown of TET1 or TET3 with siRNA did not alter 5hmC content in the promoter of *c-fos* (data not shown), suggesting that Fos expression is not regulated by hydroxymethylation. Therefore, we further investigated the effect of TET1 and TET3 on CFA-induced spinal neuron activation by detecting spinal Fos expression. The results showed Lenti-T1-siRNA and Lenti-T3-siRNA, but not Lenti-vector, inhibited CFA-induced increases of Fos expression in the superficial and deep dorsal horn at 7 days after CFA injection (2 days after 3 continuous days of lentivirus injection; 123 ± 26 *vs.* 294 ± 6; $P = 0.0031$; and 140 ± 21 *vs.* 294 ± 60; $P = 0.0043$, respectively; fig. 5A). Pretreating animals with Lenti-T1-siRNA or Lenti-T3-siRNA for 3 days before the CFA injection reversed the increase in spinal Fos expression (220 ± 44 *vs.* 464 ± 98; $P = 0.0041$; and 264 ± 54 *vs.* 464 ± 98; $P = 0.0063$, respectively; fig. 5B), suggesting that knockdown of TET1 and TET3 reversed nociceptive sensitization by inhibiting spinal neuron activation. Conversely, overexpression of TET1 and TET3 with Lenti-T1 and Lenti-T3, respectively, increased spinal Fos expression in naïve mice (189 ± 37.6 *vs.* 100 ± 21.5; $P = 0.0045$; and 186 ± 38.7 *vs.* 100 ± 21.5; $P = 0.0048$, respectively; fig. 5C). Collectively, these findings suggest that spinal TET1 and TET3 contribute to nociceptive sensitization *via* regulating neuronal activation.

TET1 and TET3 Regulate Nociception by Targeting Stat3

TET proteins convert 5mC to 5hmC; therefore, we further investigated the role of TET1 and TET3 in producing spinal

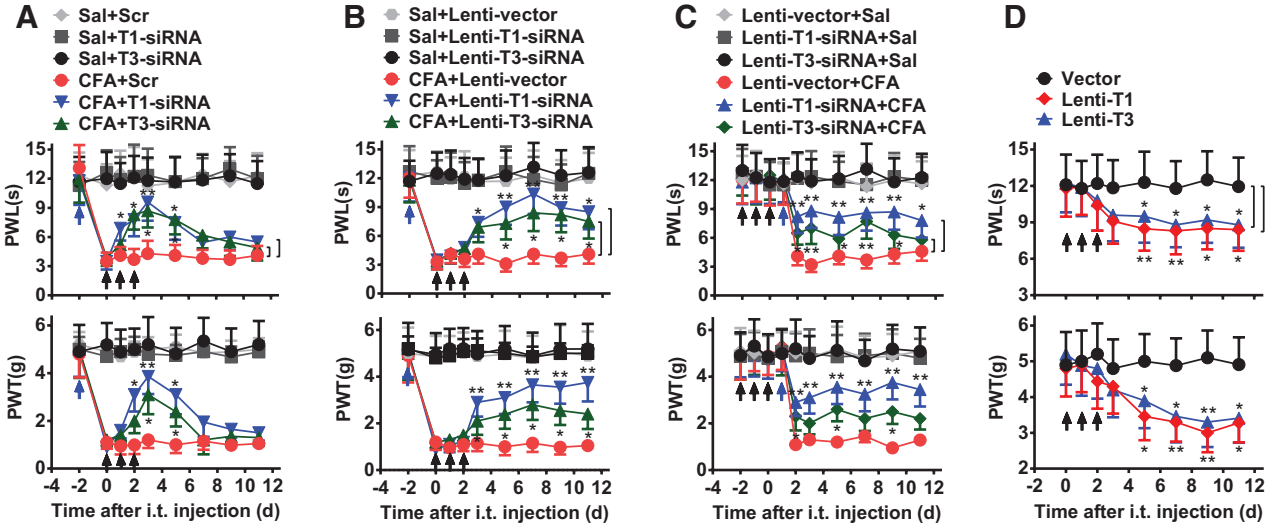

Fig. 4. The regulation of ten-eleven translocation methylcytosine dioxygenase (TET)1 and TET3 on nociceptive responses. (*A*, *B*) Intrathecal injection of *Tet1*-siRNA and *Tet3*-siRNA (*A*) or Lenti-T1-siRNA and Lenti-T3-siRNA (*B*) for 3 consecutive days reversed complete Freund's adjuvant (CFA)-induced thermal hyperalgesia (paw withdrawal latency, PWL) and mechanical allodynia (paw withdrawal threshold, PWT). n = 6/group; two-way ANOVA (effect *vs.* group × time interaction) followed by *post hoc* Tukey test, PWL: F_{group} (40, 270) = 44.61, PWT: F_{group} (40, 216) = 6.91 (*A*); PWL: F_{group} (40, 270) = 44.78, PWT: F_{group} (40, 270) = 7.55 (*B*), $^{*}P < 0.05$, $^{**}P < 0.01$. (*C*) Pretreatment with Lenti-T1-siRNA and Lenti-T3-siRNA for 3 consecutive days prevented CFA-induced thermal hyperalgesia and mechanical allodynia. *Blue arrow* indicates CFA or saline injection; *black arrow* indicates *Tet1*- and *Tet3*-siRNA/Scr or Lenti-T1-siRNA and Lenti-T3-siRNA/Lenti-vector injection. n = 6/group; two-way ANOVA (effect *vs.* group × time interaction) followed by *post hoc* Tukey test, PWL: F_{group} (45, 300) = 54.87, PWT: F_{group} (45, 300) = 24.82, $^{*}P < 0.05$, $^{**}P < 0.01$. (*D*) Intrathecal injection of Lenti-T1 and Lenti-T3 for 3 consecutive days produced thermal hyperalgesia and mechanical allodynia in naïve mice. *Black arrow* indicates Lenti-T1 and Lenti-T3 or vector injection. n = 6/group; two-way ANOVA (effect *vs.* group × time interaction) followed by *post hoc* Tukey test, PWL: F_{group} (14, 120) = 18.4, PWT: F_{group} (14, 120) = 9.18, $^{*}P < 0.05$, $^{**}P < 0.01$. Scr = scrambled; T1 = TET1; T3 = TET3.

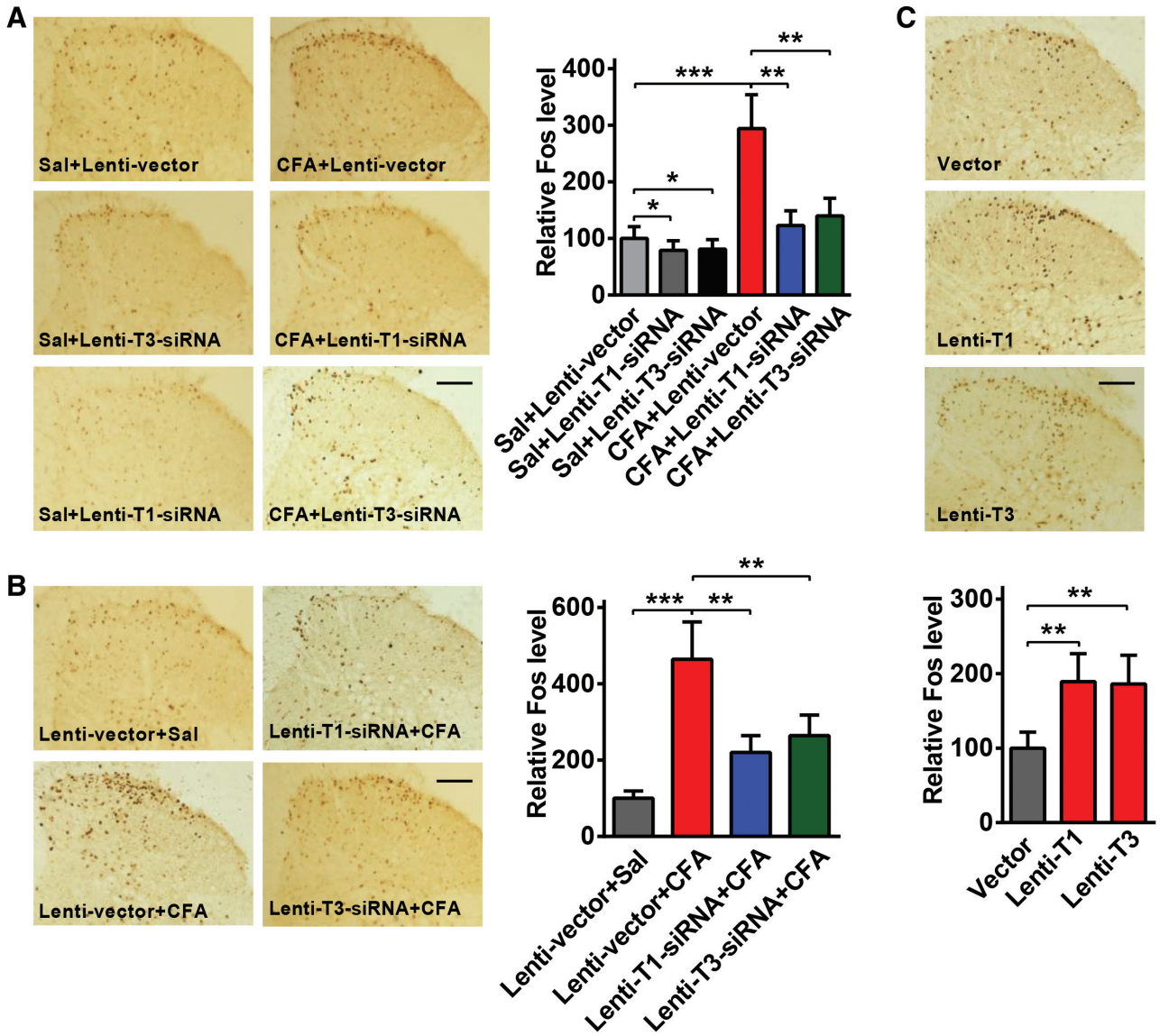

Fig. 5. Ten-eleven translocation methylcytosine dioxygenase (TET)1 and TET3 regulate spinal neuronal sensitization. (*A*) Intrathecal injection of Lenti-T1-siRNA and Lenti-T3-siRNA for 3 consecutive days reversed the increase in Fos protein in spinal dorsal horns of complete Freund's adjuvant (CFA)-treated mice (*left*, immunohistochemical staining; *right*, histogram analysis of Fos number). n = 6/group; one-way ANOVA (expression *vs.* the treated groups) followed by *post hoc* Tukey test, F_{time} (5, 30) = 161.6, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. *Scale bar*, 25 μm. (*B*) Pretreatment with Lenti-T1-siRNA and Lenti-T3-siRNA for 3 consecutive days prevented CFA-induced increase of Fos protein expression in the spinal dorsal horn. Fos protein was measured 2 days after CFA injection. n = 6/group; one-way ANOVA (expression *vs.* the treated groups) followed by *post hoc* Tukey test, F_{time} (3, 20) = 77.56, $^{**}P < 0.01$, $^{***}P < 0.001$. *Scale bar*, 25 μm. (*C*) Intrathecal injection of Lenti-T1 and Lenti-T3 for 3 consecutive days increased the Fos protein expression in spinal dorsal horns of naïve mice. Fos expression in spinal cord was counted 7 days after the first injection of lentivirus. n = 6/group; one-way ANOVA (expression *vs.* the treated groups) followed by *post hoc* Tukey test, F_{time} (2, 15) = 19.9, $^{*}P < 0.05$, $^{**}P < 0.01$. *Scale bar*, 25 μm. Sal = saline.

5hmC in specific gene loci. First, we carried out a cross-analysis for 5hmC and 5mC densities in promoter CGI with 2-fold changes in CFA and control groups. We identified 337 genes with a greater than 2-fold 5hmC gain (hMeDIP-Seq score > 30) and 3,295 genes with a greater than 2-fold 5mC loss after crossover analysis and obtained 103 genes with 5hmC gain and 5mC loss (fig. 6A). Among these 103 genes (data not shown), there were only two transcript factors, *Gata2* and *Stat3*, that play important roles in turning gene transcription on and off by binding to gene promoters. RT-qPCR analysis revealed that mRNA levels of *Gata2* and *Stat3* were up-regulated in spinal cords of CFA-treated mice (247 ± 52.1 *vs.* 100 ± 21.2; $P = 0.00057$; fig. 6B). *Stat3*, however, showed greater differences than *Gata2* in 5hmC and 5mC levels (data not shown) and mRNA expression (fig. 6B) between CFA-treated and control groups. Furthermore, we

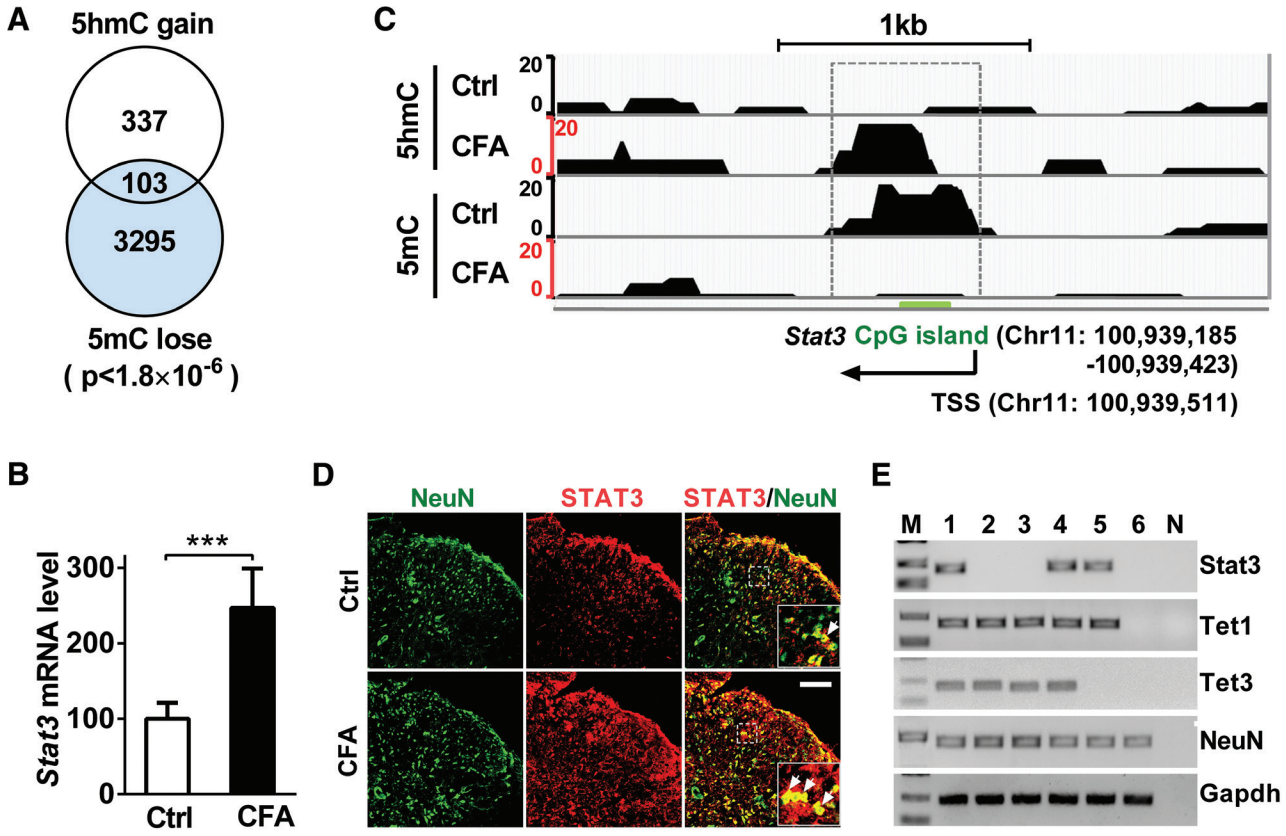

Fig. 6. Nociceptive sensitization changes spinal 5-hydroxymethylcytosine (5hmC) and 5-methylcytosine (5mC) levels in *Stat3* promoter and STAT3 expression. (*A*) Overlap of global promoters that gain 5hmC and those that lose 5mC in the spinal cords of mice with complete Freund's adjuvant (CFA)-induced nociception. The hydroxymethylated DNA immunoprecipitates (hMeDIP)-score (fragment-counts/kb) greater than 30. The *P* value was calculated using Fisher exact test. (*B*) The differential expression of *Stat3* was analyzed quantitatively by real-time quantitative polymerase chain reaction. n = 6/group; $^{**}P < 0.001$ *versus* control group by two-tailed paired Student's *t* test. (*C*) Genome browser shot showing an hMeDIP and MeDIP CGI region in spinal *Stat3* promoter in the CFA group *versus* the control group. The visualized signal profiles (at 50-base pairs resolution) were generated from hMeDIP-seq data using the UCSC genome browser. *(D)* Double staining of STAT3 with NeuN. *Scale bar*, 25 μm. *Arrows* indicate the positive overlayed signal. *(E)* Single-cell real-time polymerase chain reaction showing the colocalization of *Stat3* with *Tet1* or *Tet3* in the spinal neurons of mice. No. 7 is a negative control. Ctrl = control; M = maker; STAT3 = signal transducer and activator of transcription 3; TSS = transcript start site.

found that 5hmC was increased but 5mC decreased in a 238-bp CGI in the *Stat3* promoter in CFA *versus* control groups *via* hMeDIP-Seq profiling (fig. 6C). These results were verified by ChIP-qPCR (data not shown). A previous finding indicated that STAT3 may be involved in the development of chronic pain.[20] How the expression of *Stat3* is regulated during nociceptive sensitization, however, remains unclear. Therefore, we chose to further investigate the role of *Stat3* in regulating nociceptive sensitization mediated by TET1 and TET3.

We examined the colocalizations of STAT3 with TET1 and TET3 and the binding capacities of TET1 and TET3 to the *Stat3* promoter in spinal neurons. We first examined the expression of STAT3 in spinal neurons via immunofluorescence double staining and single-cell PCR. Double-staining results showed that there was ~28.0% overlap between NeuN and STAT3 staining in the control group and 64.7% overlap between NeuN and STAT3 in the CFA group (fig. 6D). These findings were supported by the results of single-cell PCR showing that three of six spinal neurons expressed *Stat3*, that these three cells coexpressed TET1, and that two of them coexpressed TET3 (fig. 6E). ChIP-PCR using TET1 and TET3 antibodies showed a 2-fold increases in the amounts of *Stat3* that were pulled down in CFA *versus* control groups; however, no differences were observed in the input or negative control sample (see fig. S3A, Supplemental Digital Content 2, http://links.lww.com/ALN/B420). Together, these results suggest STAT3 is coexpressed with TET1 or TET3 in spinal neurons and the promoter of Stat3 is bound by TET1 and/or TET3 in spinal cells.

To determine whether 5hmC in the *Stat3* promoter is catalyzed by TET, we used *in vitro* and *in vivo* strategies (fig. 7, A and G). First, we evaluated whether hydroxymethylation of the *Stat3* promoter increases gene transcription *in vitro*. We cloned two segments of the *Stat3* promoter, including a 1,511-bp segment containing the TSS (–1365 to

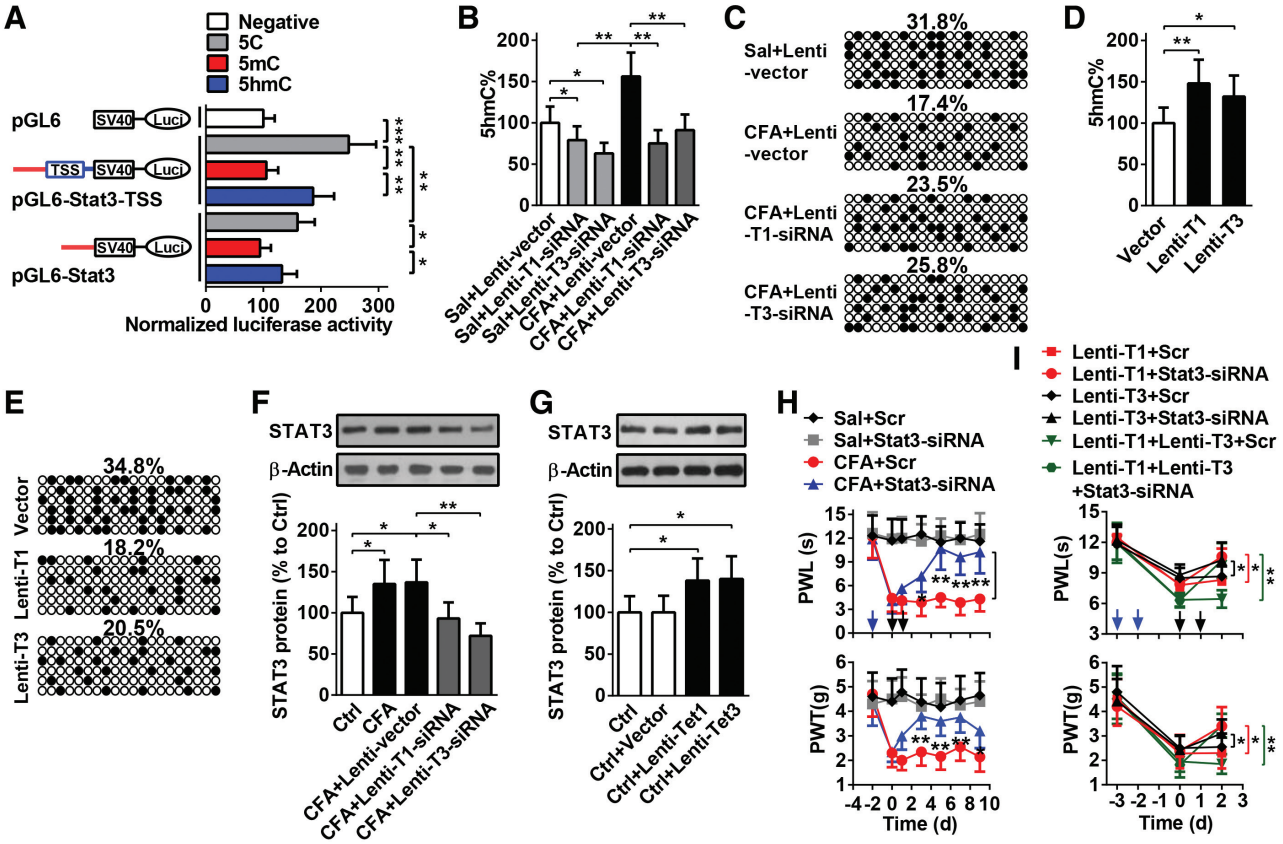

Fig. 7. Ten-eleven translocation methylcytosine dioxygenase (TET)1- and TET3-mediated DNA hydroxmethylation of *Stat3* promoter regulates spinal signal transducer and activator of transcription (STAT3) expression and nociceptive behavior. (*A*) The activities of methylated or demethylated promoter of the cloned *Stat3* promoter encompassing transcription start site (TSS) or not were detected by firefly luciferase (Luci) reporter assays in HEK 293T cells. Empty vector (pGL6) plasmid was used as the negative control. pGL6-Stat3-TSS and pGL6-Stat3 are plasmids with a *Stat3* promoter containing the ATG transcript site or not, respectively. Values of luciferase activities for each plasmid were normalized for transfection efficiency by cotransfecting with pRL-TK plasmid. n = 3 per group; two-way ANOVA (effect *vs.* plasmid × treated interaction) followed by *post hoc* Tukey test, F_{group} (4, 18) = 13.63, **P* < 0.05, ***P* < 0.01, ****P* < 0.001. (*B*, *C*) CFA increased spinal 5-hydroxymethylcytosine (5hmC) levels and decreased spinal 5-methylcytosine (5mC) level of *Stat3* promoter, which was reversed by *Tet1*-siRNA and *Tet3*-siRNA delivered by lentivirus. *White* and *black dots* represent demethylated and methylated CpG dinucleotides, respectively. Each *line* indicates an individual sequence. 5hmC represents the ratio of ($CC^{hm}GG$) to ($CC^{hm}GG$, $CC^{m}GG$, and CCGG) in the detection fragment; n = 5/group; one-way ANOVA (expression *vs.* the treated groups) followed by *post hoc* Tukey test, F_{time} (5, 24) = 28.06, **P* < 0.05, ***P* < 0.01. (*D*, *E*) Lentivirus-mediated overexpression of TET1 and TET3 increased the 5hmC level and decreased the 5 mC level in naïve mice. n = 5/group; one-way ANOVA (expression *vs.* the treated groups) followed by *post hoc* Tukey test, F_{time} (2, 12) = 9.1, **P* < 0.05, ***P* < 0.01. (*F*) The down-regulation of TET1 and TET3 mediated by lentivirus reversed the increased STAT3 expression in CFA-treated mice. n = 5/group; one-way ANOVA (expression *vs.* the treated groups) followed by *post hoc* Tukey test, F_{time} (4, 20) = 8.03, **P* < 0.05, ***P* < 0.01. (*G*) TET1 and TET3 overexpression *via* lentivirus infection increased *Stat3* expression in naïve mice. n = 5/group; one-way ANOVA (expression *vs.* the treated groups) followed by *post hoc* Tukey test, F_{time} (3, 16) = 7.52, **P* < 0.05. (*H*) Intrathecal injection of *Stat3*-siRNA for 2 consecutive days reversed CFA-induced thermal hyperalgesia and mechanical allodynia. *Blue arrow* indicates CFA or saline injection; *black arrow* indicates *Stat3*-siRNA or scrambled (Scr) injection. n = 6/group; two-way ANOVA (effect *vs.* group × time interaction) followed by *post hoc* Tukey test, paw withdrawal latency (PWL): F_{group} (18, 140) = 56.56, paw withdrawal threshold (PWT): F_{group} (18, 140) = 11.29, ***P* < 0.01. (*I*) *Stat3*-siRNA markedly inhibited thermal hyperalgesia and mechanical allodynia induced by Lenti-T1 and Lenti-T3 in naïve mice. *Stat3*-siRNA was injected intrathecally 2 days after pain behavior testing. *Blue arrow* indicates *Stat3*-siRNA or Scr injection; *black arrow* indicates Lenti-T1 and Lenti-T3 injection. n = 6/group; two-way ANOVA (effect *vs.* group × time interaction) followed by *post hoc* Tukey test, PWL: F_{group} (10, 90) = 19.72, PWT: F_{group} (10, 90) = 9.84, **P* < 0.05, ***P* < 0.01. 5C = unmethylated promoter; SV40 = SV40 promoter.

+146 bp) and a 1,361-bp segment without the TSS (–1365 to –5 bp), into a pGL6 luciferase reporter (pGL6-S3-TSS and pGL6-S3, respectively) and examined their abilities to drive luciferase expression in HEK 293T cells. We found that both segments produced greater luciferase activities than the empty vector; however, pGL6-S3-TSS generated stronger activity than pGL6-S3 (pGL6-S3-TSS: 248.1 ± 47.7 *vs.* 100 ± 19.5; *P* = 0.00068; fig. 7A), indicating the cloned

pGL6-S3-TSS region contains the efficient regulatory element and controls *Stat3* expression. Moreover, the methylation of pGL6-S3-TSS *in vitro* by the CpG methyltransferase before introducing it into cells abolished the enhancement in luciferase activity (105 ± 20.5 *vs.* 248.1 ± 47.7; $P = 0.0032$; fig. 7A). This effect of methylation was reversed by hydroxymethylation, which was generated *in vitro* from the methylated pGL6-S3-TSS *via* TET protein catalysis (186.2 ± 36.6 *vs.* 105 ± 20.5; $P = 0.006$; fig. 7A). These results suggest a direct positive role of hydroxymethylation of the pGL6-S3-TSS promoter in transcriptional efficiency. Second, we quantitatively analyzed *in vivo* the changes of 5hmC and 5mC in the *Stat3* promoter after *Tet1* and *Tet3* knockdown in CFA-treated mice and after overexpression in naïve mice. We found that lentivirus-mediated knockdown of TET1 and TET3 reversed the increase of 5hmC (75 ± 16.1 *vs.* 156 ± 28.9; $P = 0.0043$; and 91 ± 19.1 *vs.* 156 ± 28.9; $P = 0.0066$, respectively; fig. 7B) and the decrease of 5mC in spinal cords of CFA-treated mice (fig. 7C), which was accompanied by an inhibition of STAT3 expression at the mRNA (data not shown) and protein (93 ± 19.6 *vs.* 137 ± 27.5; $P = 0.035$; and 72 ± 15.2 *vs.* 137 ± 27.5; $P = 0.0028$, respectively; fig. 7F) levels. Lentivirus-mediated overexpression of spinal TET1 and TET3 significantly increased 5hmC levels (148 ± 28.7 *vs.* 100 ± 18.8; $P = 0.0085$; and 132 ± 25.5 *vs.* 100 ± 18.8; $P = 0.046$, respectively; fig. 7D) but reduced the 5mC content (fig. 7E) and increased the expression of STAT3 at the mRNA (data not shown) and protein (93 ± 19.6 *vs.* 137 ± 27.5; $P = 0.040$; and 72 ± 15.2 *vs.* 137 ± 27.5; $P = 0.038$, respectively; fig. 7G) levels in naïve mice. Collectively, these results suggest that TET1 and TET3 regulate spinal *Stat3* expression by converting 5mC to 5hmC in its promoter during nociceptive sensitization. To further determine whether the increased expression of STAT3 contributed to the regulation of nociceptive sensitization, we tested pain behaviors after knockdown of STAT3 *via* intrathecal injections of siRNA in CFA-treated mice. We found that *Stat3*-siRNA not only knocked down the mRNA and protein expressions of STAT3 in naïve mice but also reversed the increase in STAT3 protein in CFA-treated mice, demonstrating the efficiency of *Stat3*-siRNA knockdown (see fig. S3B, Supplemental Digital Content 2, http://links.lww.com/ALN/B420). Climbing tests showed that the knockdown of *Stat3* with siRNA did not affect motor function (data not shown). Compared with in the scramble group, however, *Stat3*-siRNA significantly alleviated pain induced by CFA (all $P < 0.042$; fig. 7H), suggesting that spinal STAT3 plays an important role in nociceptive sensitization.

Finally, to explore the role of STAT3 in mediating pain *via* TET1 and TET3, we pretreated or posttreated animals with siRNA to knockdown STAT3 before or after, respectively, intrathecal injections of Lenti-T1 and/or Lenti-T3, and then measured the behavioral responses. We found that knockdown of STAT3 significantly inhibited or reversed nociceptive responses induced by TET1 and/or TET3 overexpression in naïve mice (all $P < 0.046$; fig. 7I), suggesting that STAT3 mediates the regulation of pain by TET. Taken together, these results indicate that hydroxymethylation of the *Stat3* promoter mediated by TET1 and TET3 regulates nociceptive hypersensitivity *via* spinal neuron sensitization (fig. 8). In this study, 16 mice had to be excluded from the statistical analysis because of poor health or accidental death caused by anesthesia.

Discussion

Central sensitization describes a state in which central synapses become hyperresponsive to extracellular nociceptive and/or nonnociceptive stimuli, and it is thought to play a critical role in the pathogenesis of chronic pain. The induction and maintenance of central sensitization is associated closely with the release of neurotransmitters and the activation of spinal ion channels, receptors, and intracellular signal

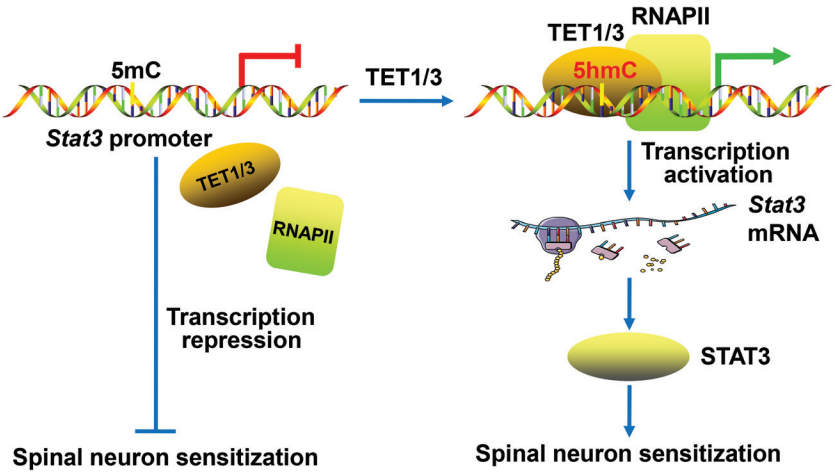

Fig. 8. Schematic of regulation of nociceptive sensitization by ten-eleven translocation methylcytosine dioxygenase (TET)1 and TET3-mediated hydroxymethylation of the Stat3 promoter. 5mc = 5-methylcytosine; RNAPII = RNA polymerase II; STAT3 = signal transducer and activator of transcription 3.

transduction pathways. Aberrant pain-related gene expression is the molecular basis for central sensitization of spinal networks. Therefore, uncovering the mechanisms of gene regulation that underlie central sensitization will improve our understanding of chronic pain and may provide potential targets for developing new therapeutic strategies. DNA demethylation mediated by pharmacologic inhibitors, as a disruptive active mechanism of DNA methylation, has attracted a great deal of attention for its regulatory function in not only diverse neurologic diseases[32,33] but also neuroinflammatory diseases.[7,34] For example, treatment with the demethylating agent 5′-aza-2′-deoxycytidine, as a passive demethylation strategy, markedly attenuates nociceptive behaviors and spinal neuronal sensitization by reducing the methylation and subsequently increasing the expression of spinal miRNA-219.[2] Interestingly, in this study, we demonstrated that knockdown of TET, an active demethylating enzyme, significantly reversed nociceptive behavior by decreasing the sensitivity of spinal neurons mediated by an increase in *Stat3* methylation. In addition, to our knowledge, this is the first evidence for an active demethylating mechanism in a chronic inflammatory pain model.

Indeed, recent growing evidence has confirmed a dependent linkage between global 5hmC alteration and neurologic processes and psychiatric diseases. Global 5hmC is reduced markedly both in terminally differentiated mouse Purkinje neurons[9] and in mouse brain tissues of the YAC128 model of Huntington disease. The loss of 5hmC impairs neurogenesis in Huntington disease brain.[28] In contrast, gains in global 5hmC have been found in different CNS areas, including in embryonic mouse brains,[35] aging mouse hippocampi,[36] and spinal cords from amyotrophic lateral sclerosis disease.[37] By demonstrating a determinating function of genomic 5hmC in the development of CNS disease, these studies provide the rationale to further study the role of 5hmC in neuronal dysfunction in a variety of CNS diseases. Interestingly, the loss or gain of 5hmC has been confirmed as a novel epigenetic feature in CNS diseases. For example, mutant huntingtin is associated with a marked reduction in the 5hmC landscape, indicating that the loss of the 5hmC marker is a new epigenetic feature of Huntington disease. Therefore, reestablishing 5hmC levels and landscape may slow/halt the progression of Huntington disease.[28] In this study, we observed a change in spinal 5hmC that was similar to the increase seen in peripheral blood during nociceptive sensitization. Interestingly, 5hmC immunostaining was distributed among the neurons of the superficial and deep dorsal horn and the ventral horn in spinal slices, suggesting 5hmC in dorsal and ventral horn spinal neurons may be involved in nociceptive information processing. Whether the alteration of 5hmC in deep dorsal or ventral horn neurons contributes to nociceptive sensitization deserves further study in the future. Interestingly, there was extremely high 5hmC in the Y sex chromosome after CFA injection. As all of the experiments were performed in male mice, future study is needed to determine if there are sex differences in 5hmC in response to nociception.

TET family proteins have been shown to specifically catalyze the demethylation of 5mC to 5hmC, as well as its further oxidation into 5-formylcytosine and 5-carboxylcytosine.[38,39] TET1 and TET3 are abundantly expressed in mouse embryonic stem cells and CNS neurons, including in the inferior parietal lobule (BA39-40) in psychotic patients.[40] *Tet1* knockout in mice reduced the 5hmC content in hippocampal neurons, resulting in abnormal hippocampus long-term depression and impaired memory extinction[15] or spatial memory deficits.[14] Although TET2 is enriched in several CNS regions, the association between TET2 and CNS-related physiologic and pathologic processes remains unclear. TET2 is a tumor suppressor and is implicated specifically in the production and development of hematopoietic stem cells from animals and humans.[41–43] It is possible that TET2 protein was expressed in low levels in mouse spinal cords in this study. In addition, it is demonstrated that TET3 plays a critical role in neural progenitor cell maintenance, terminal differentiation, and neuronal development. Embryonic stem cells lacking *Tet3* have normal self-renewal and maintenance but impaired neuronal differentiation.[44,45] In contrast, TET3 overexpression rescues miRNA-15b-induced impairment of cortical neural progenitor cell proliferation, which is responsible for neuronal differentiation.[46] Moreover, a behavioral study reveals that neocortical TET3-mediated accumulation of 5hmC contributes to a rapid behavioral adaptation in extinction learning.[47] In addition, TET1 and TET3 are increased significantly in the cerebella of autistics and are accompanied by increases in global 5hmC, suggesting a cooperative role of TET1 and TET3 in autism disease.[48] In our previous study, we found that nociceptive behavior induced by formalin increased the expression of spinal TET1 and TET3, and manipulating TET1 and TET3 alleviated nociceptive injury *via* miRNA-365-3p hydroxymethylation.[19] It was not known, however, whether TET proteins regulate chronic inflammatory pain. The results of this study show that CFA-induced nociceptive sensitization increased the expression of TET1 and TET3 in the spinal cords of mice. Conversely, knockdown of TET1 and TET3 *via* endogenous or exogenous siRNA alleviated nociceptive responses. These results are consistent with those from a recently published report in which spinal nerve ligation up-regulated TET1 expression in rat spinal neurons and knockdown alleviated nociceptive responses induced by nerve injury.[49] These data reveal a close correlation between TET1 or TET3 and nociceptive processes; consequently, our data expand the functional role of TET in the modulation of CNS diseases. It is worth noting, however, that the recent accumulating data have shown that intrathecal injections of the DNA methyltransferase inhibitor 5′-aza-2′-deoxycytidine significantly attenuated hyperalgesia behaviors induced by CFA and chronic constrictive injury.[2,3,50] Thus, compared with inhibitors of the demethylating enzyme TET in analgesic function, two proteins with converse functions in DNA methylation have the same effect of inducing hyperalgesia behaviors.

Therefore, we speculated that different methylation enzymes may be epigenetically responsible for differential gene regulation in nociceptive processes and analgesic responses, suggesting that the epigenetic mechanism underlying the pain process comprises a complicated integrated network.

In addition, in this study, our genomic Integrated Genomics Viewer results indicated that 5hmC-enriched loci occurred mainly in the promoters and bodies of different genes, suggesting that intragenic 5hmC enrichment is likely a positive epigenetic regulator for gene expression. These findings are in agreement with those from a recent report showing that 5hmC is associated with activated genes and actively transcribed genes in CNS tissues, such as the cerebellum and hippocampus of mouse brains.[12] As transcription factors regulate the rate that genetic information is transcribed from DNA to mRNA *via* binding to specific DNA sequences, they are key players in gene expression. We analyzed the 5hmC level of transcription factors among 103 screened genes and found that *Stat3* was actively transcribed by the enriched 5hmC in its promoter during nociceptive sensitization. STAT3, as a primary signal transducer and activator of transcription, plays an important role in a variety of neurophysiologic and neuropathologic processes, such as neuronal development, plasticity, and CNS diseases. Emerging lines of evidence suggest a potential role for STAT3 in developing and maintaining nociceptive sensitization in various types of chronic pain models, including neuropathic pain, inflammatory pain, and cancer pain.[20–22] Rat spinal nerve ligation or bilateral chronic constriction injury increases the mRNA and protein levels of spinal STAT3 and Janus kinase, an upstream key active enzyme of STAT3; the increased Janus kinase further amplifies the level of active phosphorylated STAT3. Intrathecal administration of STAT3 inhibitors (AG490 or WP1066) or Janus kinase inhibitor I significantly reduced established thermal hyperalgesia and mechanical allodynia.[20,21] Although a large body of molecular and functional evidence links STAT3 with nociceptive information processing, little is known about how *Stat3* expression is regulated in these processes. In this study, our data indicated that knockdown of TET1 and TET3 markedly reversed the enhanced 5hmC level, which was accompanied by a decrease in STAT3 expression and an alleviation of nociceptive behavior in CFA-treated mice. TET-mediated demethylation of *Stat3* and its functional significance in nociceptive sensitization provide a novel potential pharmacotherapeutic target. Interestingly, STAT3 is found to be frequently activated in microglial or astrocyte cells of the spinal cord in a neuropathic pain model induced by spared nerve injury,[51] spinal nerve ligation,[52] or inflammatory pain by lipopolysaccharide.[53] In this study, *Stat3* was found *via* single-cell PCR to be coexpressed with *Tet1* and *Tet3* in spinal neurons of mice. Furthermore, manipulating TET1 and TET3 expression significantly changed the 5hmC level of the *Stat3* promoter and its subsequent expression in the spinal cord; however, we found that TET3 also was expressed in some astrocytes. Whether TET3 functionally regulates 5hmC production in astrocytes in nociceptive processes needs to be investigated in the future.

In conclusion, our findings not only reveal an unknown epigenetic mechanism involved in a chronic inflammatory pain model but also provide a new sight into how TET1 and TET3 epigenetically regulate *Stat3* expression in the spinal cord. It may significantly contribute to potential treatment strategies for chronic pain.

Acknowledgments

The authors thank Hai-Lei Ding, M.D., Ph.D., Wen Shen, M.D., Lianshen Zhong, M.D., Ph.D., Yanqiang Li, Ph.D., and Zi-Peng Liu, Ph.D. (Department of Anesthesiology, The University of Hong Kong) for help with statistical analyses, informatics analyses, and for constructive discussions.

Research Support

The study was supported by grants from the National Natural Science Foundation of China (Nos. 81230025, 81671096, 81271231, and 31500855; Peking, China), the Key Project of the Natural Science Foundation of Jiangsu Education Department (No. 15KJA320004; Nanjing, Jiangsu, China), the Qing Lan Project (Nanjing, Jiangsu, China), the Six Talent Summit Project (Nanjing, Jiangsu, China), the 333 High-level Personnel Training Project (Nanjing, Jiangsu, China), the Jiangsu Provincial Special Program of Medical Science (BL2014029; Nanjing, Jiangsu, China), and the Priority Academic Program Development of Jiangsu Higher Education Institutions (Nanjing, Jiangsu, China).

Competing Interests

The authors declare no competing interests.

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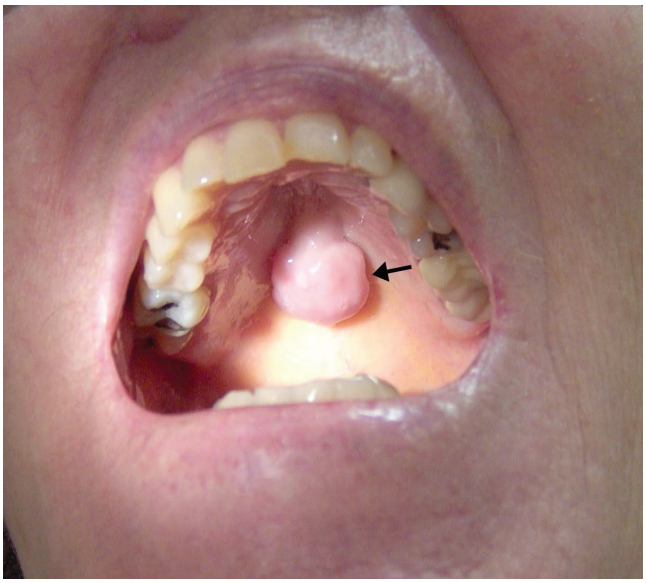
Charles D. Collard, M.D., Editor

Images in Anesthesiology: Torus Palatinus and Airway Management

Jesse Aron, M.D., Stephen J. Raithel, B.A., Andrew J. Mannes, M.D.



TORUS palatinus is a bony exostosis of the maxilla that affects 20 to 30% of people in the United States.[1] Tori can be unilobular or multilobulated and pedunculated or flat. Large tori that protrude more than 5 mm from the maxilla comprise less than 5% of tori.[2] The accompanying image is of a pedunculated torus palatinus measuring 2 cm in diameter, found during the preoperative evaluation of a patient. There is no definitive explanation as to why torus palatinus occurs, although genetic factors, superficial injuries, and palate stress from mastication are thought to play a role.[1]

These bony growths are covered by a thin mucosa that is susceptible to lacerations,[1] which could cause perioperative bleeding and swelling if traumatized. Large tori can develop sites that trap food,[2] shown in the photograph with a potential cavity between the base of the lesion and the hard palate (arrow), thus posing an aspiration risk if any trapped food material is dislodged while establishing an airway. A modified technique for insertion of a laryngeal mask airway in patients with a large palatal torus has been described, involving folding and guiding the cuff flaps around the mass.[3] Injury to the torus with other items frequently placed in the oropharynx should also be avoided, such as when placing orogastric tubes, esophageal temperature probes, oral airways, or transesophageal ultrasound transducers. With proper preoperative evaluation and care to avoid trauma to the mucosa, the risks of aspiration, perioperative bleeding, and postoperative pain for the patient can be minimized.

Research Support

Supported by intramural funding from the National Institutes of Health Clinical Center, Bethesda, Maryland.

Competing Interests

The authors declare no competing interests.

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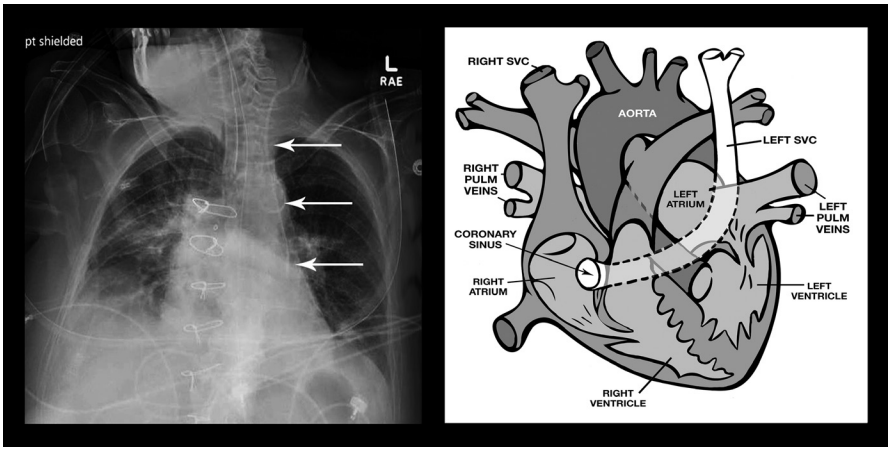
 Anesthesiology 2017; 127:164

Charles D. Collard, M.D., Editor

Persistent Left Superior Vena Cava

Unusual Catheter Position on Chest X-ray Film

Ranjit Deshpande, M.D., Matthew Band, P.A., Viji Kurup, M.D.



CONGENITAL anomalies of the great veins of the neck are relatively infrequent. Persistent left superior vena cava (PLSVC) is an embryologic remnant of the left superior cardinal vein seen in 0.1 to 0.3% of healthy adults.[1] PLSVC runs between the left pulmonary veins and the left atrial appendage enlarging the coronary sinus as it enters the atrium. When present, it can affect placement of central catheters, pacemakers, and cardiopulmonary bypass. It is important to be aware of this variation and to recognize it in imaging studies.

Patients with PLSVC are usually asymptomatic but can have associated cardiac anomalies such as atrial septal defect, cor triatriatum, and mitral atresia.[2] Diagnosis is by chest x-ray showing widening above the aortic knob or a dilated coronary sinus on echocardiography. It is confirmed by injecting agitated saline in the left arm vein and observing bubbles in the coronary sinus.

The accompanying image shows the central venous catheter (*arrows*) placed in a patient who needed central access for pressor support (in the illustration on the *right*, SVC = superior vena cava). A catheter is seen on the left of the mediastinum entering the coronary sinus. In patients with known anomaly of the great veins, the recommendation is for preoperative imaging and guidance for safe placement of catheters. In 10% of patients, the PLSVC drains into the left atrium with a potential for embolic complications.[1] PLSVC is a relative contraindication to retrograde cardioplegia as the coronary sinus may not be adequately occluded with the catheter balloon.[3]

Acknowledgments

The authors thank Garrett Sendlewski, B.F.A., Department of Anesthesiology, Yale University, New Haven, Connecticut, for his help with editing the images in this article.

Competing Interests

The authors declare no competing interests.

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 Anesthesiology 2017; 127:165

Jerrold H. Levy, M.D., F.A.H.A., F.C.C.M., Editor

Perioperative Steroid Management

Approaches Based on Current Evidence

Melanie M. Liu, M.D., Andrea B. Reidy, M.D., Siavosh Saatee, M.D., Charles D. Collard, M.D.

CHRONIC steroid therapy is a cornerstone treatment for many common conditions, including inflammatory bowel disease, rheumatologic disease, reactive airway disease, and immunosuppression for transplant recipients. Patients on chronic steroid therapy may develop secondary adrenal insufficiency that can manifest as full-blown adrenal crisis in the perioperative period. When these patients present for surgery, the anesthesiologist must decide whether to administer perioperative stress-dose steroids to mitigate this rare but potentially fatal complication of chronic steroid use. In doing so, the patient's risk for adrenal crisis must be weighed against the risks of unnecessary steroid supplementation. Unfortunately, this decision is not always clear-cut, because even the recommendations found in major textbooks are confusing, inconsistent, and lacking in class A and B evidence (table 1). Despite the lack of standardization and the widespread use of perioperative stress-dose steroids observed in clinical practice, a recent search of the Anesthesia Closed Claims Project database containing 11,247 claim narratives using the terms "stress dose," "Cushing," "Addison," and "adrenal insufficiency" revealed that failure to administer stress steroids generated only two claims that resulted in liability payments, and both of these cases were complicated by other issues (written personal communication, Karen L. Posner, Ph.D., Department of Anesthesiology and Pain Medicine, University of Washington, Seattle, Washington, December 2015). It is unclear whether this paucity of claims is due to underdiagnosis of adrenal crisis or overtreatment of perioperative patients with steroids. We now review and evaluate the current data on the use of perioperative stress-dose steroids and propose approaches to administration and dosing.

Hypothalamic-Pituitary-Adrenal Axis Suppression

Acute physiologic or psychologic stress activates the hypothalamic-pituitary-adrenal axis (HPAA). The hypothalamus produces corticotropin-releasing hormone (CRH), which stimulates production of adrenocorticotrophic hormone (ACTH) in the anterior pituitary, which in turn signals cortisol production in the adrenal glands. Cortisol has a number of roles within the body, including stimulation of gluconeogenesis, catecholamine production, and activation of antistress and antiinflammatory pathways. Cortisol is also essential for maintenance of cardiac output and contractility and enhancement of vascular tone *via* modulation of β-receptor synthesis and function and increased sensitivity to catecholamines, respectively.[1,2] Cortisol production is self-regulated *via* negative feedback loops that lead to decreased secretion of CRH and ACTH (fig. 1).[3] Normally, the adrenal gland secretes approximately 8 to 10 mg of cortisol per day. Transient increases in cortisol secretion are seen in response to stress, such as illness or surgery. The rate varies between individuals but is usually up to 50 mg/day for minor procedures and up to 75 to 150 mg/day for more complex procedures, rarely exceeding 200 mg/day (table 2).[4]

Patients on chronic steroid therapy may experience HPAA suppression, resulting in low CRH and ACTH levels that lead to atrophy of the adrenal zona fasciculata and a decrease in cortisol production. This process is known as *secondary* adrenal insufficiency. Unlike in *primary* adrenal insufficiency, the renin-angiotension-aldosterone system remains intact, and there is no mineralocorticoid deficiency. Inadequate cortisol production may predispose to vasodilatation

This article is featured in "This Month in Anesthesiology," page 1A. Figure 1 was enhanced by Annemarie B. Johnson, C.M.I., Medical Illustrator, Vivo Visuals, Winston-Salem, North Carolina.

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 Anesthesiology 2017; 127:166–72

Table 1. Published Perioperative Steroid Dosing Recommendations

Publication	Recommendations
Miller's anesthesia 8e[24]	Acknowledge "a precise amount required has not been established": IV 200 mg/day hydrocortisone phosphate per 70 kg of body weight or for minor procedure 100 mg/day hydrocortisone phosphate per 70 kg of body weight and then decreased at 25% per day until PO intake of maintenance dose can be resumed
Clinical anesthesia 7e[3]	Acknowledge both "an extensive review concluded that the best evidence was that patients should receive usual daily dose but no supplementation" and "many clinicians are unwilling to adopt the regimen until further trials have been undertaken in patients receiving physiologic steroid replacement" and ultimately give "popular regimen": 200–300 mg of hydrocortisone per 70 kg of body weight in divided dose on the day of surgery, with adjustment in dose based on extent and duration of surgery and patients are to take their daily dose of steroids
Anesthesia and coexisting disease 6e[2]	Surgery — Recommendations Superficial — Daily dose only Minor — Daily dose plus hydrocortisone (25 mg IV) Moderate — Daily dose plus hydrocortisone (50–75 mg, taper 1–2 days) Major — Daily dose plus hydrocortisone (100–150 mg, taper 1–2 days)
UpToDate: The Surgical Patient Taking Glucocorticoids[22]	Nonsuppressed (HPA) axis – defined as taking exogenous steroids for less than 3 weeks, or prednisone (<5 mg daily or its equivalent) for any duration, or less than 10 mg of prednisone or its equivalent every other day; we suggest continuing the same glucocorticoid regimen perioperatively (Grade 2C). These patients are unlikely to have a suppressed HPA axis, and neither preoperative evaluation of the HPA axis nor supraphysiologic doses of glucocorticoids are needed. In suppressed patients (defined as equivalent to prednisone 20 mg/day for 3 weeks or more), recommendations are surgery specific Surgery — Recommendations Minor — Morning dose only Moderate — Morning dose plus IV 50 mg of hydrocortisone before incision; then IV 25 mg every 8 h for 24 h and then maintenance Major — Morning dose plus IV 100 mg of hydrocortisone before induction; then IV 50 mg every 8 h for 24 h; Taper dose by half per day to maintenance level
Bornstein *et al*.: Diagnosis and treatment of primary adrenal insufficiency: An Endocrine Society Clinical Practice Guideline[6]	Acknowledge the "proposed glucocorticoid regimen in the management of adrenal crisis places a higher value on the prevention of underdosage than on reducing potential negative effects of short-term overdosage" as "under-dosing of glucocorticoids in an adrenal crisis is potentially hazardous. . . . Harm from these doses has not been shown, and direct studies indicating that lower doses are safe do not exist." Glucocorticoid dose adjustment based on severity of illness or magnitude of stressor, as follows: Surgery — Recommendations Minor to moderate — Hydrocortisone, 25–75 mg/24 h (usually 1–2 days) Major surgery, trauma, delivery, disease that requires intensive care, suspected adrenal crisis — Hydrocortisone 100 mg IV followed by continuous IV infusion of hydrocortisone 200 mg/24 h (alternatively 50 mg every 6 h IV/IM)

HPA = hypothalamic-pituitary-adrenal; IM = intramuscular; IV = intravenous; PO = per os.

and hypotension.[4] Thus, patients on chronic steroids are traditionally considered at risk for adrenal crisis during periods of stress due to their attenuated ability to mount a cortisol response.[5] In the awake patient, signs and symptoms of adrenal crisis may include altered mental status, abdominal pain, nausea/vomiting, weakness, and hypotension.[6] For the practicing anesthesiologist, however, perioperative adrenal crisis becomes a diagnosis of exclusion and requires a high index of suspicion because the signs and symptoms described above are largely absent in the anesthetized patient and nonspecific in the immediately postoperative patient. The clinical picture is one of severe, persistent hypotension that is poorly responsive to fluid and vasopressor therapy. Perioperative adrenal crisis can be life-threatening and requires prompt recognition and treatment with stress-dose steroids in addition to supportive care with fluid and vasopressor administration.

There is no universally agreed-upon dose or duration of exogenous steroids required to cause HPAA dysfunction, though prednisone 20 mg/day or its equivalent for more than 3 weeks has been cited.[1] Indeed, based on biochemical testing, neither the dosage nor duration of exogenous glucocorticoid administration corresponds well with the level of HPAA suppression or its return to normality after discontinuation of therapy.[7] The exact time course of recovery from HPAA

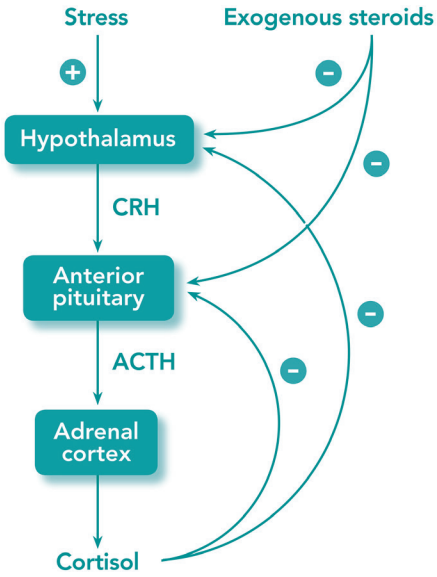

Fig. 1. Functional anatomy of hypothalamic-pituitary-adrenal axis. ACTH = adrenocorticotropic hormone; CRH = corticotropin releasing hormone; – = negative feedback; + = positive feedback.

suppression differs between individuals and is difficult to predict. Nevertheless, most agree that HPAA suppression does not continue beyond 1 yr after cessation of exogenous steroid therapy with the possible exception of patients receiving intraarticular glucocorticoid injections, for whom the time course of HPAA suppression is variable, depending on the frequency and dose of injections, and not well studied.[4]

Historical Perspectives

In 1949, cortisone was first commercially produced for the treatment of primary adrenal insufficiency and shortly thereafter was being used as an antiinflammatory and immunosuppressant.[5] The historical basis for giving perioperative stress-dose steroids lies in two case reports, each describing a single patient (n = 1), from the early 1950s in which cardiovascular collapse was attributed to secondary adrenal crisis based on autopsy findings.[8,9] However, both case reports have subsequently been criticized for confounding factors such as the withholding of aggressive fluid resuscitation, vasopressors, antibiotics, and most importantly the lack of biochemical proof of adrenal insufficiency *via* measurement of serum cortisol levels.[7] Indeed, Brown and Buie[10] found that perioperative hypotension due to adrenal crisis is rare, with an estimated incidence of 1 to 2%, and this estimate was extrapolated mainly from a 1973 Kehlet and Binder prospective study[11] of patients on chronic steroids for whom steroids were withheld. Nevertheless, these two case reports form the basis for much of the current perioperative management of patients with suspected HPAA suppression.

Current Evidence

Since these sentinel articles, there has been a growing body of literature and debate about the management of patients on chronic steroids who present for surgery. As a whole, the literature on administration of perioperative stress-dose steroids is devoid of class A or B levels of evidence and is complicated by

Table 2. Surgical Stress by Procedure and Recommended Steroid Dosing

Surgery Type	Endogenous Cortisol Secretion Rate	Examples	Recommended Steroid Dosing
Superficial	8–10 mg per day (baseline)	Dental surgery Biopsy	Usual daily dose
Minor	50 mg per day	Inguinal hernia repair Colonoscopy Uterine curettage Hand surgery	Usual daily dose *plus* Hydrocortisone 50 mg IV before incision Hydrocortisone 25 mg IV every 8 h × 24 h Then usual daily dose
Moderate	75–150 mg per day	Lower extremity revascularization Total joint replacement Cholecystectomy Colon resection Abdominal hysterectomy	Usual daily dose *plus* Hydrocortisone 50 mg IV before incision Hydrocortisone 25 mg IV every 8 h × 24 h Then usual daily dose
Major	75–150 mg per day	Esophagectomy Total proctocolectomy Major cardiac/vascular Hepaticojejunostomy Delivery Trauma	Usual daily dose *plus* Hydrocortisone 100 mg IV before incision Followed by continuous IV infusion of 200 mg of hydrocortisone more than 24 h *or* Hydrocortisone 50 mg IV every 8 h × 24 h Taper dose by half per day until usual daily dose reached *plus* Continuous IV fluids with 5% dextrose and 0.2–0.45% NaCl (based on degree of hypoglycemia)

Data from Axelrod,[4] Salem *et al.*,[13] and Bornstein *et al.*[6]
IV = intravenous.

Table 3. Steroid Choices, Potency, Dosages, and Their Conversion Charts[1,2]

Steroid	Glucocorticoid Activity	Mineralocorticoid Activity	Equivalent Dose (IV/PO)	Half-Life, h
Cortisol (hydrocortisone)	1	1	20	8–12
Cortisone	0.8	0.8	25	8–12
Prednisone	4	0.8	5	18–36
Prednisolone	4	0.8	5	12–36
Methylprednisolone	5	0.5	4	18–36
Dexamethasone	30–40	0	0.5–0.75	36–54

IV = intravenous; PO = per os.

a lack of consistency in patient selection, surgery and anesthesia type, clinical outcome, and steroid timing and dose. Randomized double-blinded, placebo-controlled trials addressing this topic are few in number and insufficiently powered (most involve 20 or fewer patients).[10] This has made statistical analysis of the current steroid stress-dose literature close to impossible.

Kehlet and Binder[11] published perhaps the most robust data on stress-dose steroids. They looked at 73 patients on chronic steroid therapy ranging from 5 to 80 mg/day of prednisone or equivalent undergoing minor and major surgery for whom all steroids were withheld for 36 h preoperatively and not resumed until at least 24 h postoperatively. Plasma cortisol levels and vitals were followed. Unexplained hypotension (defined as systolic blood pressure less than 80 mmHg not due to sepsis, anaphylaxis, or bleeding) was found in 7 of 18 hypotensive patients. However, only 3 of the 7 patients with unexplained hypotension had low cortisol levels, defined in this study as less than 15 μg/100 ml, and these patients did not respond to treatment with rescue steroids. Additionally, patients who had low plasma cortisol levels before surgery were not significantly more likely to have hypotension. The authors concluded that preoperative plasma cortisol is "not the prime determinant of the level of blood pressure in the glucocorticoid-treated patients during and after surgery, and acute stress-induced adrenocortical insufficiency is rare even when steroids are withheld."[11] It must be noted, though, that there is no agreed-upon definition of what constitutes a low cortisol level in physiologically stressed individuals, which makes these data difficult to interpret. Moreover, the method used to measure cortisol levels in this study is a fluorometric assay rarely used today, which further calls into question the applicability of these findings.

In 1997, Glowniak and Loriaux[12] studied 18 male patients taking prednisone for at least 2 months for various conditions with baseline secondary adrenal insufficiency as determined by cosyntropin study (also known as the short ACTH stimulation test). Cosyntropin, a synthetic analog of ACTH, is administered as a bolus of 250 μg IV or intramuscularly at least 24 h after the last dose of exogenous glucocorticoids. Plasma cortisol levels are then measured 30 to 60 min postadministration, with 18 μg/dl or higher, indicating a normal response.[4] In this study, patients underwent various surgical procedures using different anesthetic techniques, including local, neuraxial, and general. Patients were randomized to receive stress-dose steroid injections (100 mg of cortisol in normal saline based on the Salem *et al.* guidelines) *versus* control (normal saline).[13] No significant perioperative differences in hemodynamic parameters were found between groups. The authors concluded that patients with secondary adrenal insufficiency as a result of chronic steroid therapy do not experience hypotension in the absence of stress-dose steroid administration and can be maintained on their usual daily dose of steroids in the perioperative period.[12] However, it should be noted that this study may not have been sufficiently powered (total n = 18) to detect statistical differences (*i.e.*, type II error).

Thomason *et al.*[14] studied 20 organ transplant patients on chronic steroid therapy for immunosuppression presenting for gingival surgery under local anesthesia. Patients were randomized to receive stress-dose steroids *versus* placebo. Each patient required at least two operations and thus served as their own control. Serum ACTH levels were drawn pre- and postoperatively, and blood pressure was measured at set intervals throughout. No significant differences in blood pressure or ACTH measurements were found between groups. The authors concluded that patients on chronic steroids do not require stress-dose steroids before undergoing gingival surgery.[14] Not only was this study underpowered, it is also unclear whether these conclusions would apply to major surgery performed under general anesthesia. Additionally, measurement of random plasma ACTH levels as an indicator of adrenal insufficiency is neither a standard nor valid method of assessing adrenocortical function and further decreases the applicability of the study findings

Further complicating this muddied picture is the retraction of a Cochrane review in 2013[16] that had concluded, largely based on the articles by Glowniak and Loriaux[12] and Thomason *et al.*,[14] that there is "currently inadequate evidence to support the use of supplemental perioperative steroids in patients with adrenal insufficiency. It is likely that in the majority of adrenally suppressed patients undergoing surgery, administration of the patient's daily maintenance dose of corticosteroid may be sufficient and that supplemental doses are not required."[15] This Cochrane review was

retracted after comments received "*via* direct correspondence which have challenged the eligibility criteria and interpretation of the evidence summarized in this review."[16]

In contrast to the historical recommendations for perioperative stress-dose steroids, recent data suggest that the patient's usual dose of steroids can be maintained preoperatively and taken the day of surgery, with vigilance to signs and symptoms (*e.g.*, hypotension) of adrenal insufficiency intraoperatively.[5,10,17] Intraoperative hypotension that cannot be adequately managed by conservative means (*e.g.*, decreasing depth of anesthesia, fluid resuscitation, vasopressor administration, and managing metabolic abnormalities) should raise suspicion for adrenal crisis, and a rescue dose of 100 mg of hydrocortisone IV should be administered, followed by continued supplementation of 50 mg of hydrocortisone IV every 6 h.[6]

Chronic steroid therapy is well known to be associated with risk of immunosuppression, impaired wound healing, hyperglycemia, and psychologic disturbances in the postoperative period.[18] Whether perioperative stress-dose steroids further increase these risks is debatable, especially as there are currently no randomized controlled trials that address their adverse effects. Elevated circulating levels of glucocorticoids are associated with a range of psychiatric symptoms including acute psychosis.[19] One retrospective study on renal transplant patients undergoing lymphocele surgery showed that administration of stress-dose steroids resulted in elevated blood glucose, but there was otherwise no statistically significant difference between clinical outcome in patients treated with stress-dose steroids and those who were not. The authors concluded that stress-dose steroids increased the risk of hyperglycemia without apparent clinical benefit.[20]

Our Approach

Recent data suggest that stress-dose steroids may not be necessary, even in patients with confirmed preoperative secondary HPAA suppression.[12] Instead, these patients may be maintained on their usual preoperative dose and treated with rescue dose steroids only if refractory hypotension presents in the perioperative period.[5,10,17] Nonetheless, some authors advocate for the administration of stress-dose steroids for at-risk patients despite the lack of class A and B evidence given the rare, but possibly fatal, consequences of adrenal crisis.[10,13] Indeed, the recent 2016 Endocrine Society Clinical Practice Guideline[6] on primary adrenal insufficiency notes that harm has not been shown from recommended doses of perioperative stress-dose steroids and thus places a higher value on preventing adrenal crisis rather than reducing the potential adverse effects of short-term overtreatment.[21]

Marik and Varon[17] suggest that most patients receiving chronic steroid therapy do not need preoperative evaluation of their adrenocortical function unless there is clinical reason to believe that it might affect perioperative management, as this testing does not reliably predict which patients will develop adrenal crisis. For example, a patient experiencing complications of chronic steroid therapy (*e.g.*, gastrointestinal bleeding) who may otherwise benefit from rapid taper and cessation of steroid treatment may instead need to continue on glucocorticoids throughout the perioperative period if HPAA suppression is present.[4] Patients on chronic steroids who are at low risk for HPAA suppression (*i.e.*, those taking any dose of glucocorticoid for less than 3 weeks, morning doses of prednisone 5 mg/day or less, or prednisone 10 mg/day or less every other day) need neither preoperative testing nor stress-dose steroid administration. Patients who are at high risk for HPAA suppression (*i.e.*, those with clinical Cushing syndrome due to exogenous glucocorticoid use or those taking more than 20 mg/day of prednisone for more than 3 weeks) require stress-dose steroid administration but also do not need preoperative testing. Preoperative evaluation may be helpful for patients on chronic steroid therapy who do not fall into either of the above categories, as stress-dose steroids can be safely withheld with proof of non-suppressed HPAA.[22]

When preoperative evaluation is clinically warranted, the short ACTH stimulation test is the test of choice for assessing the integrity of the HPAA and its function. Patients with normal response to administration of cosyntropin do not require further evaluation or perioperative glucocorticoid treatment. Other diagnostic methods (*e.g.*, insulin-induced hypoglycemia, or low dose [1 μg] ACTH stress test) are neither practical nor validated and are not recommended.[4] Nevertheless, the short ACTH stimulation test is not without its pitfalls, because it measures serum total cortisol levels rather than serum free cortisol levels. Free cortisol, not the protein-bound fraction, is responsible for the physiologic effects of cortisol. A recent study by Hamrahian *et al.*[23] on nutritionally deficient, critically ill patients with hypoproteinemia showed that these patients can have elevated serum free cortisol levels with concurrently lower-than-expected serum total cortisol levels. The diagnostic value of free cortisol levels, however, is not definitively proven, and the test itself is also not yet widely available.

An additional approach to management of the patient presenting for surgery on chronic steroids is to assess the anticipated surgical stress to determine the appropriate perioperative stress dose (table 2). If the estimated surgical stress requirement does not exceed the maintenance dose of exogenous steroids, stress-dose steroid administration is not warranted during the perioperative period unless the patient exhibits signs of adrenal suppression (*e.g.*, vasoplegia of unclear origin).

So the practical question remains: Which chronic steroid-treated patients require perioperative stress-dose steroids? Our approach involves categorizing patients into four groups based on the current available evidence:

1. Patients who have diagnosed secondary adrenal insufficiency as demonstrated by the short acting ACTH test. These patients will require perioperative stress-dose steroids with dosing based on surgical stress risk (table 2).
2. Patients at high risk of HPAA suppression, including patients who have been treated with a glucocorticoid in doses equivalent to at least 20 mg/day of prednisone for more than 3 weeks or who have clinical features of Cushing syndrome. Unless data confirming the integrity of the HPAA is available, these patients would benefit from perioperative stress-dose steroids with dosing based on surgical stress (table 2).
3. Patients at low risk of HPAA suppression, including patients who have been treated with any dose of glucocorticoid for less than 3 weeks, morning doses of prednisone 5 mg/day or less, or prednisone 10 mg/day every other day. Perioperative stress-dose steroids are not required unless they exhibit signs of HPAA suppression.
4. Patients at intermediate risk of HPAA suppression, including any patient on chronic steroid therapy who does not fall into one of the above categories. If time permits, consider referring these patients for preoperative testing to determine their HPAA integrity. If testing is unavailable, the anesthesiologist must exercise his/her clinical judgment as to whether to administer stress-dose steroids based on the patient's perioperative condition (*e.g.*, degree of hemodynamic stability) and surgical risk. It is reasonable, for example, to withhold glucocorticoids if the patient is otherwise healthy and stable preoperatively without signs or symptoms of Cushing disease, with a low threshold for administration of a rescue dose of steroids in the event of unexplained intra- or postoperative hypotension.

Hydrocortisone is the drug of choice for stress and rescue dose steroid coverage.[2,4,13,22] When selecting a drug to use as a perioperative stress dose, it is important to remember that in secondary adrenal insufficiency, the problem is a glucocorticoid deficiency (as opposed to a mineralocorticoid deficiency); therefore, the relative glucocorticoid and mineralocorticoid activity of the chosen drug must be taken into consideration.[2,5] Moreover, the mineralocorticoid properties of the drug may result in dose-dependent edema/fluid retention and hypokalemia. For example, if hydrocortisone dosages more than 100 mg are required, it is prudent to consider switching to methylprednisolone, because this drug has a higher glucocorticoid to mineralocorticoid activity ratio.[2,4] Steroid equivalent dosages and their relative glucocorticoid and mineralocorticoid activities are outlined in table 3.

Conclusions

Patients on chronic steroid therapy should receive their usual preoperative dose of steroids on the day of surgery. However, existing evidence on the necessity of administering perioperative stress-dose steroids for patients with suspected, or even confirmed, secondary adrenal insufficiency is inadequate to fully support or refute this practice. If HPAA suppression is a clinical concern, perioperative stress-dose steroid administration appears to carry minimal risk compared to the risk of adrenal crisis. However, the lack of class A and B evidence makes it controversial as to whether the administration of perioperative stress-dose steroids is the standard of care, even for patients with known HPAA suppression. The paucity of evidence highlighted by our examination of the available literature should serve as a call for more adequately powered studies comparing different strategies for perioperative steroid management that can generate robust, high-quality data. Until such time that class A and B evidence is available for determining an agreed-upon standard of care, we support this practical approach to the perioperative management of patients on chronic steroid therapy presenting for surgery based on our review of the currently available evidence.

Acknowledgments

The authors acknowledge Karen L. Posner, Ph.D., Department of Anesthesiology and Pain Medicine, University of Washington, Seattle, Washington. The authors also acknowledge the insightful comments of endocrinologists Pouneh Fazeli, M.D., M.P.H, Neuroendocrinology Unit, Massachusetts General Hospital, Boston, Massachusetts; Nicholas Tritos, M.D., D.Sc., Neuroendocrine and Pituitary Tumor Clinical Center, Massachusetts General Hospital, Boston, Massachusetts; and Guillermo Umpierrez, M.D., Clinical Research Center of Diabetes and Metabolism, Department of Endocrinology, Emory University School of Medicine, Atlanta, Georgia.

Research Support

Support was provided solely from institutional and/or departmental sources.

Competing Interests

The authors declare no competing interests.

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Deborah J. Culley, M.D., Editor

Phrenic Nerve Palsy and Regional Anesthesia for Shoulder Surgery

Anatomical, Physiologic, and Clinical Considerations

Kariem El-Boghdadly, F.R.C.A., Ki Jinn Chin, F.R.C.P.C., Vincent W. S. Chan, F.R.C.P.C.

ABSTRACT

Regional anesthesia has an established role in providing perioperative analgesia for shoulder surgery. However, phrenic nerve palsy is a significant complication that potentially limits the use of regional anesthesia, particularly in high-risk patients. The authors describe the anatomical, physiologic, and clinical principles relevant to phrenic nerve palsy in this context. They also present a comprehensive review of the strategies for reducing phrenic nerve palsy and its clinical impact while ensuring adequate analgesia for shoulder surgery. The most important of these include limiting local anesthetic dose and injection volume and performing the injection further away from the C5–C6 nerve roots. Targeting peripheral nerves supplying the shoulder, such as the suprascapular and axillary nerves, may be an effective alternative to brachial plexus blockade in selected patients. The optimal regional anesthetic approach in shoulder surgery should be tailored to individual patients based on comorbidities, type of surgery, and the principles described in this article. **(ANESTHESIOLOGY 2017; 127:173-91)**

SURGERY for shoulder pathology is increasingly common,[1,2] with regional anesthesia playing an important role in multimodal analgesia for these painful procedures.[3] Interscalene brachial plexus block is the most common regional anesthetic technique; however, phrenic nerve palsy and hemidiaphragmatic paresis have traditionally been inevitable consequences, which limit its utility in the population of patients at high risk of respiratory complications. A range of modifications and alternatives to interscalene block have been proposed to minimize the respiratory impact of phrenic nerve palsy, but to date there has been no thorough assessment of the clinical value offered by each of these strategies. In this article, we aim to describe the anatomical, physiologic, and clinical principles governing phrenic nerve palsy in the context of regional anesthesia for shoulder surgery. We also review the various techniques that seek to provide adequate regional anesthesia of the shoulder while minimizing the risk of phrenic nerve palsy, as well as methods for assessing their impact on diaphragmatic function, and thus provide a comprehensive narrative of their value in achieving these two objectives.

Materials and Methods

For this narrative review, we systematically searched electronic databases including MEDLINE, PubMed-not-MEDLINE, Excerpta Medica database (Embase), Cochrane Central Controlled Trials Database Register, and Cumulative Index to Nursing and Allied Health Literature (CINAHL), supplemented by a manual search. Search terms in medical subject headings, text words, and controlled vocabulary terms were used in permutations relevant to the components of this review. Search terms included (1) regional anesthesia; (2) local anesthesia; (3) shoulder; (4) surgery; (5) phrenic; (6) nerve; (7) diaphragm; and (8) diaphragmatic. Filters applied included (1) publication date January 1, 1946, to November 1, 2016; (2) English language; (3) human studies; and (4) adult studies. Eligible trials included randomized or quasirandomized controlled trials, controlled trials, case series, or pertinent correspondence that were deemed relevant or providing new knowledge on the subject in question. Trials were excluded if they produced no original empirical data, or if they were not directly relevant to phrenic nerve palsy related to regional anesthesia for shoulder surgery (fig. 1).

Studies were supplemented qualitatively with an informal literature search for relevant articles describing anatomical, physiologic, clinical, and diagnostic concepts so as to provide a comprehensive insight into the subject.

This article is featured in "This Month in Anesthesiology," page 1A. Figure 1 was enhanced by Annemarie B. Johnson, C.M.I., Medical Illustrator, Vivo Visuals, Winston-Salem, North Carolina.

Submitted for publication December 5, 2016. Accepted for publication March 1, 2017. From the Department of Anaesthesia, Guy's and St. Thomas' National Health Service Foundation Trust, London, United Kingdom (K.E.-B.); and Department of Anesthesia and Pain Medicine, Toronto Western Hospital, Toronto, Ontario, Canada (K.J.C., V.W.S.C.).

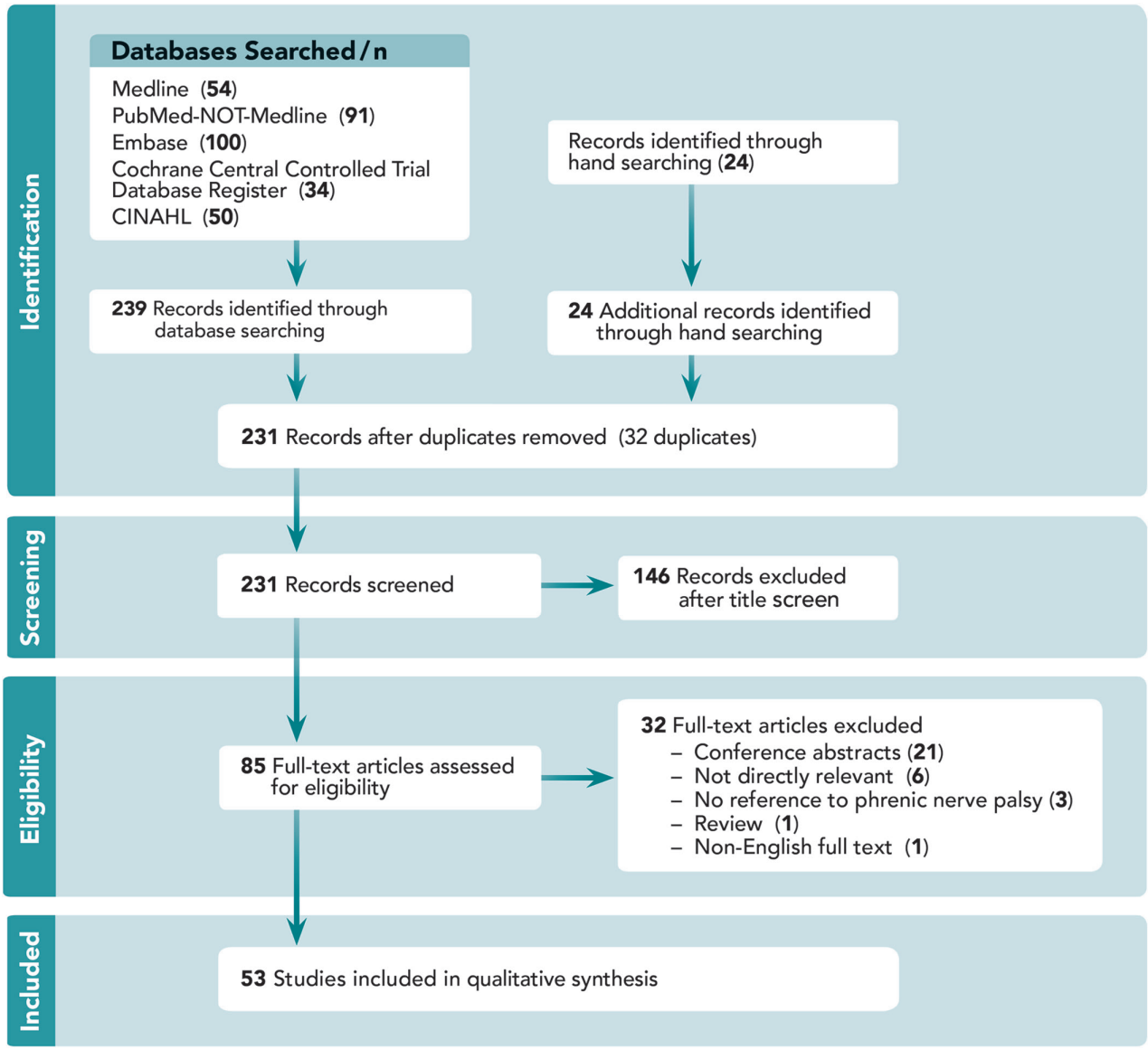

Fig. 1. Flowchart of study selection. CINAHL = Cumulative Index to Nursing and Allied Health Literature.

Discussion

Regional Anesthesia Targets for Shoulder Analgesia

Innervation of the cutaneous, muscular, bony, and capsular components of the shoulder is complex. Cutaneous innervation is provided by the axillary (C5–C6), suprascapular nerve (C5–C6), and supraclavicular nerves of the cervical plexus (C3–C4). Bony and capsular components are innervated by the suprascapular, axillary, lateral pectoral (C5–C7), musculocutaneous (C5–C7), and long thoracic (C5–C7) nerves (fig. 2). The suprascapular nerve provides up to 70% of the innervation to the glenohumeral joint,[4] with the axillary nerve supplying the majority of the remaining joint capsule. Sensory contributions to the muscles of the shoulder comprise the following: the ventral rami of the third and fourth cervical nerves to the trapezius muscle, the pectoral nerves (C5–C7) to the pectoral muscles, the dorsal scapular nerve (C5) to the levator scapulae and rhomboid muscles, and the axillary nerve (C5–C6) to the deltoid muscle. The rotator cuff muscles are innervated by the suprascapular, upper and lower subscapular (C5–C6), and axillary nerves.

The clinical aim of regional anesthesia or analgesia is to deliver local anesthetic to some or all of these key nerves that contribute to pain after shoulder surgery. The specific nerves to be targeted will depend in part on the surgical approach that is used (fig. 2). This traditionally has been achieved by performing an interscalene block, which targets the C5 and C6 roots of the brachial plexus in the interscalene region. However, conventional interscalene block is associated with several complications, the most common of which is phrenic nerve palsy with ensuing hemidiaphragmatic paresis, and this has driven the development of modifications to the interscalene block as well as alternative techniques that target the peripheral sensory supply to the shoulder at sites distal to the C5 and C6 roots.

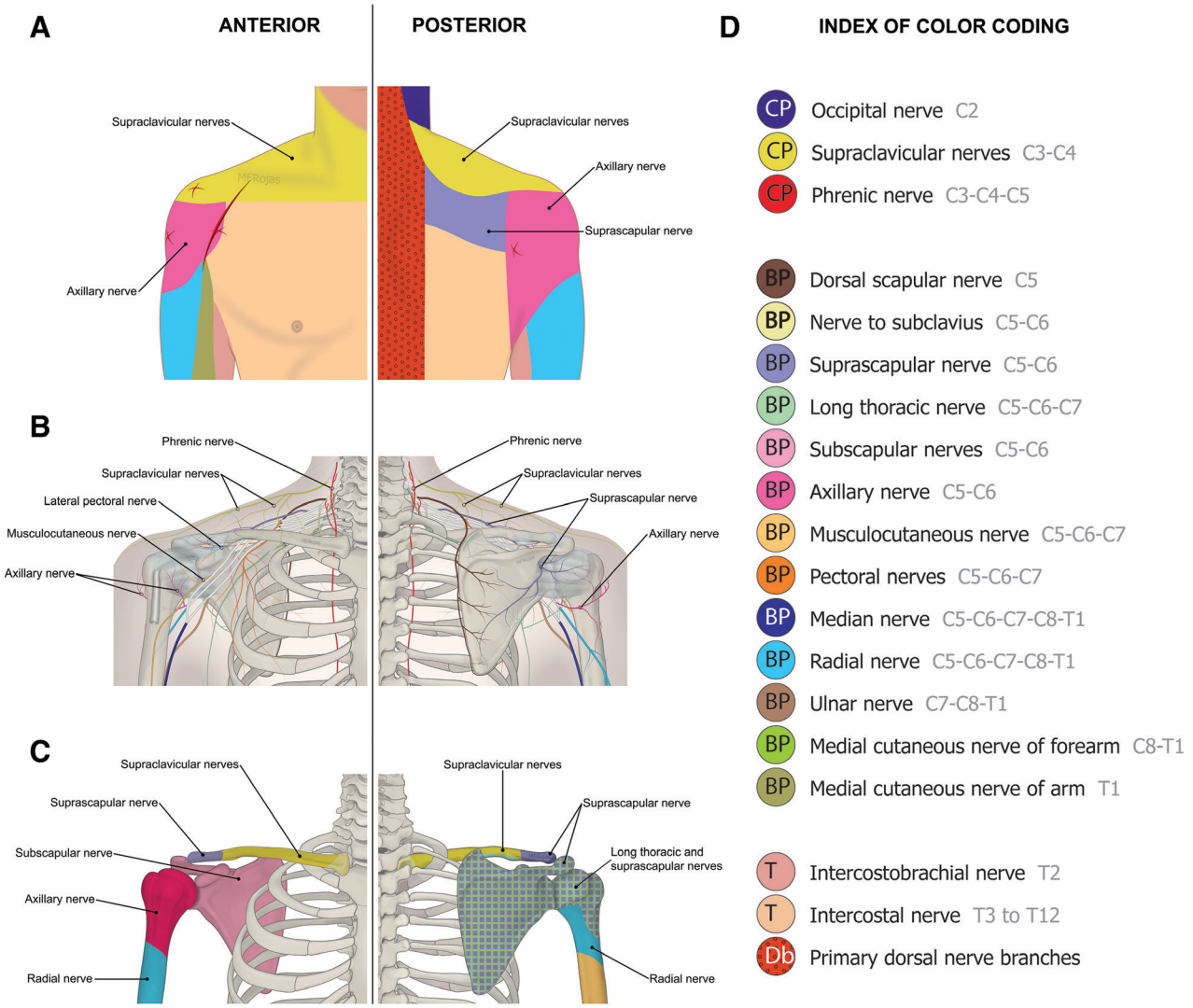

Fig. 2. Anterior (*left*) and posterior (*right*) innervation of the shoulder. (*A*) The distribution of cutaneous innervation of the shoulder. Common port-hole incisions (*red crosses*), including superior, anterior, lateral, and posterior incisions made for arthroscopic shoulder surgery and the deltopectoral incision (*red line*) for open shoulder surgery are represented. (*B*) The route taken by nerves to supply both skin and bone in the shoulder. (*C*) The osteotomal supply of the shoulder. (*D*) An index of color coding of dermatomes, nerves, and osteotomes. Images adapted with permission from Maria Fernanda Rojas Gomez and reproduced with permission from Ultrasound for Regional Anesthesia (USRA; http://www.usra.ca).

Anatomy of the Phrenic Nerve

The anatomy of the phrenic nerve is key to understanding the basis for the strategies to reduce the risk of phrenic nerve palsy. The phrenic nerve originates primarily from the fourth cervical ventral ramus but also receives contributions from both third and fifth ventral rami, as well as the cervical sympathetic ganglia or thoracic sympathetic plexus.[5] This small nerve forms at the upper lateral border of the anterior scalene muscle and descends obliquely across the anterior surface of the muscle toward its medial border (fig. 3). The phrenic nerve lies deep to the prevertebral fascia here and remains posterior to the sternocleidomastoid muscle, the inferior belly of the omohyoid, the internal jugular vein, the dorsal scapular and transverse cervical arteries, and the thoracic duct on the left. The phrenic nerve courses in close proximity to the brachial plexus, initially lying 18 to 20 mm medial to the C5 nerve root at the level of the cricoid cartilage (C5/C6) but diverging an additional 3 mm further away for every centimeter that it descends over the anterior scalene muscle (fig. 3).[6] As it approaches the root of the neck, the phrenic nerve usually lies between the subclavian artery and vein, before coursing medially in front of the internal thoracic artery (fig. 4).

An accessory phrenic nerve is present in 60 to 75% of individuals and provides an independent contribution to the phrenic nerve. The fibers of the accessory phrenic nerve arise primarily from C5 and run within the nerve to subclavius, the ansa cervicalis, or the nerve to sternohyoid.[7] These fibers then emerge from any one of these nerves to form the accessory phrenic nerve, which then joins the phrenic nerve

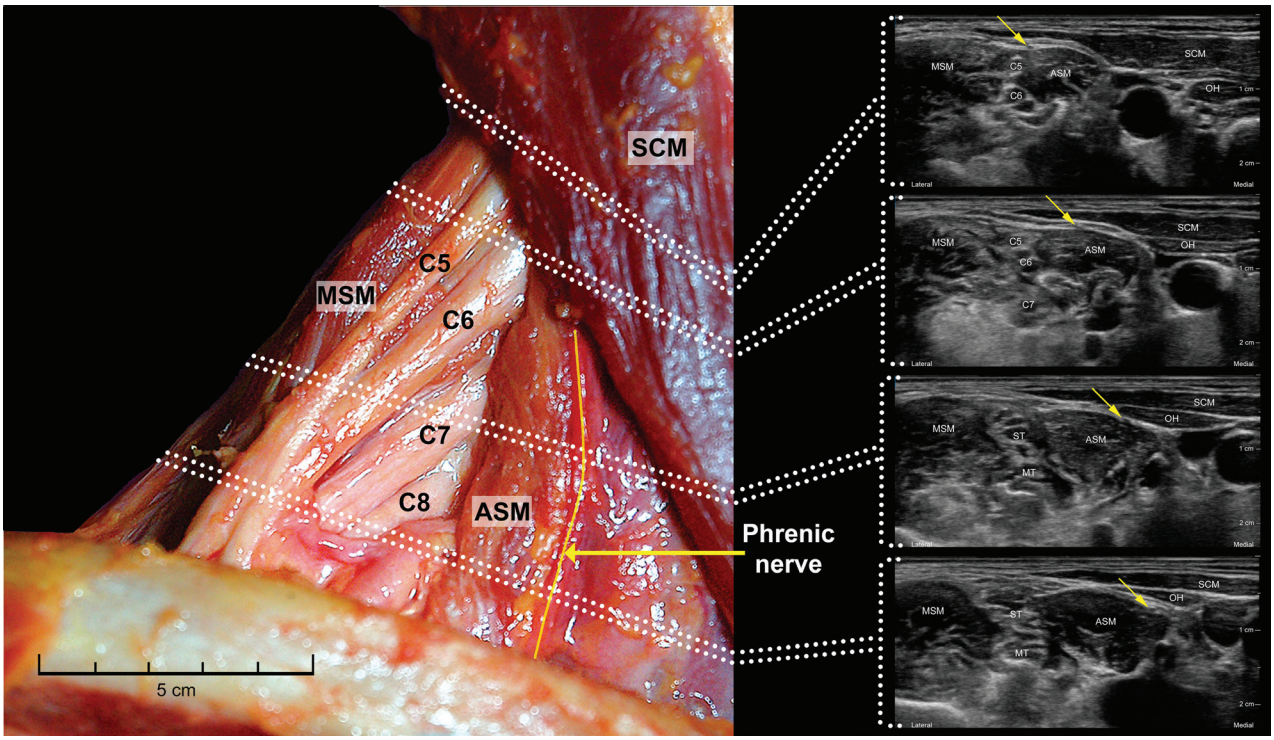

Fig. 3. Cadaveric (*left*) and corresponding sonographic images (*right*) demonstrating the course of the right phrenic nerve as it emerges beneath the lateral margin of the sternocleidomastoid muscle (SCM), between the middle scalene muscle (MSM) and the anterior scalene muscle (ASM). It begins near to the roots of the brachial plexus, then travels inferomedially away from the brachial plexus. The C5–C7 roots of the brachial plexus emerge deep to the ASM, coursing laterally, where C5 and C6 roots merge to form the superior trunk. The sonographic images of the right interscalene area descending sequentially caudally, with the brachial plexus found between the MSM and the ASM. In the *upper image*, the phrenic nerve (*yellow arrow*) can be seen above (superficial to) the ASM in close proximity to the C5 nerve root. More caudally, the phrenic nerve (*yellow arrow*) can be seen to travel medially over the ASM and beneath the omohyoid (OH) muscle until it lies nearly 2 cm away from the brachial plexus. MT = middle trunk; ST = superior trunk. *Left image* adapted with permission from Danilo Jankovic and reproduced with permission from Ultrasound for Regional Anesthesia (USRA; http://www.usra.ca).

at a variable location along its course.[8,9] Isolated damage to the accessory phrenic nerve is associated with diaphragmatic dysfunction,[10] and similarly, reports suggest that local anesthetic blockade of the accessory nerve also may lead to diaphragmatic paresis.[11,12]

Mechanisms of Phrenic Nerve Palsy after Regional Anesthesia

Transient Phrenic Nerve Palsy. Phrenic nerve palsy leading to hemidiaphragmatic paresis may be a temporary or persistent phenomenon after interscalene block or other injections of local anesthetic in the neck. Transient phrenic nerve palsy is caused by local anesthetic spreading directly to the phrenic nerve and its contributing nerves (including the accessory phrenic nerve) or proximally to the roots of the phrenic nerve. The duration of phrenic nerve palsy is determined by the duration of local anesthetic effect, which in turn is related primarily to the type and mass of local anesthetic administered. The incidence of transient phrenic nerve palsy is virtually 100% after landmark- and paresthesia-guided interscalene block techniques that use a large-volume injection of 20 ml or greater.[13,14]

Despite this, the vast majority of patients in clinical trials of interscalene block exhibit few symptoms and require no specific treatment.[15–17] Thus, on the surface, transient phrenic nerve palsy appears to have little clinical significance in terms of both objective (respiratory support) and subjective (dyspnea) features. However, randomized controlled trials generally exclude patients with pulmonary disease, obesity, or obstructive sleep apnea, and this therefore hinders the generalizability of the results reported in the literature. A significant proportion of these subgroups of patients are likely to develop symptoms or require treatment after phrenic nerve palsy, but unfortunately data on these high-risk populations usually are confined to the realm of case reports.

There is also a lack of studies formally examining clinical predictors of symptomatic phrenic nerve palsy after interscalene block, and thus it remains difficult to determine which patients, healthy or otherwise, will benefit most from avoidance of phrenic nerve palsy. It therefore falls to the individual anesthesiologist to assess the likely impact of phrenic nerve palsy in any given patient undergoing shoulder surgery and to select the appropriate regional anesthetic technique accordingly.

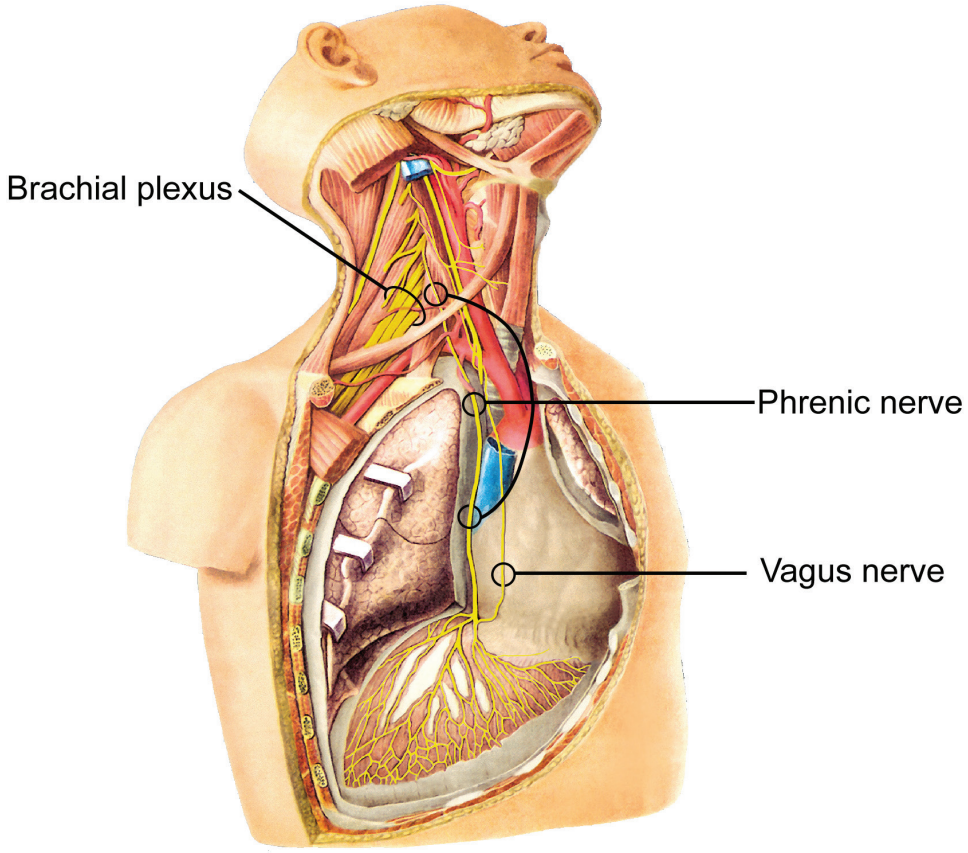

Fig. 4. Illustration demonstrating the course of the phrenic nerve from the root of the neck, through the thorax, and terminating at the diaphragm. Image adapted with permission from Danilo Jankovic and reproduced with permission from Ultrasound for Regional Anesthesia (USRA; http://www.usra.ca).

Persistent Phrenic Nerve Palsy. Persistent phrenic nerve palsy after interscalene block is a complication that has recently gained wider recognition, and its incidence has been estimated from case series data to range from 1 in 2,000[18] up to 1 in 100.[19] There are several potential causes of persistent phrenic nerve palsy that have been put forth in the literature. Nerve damage due to direct needle trauma or intraneural injection has been implicated in case reports of persistent phrenic nerve palsy after landmark-guided interscalene block techniques,[20–23] but not so far with ultrasound-guided interscalene block. Inflammatory scarring causing nerve entrapment has been reported with both landmark-guided and ultrasound-guided interscalene block, and although it has been suggested that this scarring may be related to local anesthetic myotoxicity,[24,25] these are postulated mechanisms without direct supporting evidence at present. A "double crush" syndrome[26] due to previous cervical spine stenosis along with nerve trauma also may contribute to persistent phrenic nerve palsy.[18] Finally, a "triple crush" mechanism that includes pressure ischemia resulting from high volumes of local anesthetic injected within the tight confines of the interscalene sheath also has been postulated.[27] It must be noted that these causes of persistent phrenic nerve palsy differ from those implicated in transient phrenic nerve palsy, and thus it cannot be assumed that strategies to reduce the risk of the latter will also reduce the risk of the former.

Physiologic Effects of Phrenic Nerve Palsy

The diaphragm is the most important inspiratory muscle, accounting for 75% of the increase in lung volume during quiet inspiration; intercostal, scalene, and sternocleidomastoid muscles contribute the remaining 25%. There is little crossover innervation of the right and left hemidiaphragms, and each can contract independently of the other in the event of unilateral phrenic nerve palsy. In the presence of diaphragmatic paresis, inspiration is achieved largely by contraction of intercostal and accessory muscles and expansion of the rib cage.[28] Pleural pressure is reduced, which leads to air intake and expansion of intrathoracic volume.[29] However, this reduction in pleural pressure during inspiration also causes the paralyzed diaphragm to move cephalad and the abdominal muscles inward. Consequently, there is reduced lung ventilation on the affected side, particularly of the lower lobe.[28,30] In healthy individuals, however, tidal volumes remain unchanged due to a greater contribution from the rib cage.[11,28] In higher-risk patient groups, hypoxia and dyspnea may ensue and require treatment by sitting the patient upright and administering supplemental oxygen

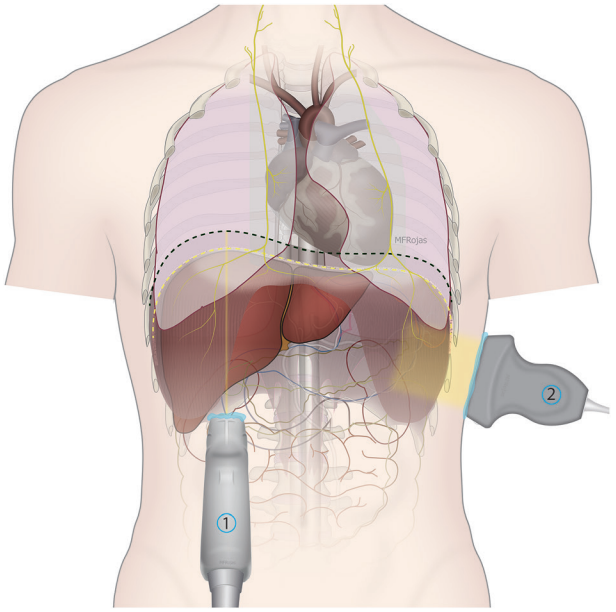

Fig. 5. Diaphragmatic ultrasound. Curved array transducer (1) position for scanning the diaphragm in the midclavicular, right subcostal margin using the liver as an acoustic window, and linear array transducer (2) on the left in the midaxillary line at the level of ribs eight to nine. Image reproduced with permission from Maria Fernanda Rojas Gomez and Ultrasound for Regional Anesthesia (USRA; http://www.usra.ca).

therapy or, in severe cases, instituting noninvasive or invasive ventilatory support to augment tidal volumes.

Subjectively, dyspnea is the cardinal symptom of phrenic nerve palsy after interscalene block. However, just as phrenic nerve palsy does not always result in dyspnea, dyspnea may also be experienced in the absence of phrenic nerve palsy.[31–38] Although up to 40% of patients complain of dyspnea after interscalene block or supraclavicular block,[14,17,39] only one third[40] to three quarters[41] of these patients have objective evidence of phrenic nerve palsy. Patients who are obese are more likely to experience dyspnea in association with phrenic nerve palsy.[42] Thus, although dyspnea clearly is more prevalent in the presence of phrenic nerve palsy,[42] it is neither sensitive nor specific for phrenic nerve palsy. Dyspnea after interscalene block might not be related to the block itself, and other causes must be sought and excluded.

Assessing the Severity of Phrenic Nerve Palsy

The impact of phrenic nerve palsy on respiratory function may be quantified by several bedside methods, including pulse oximetry, pulmonary function tests, and sonographic evaluation of the diaphragm.

Oxygen Saturation. Hypoxemia secondary to unilateral phrenic nerve palsy after regional anesthesia has a low diagnostic sensitivity due to the mechanics of respiratory compensation. Accessory muscles and the contralateral diaphragm both contribute to maintaining gas exchange.

There are conflicting data regarding the incidence and extent of hypoxemia after unilateral phrenic nerve palsy, which probably reflects its multifactorial etiology. Contemporary studies in healthy patients with unilateral phrenic nerve palsy suggest that oxygen saturations may remain unchanged[40] or decrease by less than 7%.[16,43,44] The limited extent of this change correlates with a reduction in Pa_{O_2} of 6 to 7 mmHg and an increase in Pa_{CO_2} of only 3 mmHg.[28] In contrast, hypoxemia may be more significant after interscalene block in patients with multiple comorbidities and who receive higher volumes and/or concentrations of local anesthetic.[33,34,45] In one study of patients with chronic renal failure undergoing arteriovenous fistula surgery, 10% had oxygen saturations less than 85% on room air after a high-volume (30 ml) interscalene block.[45] In another study, brief episodes of oxygen saturations less than 85% after interscalene block with 20 to 28 ml bupivacaine, 0.75%, were seen in 4 of 10 patients, three of whom were obese.[33]

Pulmonary Function Tests. Pulmonary function tests using bedside spirometry to assess diaphragmatic function should be performed with the patient in the semirecumbent position, with the head up at 45°. Baseline pulmonary function tests ideally should be performed before block performance to place postblock values into context and more accurately quantify any deterioration. However, isolated testing after block performance may be compared with predicted values based on patient demographics, although this is less accurate than a comparison with baseline values. Unilateral phrenic nerve injury not related to regional anesthesia reduces the total lung capacity (by 14 to 29%), forced vital capacity (by 23 to 27%), and inspiratory capacity (by 10 to 20%) compared with baseline or predicted parameters.[46–49] Unilateral phrenic nerve palsy after interscalene block reduces the forced expiratory volume in 1 s (FEV_1) by 16 to 40%,[17,50] the forced vital capacity by 13 to 40%,[36,50] and the peak expiratory flow rates by 15 to 43% (tables 1 and 2).[36,40]

Ultrasound. The assessment of phrenic nerve palsy using ultrasound relies on visualizing the diaphragm and quantifying the magnitude and direction of its movement with respiration. The most common method involves placing a 3- to 5-MHz curved array transducer inferior to the costal margin and in a longitudinal parasagittal orientation in the anterior axillary line on the left or in the midclavicular line on the right (fig. 5). The ultrasound beam is directed medially and cephalad to visualize the posterior third of the hemidiaphragm by using either the spleen or the liver as an acoustic window (fig. 6) in a two-dimensional B-mode. Visualization on the left often is technically more challenging due to the smaller acoustic window of the spleen and the presence of the air-filled stomach. Once a view of the curved, hyperechoic diaphragmatic line has been obtained, M-mode sonography is used to quantify the extent of diaphragmatic excursion. In men, the normal displacement of an unaffected diaphragm is 1.8 ± 0.3, 7.0 ± 0.6, and 2.9 ± 0.6 cm in quiet breathing, deep breathing, and sniffing, respectively,

Table 1. Characteristics of Relevant Studies of Regional Anesthesia Techniques for Shoulder Surgery

	Characteristics						
Study	Interscalene Block Technique	Comparison	Target	Drug	Concentration	Volume	Dose
Noncomparative studies							
Urmey *et al.*,[14] 1991, n = 13	SS, LM	—	IS (Winnie approach, C5, C6)	Mepivacaine	1.5%	34–52 ml	510–780 mg
Urmey and McDonald,[13] 1992, n = 8	SS, LM	—	IS (Winnie approach, C5, C6)	Mepivacaine	1.5%	45 ml	675 mg
Neal *et al.*,[11] 1998, n = 8	SS, LM	—	SC (plumb-bob technique)	Lidocaine	1.5%	30 ml	450 mg
Volume effect							
Urmey and Gloeggler,[50] 1993, n = 20	SS, LM	LA volume 20 ml *vs.* 45 ml	IS (Winnie approach, C5, C6)	Mepivacaine	1.5%	45 ml	675 mg
						20 ml	300 mg
Sinha *et al.*,[51] 2011, n = 30	SS, USG	LA volume 10 ml *vs.* 20 ml	IS, intraplexus C5, C6, or C7 roots	Ropivacaine	0.5%	20 ml	100 mg
						10 ml	50 mg
Riazi *et al.*,[16] 2008, n = 40	SS, USG	LA volume 5 ml *vs.* 20 ml	IS, intraplexus C5, C6 roots	Ropivacaine	0.5%	20 ml	100 mg
						5 ml	25 mg
Lee *et al.*,[52] 2011, n = 60	SS, USG	LA volume 5 ml *vs.* 10 ml	IS, intraplexus C5, C6 roots	Ropivacaine	0.75%	10 ml	75 mg
						5 ml	37.5 mg
Concentration effect							
Al-Kaisy *et al.*,[36] 1991, n = 11	SS, NS	LA concentration 0.25% *vs.* 0.5%	C5, C6	Bupivacaine	0.5%	10 ml	50 mg
					0.25%	10 ml	25 mg
Casati *et al.*,[53] 1999, n = 30	SS, NS	LA concentration ropivacaine 0.5% *vs.* ropivacaine 0.75% *vs.* mepivacaine 2%	IS (Winnie approach, C5, C6)	Mepivacaine	2%	20 ml	400 mg
				Ropivacaine	0.75%	20 ml	150 mg
				Ropivacaine	0.5%	20 ml	100 mg
Wong *et al.*,[43] 2016, n = 50	SS, USG	LA concentration 0.1% *vs.* 0.2%	IS, intraplexus around trunks	Ropivacaine	0.2%	20 ml	40 mg
					0.1%		20 mg
Al-Kaisy *et al.*,[54] 1998, n = 30	SS, NS	LA concentration 0.125% *vs.* placebo	C5, C6	0.9% saline	Placebo	10 ml	0 mg
				Bupivacaine	0.125%	10 ml	12.5 mg
Concentration and volume effect							
Pippa *et al.*,[55] 2006, n = 60	SS, NS	LA concentration and volume 0.25/1% *vs.* 0.5/2% 60 (30/30) ml *vs.* 30 (15/15) ml	IS (Winnie approach, C5, C6)	Bupivacaine/ lidocaine	0.25/1%	60 (30/30) ml	75/300 mg
					0.5/2%	30 (15/15) ml	75/300 mg
Zhai *et al.*,[56] 2016, n = 95	SS, USG	LA concentration and volume 0.25% *vs.* 0.5% *vs.* 0.75% 20 ml *vs.* 10 ml *vs.* 6.7 ml	IS, intraplexus C5, C6 roots	Ropivacaine	0.25%	20 ml	50 mg
					0.5%	10 ml	
					0.75%	6.7 ml	
Injection below C6							
Renes *et al.*,[15] 2009, n = 30	SS, USG/NS	Localization method USG *vs.* NS	IS, intraplexus C7 root	NS ropivacaine	0.75%	10 ml	75 mg
				US ropivacaine		10 ml	75 mg
Petrar *et al.*,[41] 2015, n = 64	SS, USG	Injection site supraclavicular *vs.* infraclavicular	IS, intraplexus	SC ropivacaine	0.5%	30 ml	150 mg
			IC, posterior and lateral cords	IC ropivacaine		30 ml	150 mg

(*Continued*)

Table 1. (Continued)

Study	Characteristics						
	Interscalene Block Technique	Comparison	Target	Drug	Concentration	Volume	Dose
Extrafascial injection							
Palhais *et al.*,[17] 2016, n = 40	SS, USG	Injection site extrafascial *vs.* intraplexus	IS, intraplexus C5, C6 roots	Bupivacaine	0.5%	20 ml	100 mg
			IS, extrafascial 4 mm lateral to C5, C6 roots	Bupivacaine		20 ml	100 mg
Catheter technique							
Renes *et al.*,[57] 2010, n = 20	Catheter, USG	Minimum effective volume	IS, intraplexus C7 root	Ropivacaine	0.75%	2–6 ml	15–45 mg
Stundner *et al.*,[58] 2016, n = 30	Catheter, USG	LA volume 5 ml *vs.* 20 ml	IS, intraplexus C5, C6 roots	Ropivacaine	0.75%	20 ml	150 mg
						5 ml	37.5 mg
Hartrick *et al.*,[42] 2012, n = 36	Catheter, USG	LA volume 20 ml *vs.* 10 ml *vs.* 5 ml	IS, intraplexus C5 root	Ropivacaine	0.75%	20 ml	150 mg
						10 ml	75 mg
						5 ml	37.5 mg
Thackeray *et al.*,[44] 2013, n = 30	Catheter, USG	LA concentration 0.125% *vs.* 0.25%	IS, intraplexus C6	Bupivacaine	0.25%	20 ml	50 mg
					0.125%		25 mg
Koh *et al.*,[59] 2016, n = 75	Catheter, USG	Injection site interscalene *vs.* supraclavicular	IS, intraplexus C5, C6 roots	Ropivacaine	0.375%	20 ml	75 mg
			SC, intraplexus	Ropivacaine		20 ml	75 mg
Wiesmann *et al.*,[40] 2016, n = 114	Catheter, USG	Injection site interscalene *vs.* supraclavicular	SC, between upper and middle trunks	Ropivacaine	0.2%	10 ml + 4 ml/h + 4-ml bolus	20 mg
			IS, between upper and middle trunks				

Studies are divided into those assessing outcomes noncomparatively, as well as those describing outcomes by comparing volume effect, concentration effect, injection below C6, extrafascial injection, and catheter techniques.

IC = infraclavicular; IS = interscalene; LA = local anesthetic; LM = landmark; NS = nerve stimulator; SC = supraclavicular; SS = single shot; US = ultrasound; USG = ultrasound-guided.

Table 2. Incidence and Magnitude of Phrenic Nerve Palsy and Analgesic Outcomes of Relevant Studies of Regional Anesthesia Techniques for Shoulder Surgery

Study	Monitoring for PNP	Incidence/Magnitude						Analgesic Outcomes		Remarks
		US Evidence of PNP	FEV_1 Change	FVC Change	PEFR Change	Oxygen Saturation	Dyspnea	Pain Scores	Analgesic Consumption	
Noncomparative studies										
Urmey *et al.*,[14] 1991, n = 13	US	Sniff +5.96 cm to –4.53 cm (176% reduction)	—	—	—	—	38%	—	—	—
Urmey and McDonald,[13] 1992, n = 8	US, PFTs	100%	–26.4%	–27.2%	–15.4%	—	—	—	—	Magnetometry demonstrating increased chest wall movement (indicating use of intercostal and accessory muscles) with reduced ipsilateral abdominal movement
Neal *et al.*,[11] 1998, n = 8	US, PFTs	50%	Paralyzed: +6% Nonparalyzed: 0%	+2% +2%	+7% +6%	0%	0%	—	—	—
Volume effect										
Urmey and Gloeggler, [50] 1993, n = 20	US, PFTs	100%	–39.9%	–40.9%	–35.9%	—	—	—	—	—
		100%	–31.6%	–32%	–32%	—	—	—	—	
Sinha *et al.*,[51] 2011, n = 30	US, PFTs	93%	–35%	–32%	–19%	–	—	—	PACU fentanyl, 0 μg; home hydrocodone, 25 mg	No significant difference in analgesic consumption
		93%	–29%	–31%	–23%	–	—	—	0 μg ,30 mg	
Riazi *et al.*,[16] 2008, n = 40	US, PFTs	100%	–1.23 l	–1.59 l	–2.50 l/min	–5.85%	5%	VAS at 30 min, 0.3; 60 min, 1; 120 min, 1.3; 12 h, 3.1; 24 h, 4.7	Intraoperative fentanyl, 107.5 μg; PACU MEQ, 1.3 mg; 24-h MEQ, 26.5 mg	No significant difference in pain scores or analgesic consumption
		45%	–0.83 l	–0.7 l	–0.83 l/min	–1.5%	0%	1.1, 1.1, 0.5, 3.4, 3.6	140.3 μg, 2.9 mg, 23.3 mg	
Lee *et al.*,[52] 2011, n = 60	CXR	—	—	—	—	—	—	—	—	CXR diaphragmatic paresis 60% *vs.* 33%
Concentration effect										
Al-Kaisy *et al.*,[36] 1999, n = 11	US, PFTs	100%	–21.8%	–25.4%	–15.2%	—	0%	—	—	Volunteer study
		16%	–9.4%	–13.4%	–16.7%	—	0%	—	—	
Casati *et al.*,[53] 1999, n = 30	US, PFTs	100%	–40%	–39%	—	—	33%	—	No analgesia, 0 patients	No difference in pain scores. Longer time to first analgesic with both ropivacaine groups.
		100%	–38%	–41%	—	—	0%	—	No analgesia, 0 patients	
		100%	–30%	–40%	—	—	8.3%	—	No analgesia, 2 patients (20%)	

(*Continued*)

Table 2. (Continued)

Study	Monitoring for PNP	Incidence/Magnitude: US Evidence of PNP	Incidence/Magnitude: FEV_1 Change	Incidence/Magnitude: FVC Change	Incidence/Magnitude: PEFR Change	Incidence/Magnitude: Oxygen Saturation	Incidence/Magnitude: Dyspnea	Analgesic Outcomes: Pain Scores	Analgesic Outcomes: Analgesic Consumption	Remarks
Wong *et al.*,[43] 2016, n = 50	US, PFTs	66%	–28%	–33%	–34%	No desaturations	0%	NRS at 30 min, 0; 60 min, 0; AUC POD 0-3, 8.4	PACU fentanyl, 18 μg; 72-h codeine, 55 mg	Only difference in 72-h codeine requirement, no other pain score or analgesic consumption differences
		35%	–20%	–22%	–27%	No desaturations	0%	0, 0, 12.8	25, 102	
Al-Kaisy *et al.*,[54] 1998, n = 30		—	—	—	—	—	—	VAS at 20 min, 8.1; 30 min, 6.9; 60 min, 5.0; 120 min, 3.6	PACU morphine, 9.5 mg	—
		—	—	—	—	—	—	3.4, 3.5, 2.5, 2.2	PACU morphine, 2.7 mg	
Concentration and volume effect										
Pippa *et al.*,[55] 2006, n = 60	US	0%	—	—	—	—	—	—	—	Significantly better clinical outcomes and degree of analgesia in high-volume, low-concentration group in all regions of upper limb except for lateral neck and arm
		27%	—	—	—	—	—	—	—	
Zhai *et al.*,[56] 2016, n = 95	US	70%	—	—		—	—	NRS at PACU, 0; 4 h, 0; 8 h, 0; 24 h, 0; worst, 3	—	No significant difference in diaphragmatic paresis, pain scores, or satisfaction
		69%	—	—		—	—	0, 0, 0, 1, 3	—	
		58%	—	—		—	—	0, 0, 0, 1, 3	—	
Injection below C6										
Renes *et al.*,[15] 2009, n = 30	US, PFTs	Sigh, –95%; sniff, –87%	–0.9 l (–33%)	–1.2 l (–36%)	–105.1 l/min (–28%)	—	—	NRS at 30 min, 2	13% needing morphine	No significant difference in pain scores or analgesic consumption
		–11%, + 60%	–0.1 l (–4%)	–0.1 l (–3%)	–8 l/min (–2%)			NRS at 30 min, 2	13% needing morphine	
Petrar *et al.*,[41] 2015, n = 64	US	Partial, 9%; complete, 34%	—	—	—	—	25%	—	—	Supraclavicular *vs.* infraclavicular blocks
		Partial, 9%; complete, 3%	—	—	—	—	16%	—	—	
Extrafascial injection										
Palhais *et al.*,[17] 2016, n = 40	US, PFTs	90%	–0.9 l (–28%)	–1.2 l (–28%)	–2.1 l/s (–24%)	—	30%	NRS at PACU, 0.5; 24 h, 1.6	PACU rescue (n), 0; 24-h MEQ, 5 mg; 48-h MEQ, 7.5 mg	No significant difference in analgesic consumption after PACU
		21%	–0.6 l (–16%)	–0.8 l (–17%)	–0.7 l/s (–8%)	—	0%	0.4, 1.6	5, 5, 10 mg	

(*Continued*)

Table 2. (Continued)

Study	Monitoring for PNP	Incidence/Magnitude: US Evidence of PNP	Incidence/Magnitude: FEV_1 Change	Incidence/Magnitude: FVC Change	Incidence/Magnitude: PEFR Change	Incidence/Magnitude: Oxygen Saturation	Incidence/Magnitude: Dyspnea	Analgesic Outcomes: Pain Scores	Analgesic Outcomes: Analgesic Consumption	Remarks
Catheter technique										
Renes *et al.*,[57] 2010, n = 20	US, PFTs	0% in all patients at 2 h	0%	0%	0%	—	—	—	—	No changes in respiratory function with ≤6 ml injection, but changes after 24 h of infusion of 6 ml/h 0.2% ropivacaine in 100% of patients
Stundner *et al.*,[58] 2016, n = 30	US, PEFR	53%	—	—	−2.66 l/min	—	—	—	Intraoperative fentanyl, 200 μg	Nonsignificant difference in PNP. No significant difference in rest or dynamic pain scores or analgesic consumption.
		27%	—	—	−1.69 l/min		—	—	150 μg	
Hartrick *et al.*,[42] 2012, n = 36	US	−65% excursion	—	—	—	—	33%	NRS at PACU D/C, 0.62; 24 h, 2.54; 48 h, 2.15; 12 wk, 1.15	24-h MEQ, 13.4 mg	Only difference at PACU D/C between 5 ml and 20 ml, no other pain score or analgesic consumption differences
		−60% excursion	—	—	—	—	0%	1.58, 3.33, 2.08, 2.42	24-h MEQ, 29.8 mg	
		−66% excursion	—	—	—	—	8.3%	2.67, 3.67, 2.67, 1.5	24-h MEQ, 27.7 mg	
Thackeray *et al.*,[44] 2013, n = 30	US, oxygen saturations	78%	—	—	—	−4.3%	0%	0	Fentanyl, 0 μg	
		21%	—	—	—	−2.6%	0%	1	Fentanyl, 7 μg	
Koh *et al.*,[59] 2016, n = 75	US	Partial, 32%; complete, 63%	—	—	—	—	—	Mean NRS, 2.84	PACU morphine, 1 mg; 24-h MEQ, 8 mg	No significant difference in pain scores or analgesic consumption
		Partial, 26%; complete, 24%	—	—	—	—	—	Mean NRS, 2.57	0 mg, 7 mg	
Wiesmann *et al.*,[40] 2016, n = 114	US, PFTs	PACU, −82%; POD1, −46%	−33%	−38%	−32%	+1%	8%	0–1	Ward rescue opioids, 16%	No significant difference in pain scores or analgesic consumption
		PACU, −55%; POD1, −34%	−32%	−30%	−43%	+1%	7%	0–0	Ward rescue opioids, 5.2%	

Studies are divided into those assessing outcomes noncomparatively, as well as those describing outcomes by comparing volume effect, concentration effect, injection below C6, extrafascial injection, and catheter techniques.

CXR = chest X-ray; D/C = discharge; FEV_1 = forced expiratory flow rate in 1 s; FVC = forced vital capacity; MEQ = morphine equivalents; NRS = numerical rating scale; PACU = postanesthetic care unit; PEFR = peak expiratory flow rates; PFTs = pulmonary function tests; PNP = phrenic nerve palsy; POD = postoperative day; VAS = visual analog score; US = ultrasound.

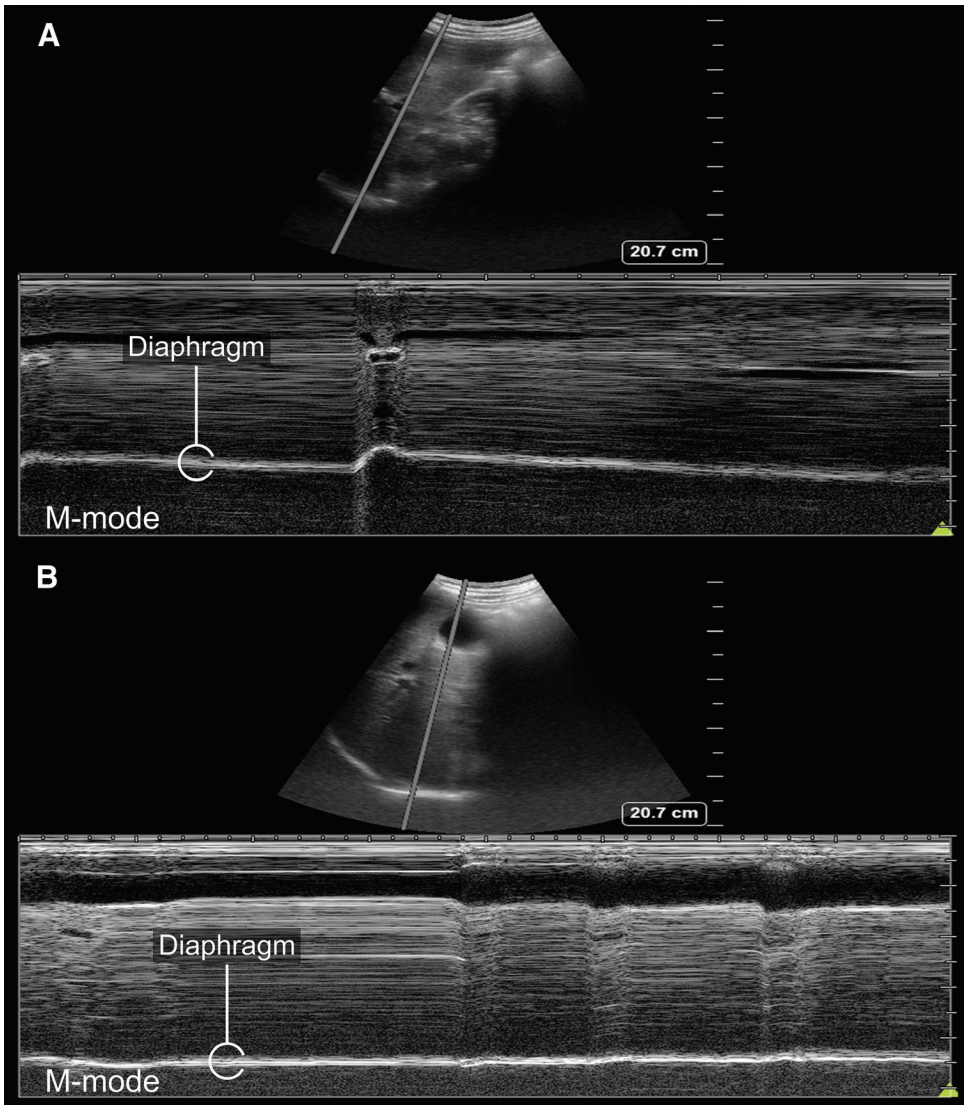

Fig. 6. Curved array transducer ultrasound image of the right diaphragm using the liver as an acoustic window in two-dimensional B-mode and M-mode. (*A*) Preblock sniff test assessment for phrenic nerve palsy. The diaphragm (*white circle*) is seen to move caudally, toward the probe, in M-mode. (*B*) Postblock sniff test assessment for phrenic nerve palsy. There is no movement of the diaphragm seen in M-mode, indicating phrenic nerve palsy.

and 1.6 ± 0.3, 5.7 ± 1.0, and 2.6 ± 0.5 cm in women.[60] Once again, it is ideal to obtain baseline measures of diaphragmatic excursion before block performance. Although evidence of hemidiaphragmatic paresis may be seen within 5 min of local anesthetic injection, measurement should be repeated 15 to 30 min after block completion to allow time for the full extent of phrenic nerve palsy to develop.[14,50] In the presence of partial phrenic nerve palsy, a forceful rapid sniff (the sniff test) can demonstrate partial diaphragmatic paresis with a 25 to 75% reduction in caudal movement (toward the transducer) of the diaphragm. Complete phrenic nerve palsy may be diagnosed by paradoxical cephalad movement of the diaphragm[61,62] or a 75% or greater reduction in diaphragmatic movement.[15,41] Diaphragmatic ultrasound has been shown to have high sensitivity (93%) and specificity (100%) in diagnosing phrenic nerve dysfunction.[63]

An alternative, simpler ultrasound approach that may be used involves placing a high-frequency (10 to 15 MHz) linear array transducer in the coronal plane at the midaxillary line to obtain an intercostal view.[64] At the level of ribs eight to nine on the left and seven to eight on the right, the spleen or liver are centered with the rib shadows on either side (fig. 5). On deep inspiration, caudal descent of the liver or spleen precedes descent of the bright pleural line (fig. 7). The transducer is then moved in both caudal and cephalad directions to visualize the end-inspiratory and end-expiratory levels of the pleural line, respectively, which are then marked on the patient's skin. This process is repeated before and after the chosen regional anesthetic technique with the patient in

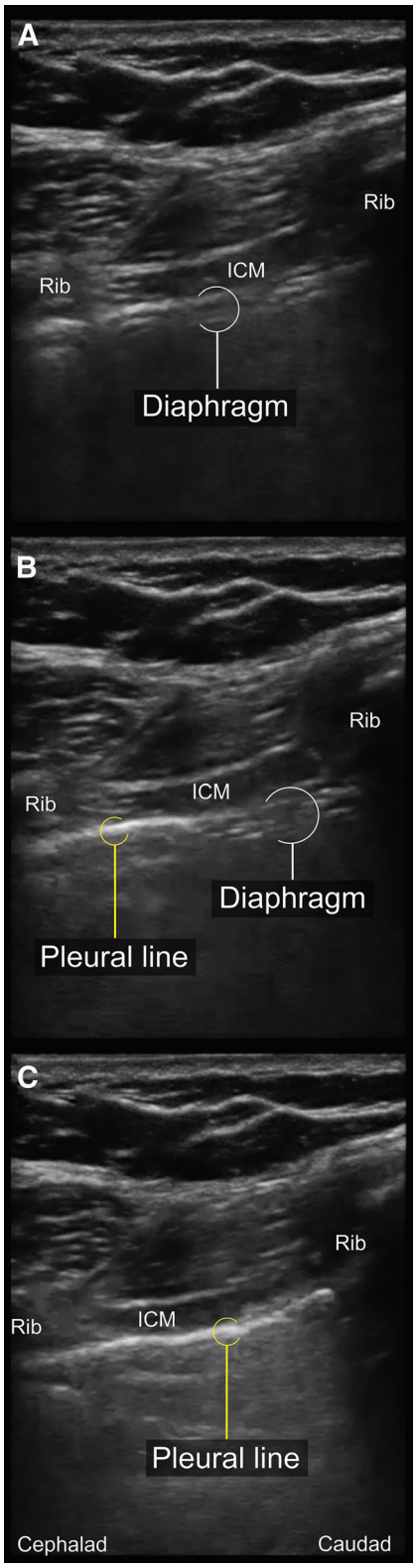

Fig. 7. Linear array transducer ultrasound image of the pleura in the right midaxillary line at the level of the seventh and eighth ribs in (*A*) early inspiration; (*B*) mid-inspiration; and (*C*) end-inspiration. The pleural line (*yellow circle*) can be seen progressively descending with inspiration. The intercostal muscles (ICM) lie superficial to the diaphragm (*white circle*).

the same position. Minimal change signifies no block, but a reduction in this distance represents phrenic nerve palsy.[65] Although this technique is yet to be validated, it is a simple qualitative assessment that relies on the gross caudad movement of the pleural line during inspiration representing diaphragmatic excursion and thus phrenic nerve function.

Correlation between Parameters

Quantified sonography of the diaphragm is more sensitive to changes in unilateral diaphragmatic dysfunction than pulmonary function tests and oxygen saturation because the latter two variables assess bilateral pulmonary function simultaneously, including the use of accessory muscles and contralateral diaphragmatic activity. However, there may be some correlation between these parameters. Borgeat *et al.*[66] demonstrated that a 60 to 80% reduction in unilateral diaphragmatic excursion with forced respiration was associated with a 30 to 40% reduction in both vital capacity and FEV_1. Similarly, our group has demonstrated that hemidiaphragmatic paresis resulted in a decrease in forced vital capacity and FEV_1 to 75 and 78% of baseline, respectively.[36] However, these patients remain asymptomatic and require no treatment. Although there is clearly some correlation between pulmonary function test changes and ultrasound evidence of unilateral diaphragmatic paresis, no study has explicitly and specifically assessed the correlation between ultrasound, pulmonary function test, oxygen saturations, and subjective symptoms of dyspnea.

Strategies to Reduce Phrenic Nerve Palsy in Regional Anesthesia of the Shoulder

Transient phrenic nerve palsy after regional anesthesia for shoulder surgery results from a direct inhibitory effect of local anesthetic on the phrenic nerve or its roots (C3–C5), and thus minimizing its occurrence depends on reducing the dose of local anesthetic reaching these neural structures. This can be achieved by modifying the local anesthetic dose (volume and concentration),[54] injection site and technique in interscalene block, or by modifying the location of local anesthetic injection and using a different regional anesthetic technique altogether. Ultrasound has been instrumental in the development of these modifications: the increased accuracy of local anesthetic deposition allows the use of lower doses, and direct visualization increases the range of available sites for injection. In the following section, we review the evidence for the effectiveness of these various modifications in minimizing the risk of phrenic nerve palsy while preserving analgesic efficacy.

Modifications of Interscalene Block

Local Anesthetic Volume. There is a clear relationship between the volume of local anesthetic injected during interscalene block and the occurrence of phrenic nerve palsy. This is likely to be related to the greater extent of spread that occurs with larger volumes. An injection around the C5–C6 nerve roots with

volumes of 20 ml or greater inevitably produces phrenic nerve palsy, regardless of localization technique.[16,50,51,53] When an ultrasound-guided technique is used, a volume of 10 ml reduces the incidence of phrenic nerve palsy to as low as 60%,[52] whereas a volume of 5 ml reduces it still further to between 27[58] and 45%,[16] without compromising analgesic efficacy up to 24 h postoperatively.[16,52] Although McNaught *et al.*[67] determined that the minimum effective volume for achieving analgesia for shoulder surgery with ultrasound-guided interscalene block at the C5–C6 nerve root level is as low as 0.9 ml ropivacaine, 0.5%, it must be noted that the duration of analgesia was not assessed formally beyond the first 30 min after surgery. The incidence of respiratory compromise was not reported in this study, so it is unclear whether there are further reductions in the incidence of phrenic nerve palsy at volumes less than 5 ml.

Local Anesthetic Concentration. Several studies have shown that reducing local anesthetic concentration independent of volume, thus reducing the dose of drug delivered, also produces a significant decrease in the incidence of phrenic nerve palsy and an improvement in pulmonary function after landmark- or ultrasound-guided interscalene block.[36,43,44] With a nerve stimulator-guided interscalene block, halving the concentration of a 30-ml mixture of 0.5% bupivacaine and 2% lidocaine but doubling the volume led to a reduction in phrenic nerve palsy from 27% to 0%.[55] Halving the concentration of bupivacaine from 0.5% to 0.25% reduced the incidence of phrenic nerve palsy from 100% to 17% when 10 ml was administered *via* a landmark approach[36] and from 78% to 21% when 20 ml was administered with nerve-stimulator localization.[44] Similarly, the incidence of phrenic nerve palsy was reduced from 71% to 42% by halving the concentration of 20 ml ropivacaine from 0.2% to 0.1% in an ultrasound-guided interscalene block.[43] Unfortunately, this reduction in phrenic nerve palsy generally appears to come at the expense of reduced analgesic efficacy. The reduction in local anesthetic concentration and dose decreased duration of sensory blockade by 34% and increased postoperative opioid requirements by up to 50%.[43,44] Zhai *et al.*[56] demonstrated that there is no significant difference in the incidence of phrenic nerve palsy with a fixed 50-mg dose of ropivacaine for ultrasound-guided interscalene block using concentrations of 0.25, 0.5, or 0.75%, with minimal effect on analgesic outcomes.

Site of Injection.

Periplexus Injection. Recently, the concept of ultrasound-guided periplexus (between the interscalene muscles and brachial plexus nerve sheath) injection of local anesthetic has been introduced for interscalene block. Palhais *et al.*[17] recently reported that an ultrasound-guided extrafascial (periplexus) injection of 20 ml bupivacaine 0.5%, performed 4 mm lateral to the brachial plexus sheath not only provided similar analgesia compared with an intraplexus injection between the C5 and C6 roots but also reduced the incidence of diaphragmatic paresis from 90% to 21%. In addition, FEV_1, forced vital capacity, and peak expiratory flow rates were less affected in the extrafascial group compared with an intraplexus injection, decreasing by 16 *versus* 28%, 17 *versus* 28%, and 8 *versus* 24%, respectively.[17]

Intrafascial Injection Below C6 Level. Another strategy to avoid phrenic nerve palsy involves injecting local anesthetic further away from the C5 and C6 roots and phrenic nerve. Renes *et al.*[15] showed that ultrasound-guided injection of 10 ml ropivacaine 0.75%, around the C7 nerve root resulted in similar analgesia, but only a 13% incidence of phrenic nerve palsy compared with 93% with a neurostimulation-guided interscalene block using the same dose of local anesthetic. Recovery of diaphragmatic function also was faster in the patients who received the C7 root injection. In a subsequent study, the same authors reported that the minimum effective anesthetic volume to achieve complete sensory block of C5 and C6 dermatomes within 30 min in 50% of patients using this technique was 2.9 ml ropivacaine 0.75%. They noted that none of the 20 patients who received 6 ml ropivacaine 0.75%, or less had any evidence of diaphragmatic paresis up to 2 h after injection.[57]

Injection Posterior versus Interior to the C5–C6 Nerve Roots. A recent study compared the effect of performing an ultrasound-guided intraplexus injection on the anterior *versus* posterior aspect of the C5–C6 nerve roots with 15 ml ropivacaine 1%.[35] There was a similar reduction of 12 to 28% in all pulmonary function parameters in both groups. Once again, this suggests that the dose and volume of local anesthetic and the caudocephalad level at which it is injected are the most significant factors affecting incidence of phrenic nerve palsy.

Injection Method. There are no studies reporting the impact of injection dynamics on phrenic nerve palsy. It is possible that a slower, lower-pressure, titrated injection of low-volume aliquots also may limit spread of injectate to the phrenic nerve, but this is yet to be supported by published evidence. Interestingly, injection of local anesthesia through a catheter appears to produce a less dramatic change in diaphragmatic sonographic excursion than if the same large-volume bolus was injected directly through a needle, possibly supporting a benefit of titrated injection.[66] This suggests that injection dynamics may play an important role in development of diaphragmatic dysfunction and should be investigated further.

Alternatives to Interscalene Block

The conventional ultrasound-guided interscalene block is a direct carryover from the landmark-guided approach, which relied on the interscalene groove and the anterior tubercle of the C6 transverse process as key landmarks, and thus necessitated a needle approach to the brachial plexus at the root level. This restriction no longer exists; ultrasound allows visualization of the entire brachial plexus and its individual branches, and thus similar analgesic effects can be achieved with more selective injection further away from the phrenic nerve and the C5 and C6 roots.

Superior Trunk Block. The superior trunk is formed by the union of C5 and C6 roots and is an appealing alternative target for local anesthetic injection, given that the phrenic nerve has diverged a considerable distance away from the brachial plexus at this level. All the terminal nerves supplying the shoulder arise distal to the origin of the superior trunk and hence analgesic efficacy is not compromised. However, to date, only two case reports of this technique have been published,[68,69] and data supporting its effectiveness in minimizing phrenic nerve palsy are still awaited.

The superior trunk also can be targeted at the supraclavicular brachial plexus level. Injection with 20 to 30 ml local anesthetic is associated with a 25 to 51% incidence of phrenic nerve palsy in both landmark- and ultrasound-guided studies.[11,40,41,59] Nonetheless, inferior sensory block and greater analgesic requirements in shoulder surgery have been reported with supraclavicular brachial plexus block compared with interscalene block.[37,59] This may reflect incomplete blockade of the suprascapular nerve, which has left the brachial plexus at this point. Related to this, placement of a supraclavicular brachial plexus catheter just proximal to the exit of the suprascapular nerve from the common superior trunk sheath has been described; there were apparently no episodes of phrenic nerve palsy with this technique reported in published correspondence.[70] Clinical trials to verify its efficacy are awaited.

Suprascapular and Axillary Nerve Block. The risk of phrenic nerve palsy might be eliminated by avoiding injection around the brachial plexus and performing a suprascapular nerve and axillary nerve block instead. The suprascapular nerve provides sensory fibers to approximately 70% of the shoulder joint capsule, and blocking this peripheral nerve can be performed with either a landmark-guided[71–73] or ultrasound-guided technique.[4,74,75] The suprascapular nerve can either be blocked in the suprascapular fossa or in the root of the neck distal to where it arises from the superior trunk of the brachial plexus.[76] However, large volumes of injection in the latter approach may still potentially lead to local anesthetic spread to the phrenic nerve and its roots.

The axillary nerve is a terminal branch of the posterior cord of the brachial plexus. It may be blocked in the anterior chest where it arises from the posterior cord of the brachial plexus in the infraclavicular and proximal axillary area[77] or posterior to the humerus as it emerges from the quadrangular space.[74,75] This latter approach may occasionally miss the articular branches of the axillary nerve and may be responsible for inferior analgesic outcomes.[78]

In arthroscopic shoulder surgery, suprascapular nerve block alone or combined with an axillary nerve block has been shown to provide superior analgesia compared with placebo or subacromial local anesthetic infiltration[79–81] but is less effective compared with interscalene block.[78,79] Because this peripheral nerve block technique primarily targets the capsular innervation of the shoulder, it also may be less useful in open or extensive shoulder surgery.[82] Nevertheless, this technique has a good safety record in chronic pain practice[83] and has not been associated with any reported episodes of phrenic nerve palsy to date. In view of the trade-off in analgesic efficacy, suprascapular and axillary nerve blocks are probably best reserved for patients with preexisting respiratory dysfunction or who have other comorbidities (*e.g.*, obesity) that are likely to lead to clinically significant dyspnea and hypoxemia in the presence of unilateral phrenic nerve palsy.

Catheter Techniques

The risk of phrenic nerve palsy appears to be different between single-shot and continuous interscalene block. Infusion rates of 4 to 6 ml/h will invariably lead to phrenic nerve palsy over the first 24 h, regardless of the concentration of the local anesthetic.[84] Similarly, Renes *et al.*[15] found that although there was no evidence of phrenic nerve palsy 2 h after a small bolus injection of 0.75% ropivacaine at the C7 nerve root, all patients developed either partial or complete phrenic nerve palsy after a 24-h infusion of 0.2% ropivacaine at 6 ml/h.

Strategies that have been proposed to reduce phrenic nerve palsy while preserving analgesic efficacy include limiting infusion rates to 2 ml/h.[85] In a letter to the editor, Tsui and Dillane[86] reported that using an intermittent bolus regimen reduces phrenic nerve palsy; however, this has yet to be confirmed in a formal clinical trial. Another approach is to use a short-acting agent such as lidocaine instead of ropivacaine or bupivacaine. In the event of respiratory compromise due to phrenic nerve palsy, cessation of the infusion should result in a more rapid return of phrenic nerve function.[87] It also may be possible to speed up the resolution of phrenic nerve palsy by administering a bolus of 0.9% sodium chloride through the catheter to "wash off" residual local anesthetic.[86]

Future Trends

As discussed previously, the use of ultra-low volumes and doses of local anesthetic will minimize the risk of phrenic nerve palsy but at the expense of reduced duration of analgesia. The use of intravenous dexamethasone or perineural local anesthetic adjuvants that prolong the duration of sensory-motor blockade and analgesia[88–90] are a promising way to address this issue and should be specifically studied in this context. However, the potential risk and impact of a prolonged phrenic nerve block must still be considered, because none of the described techniques to date guarantee that this can be avoided completely. Liposomal bupivacaine[91,92] is not approved presently for perineural injection, but using it in local wound infiltration may be an alternative worthy of further study.[93,94] Continued investigation also is needed into the optimal dosing strategy in continuous catheter techniques, as well as the impact of different injection methods, including titrated dosing, the use of low injection pressures, as well as the concept of reversal of phrenic nerve palsy by local anesthetic washout.[86]

Conclusions

Regional anesthesia continues to be of value in providing analgesia for shoulder surgery, but its benefits must be weighed against the risks, including phrenic nerve palsy. The high incidence of phrenic nerve palsy associated with the conventional technique of interscalene block have led some to propose that "the safest option to avoid phrenic nerve block would be to avoid performing an interscalene block" altogether.[3] However, the evidence indicates first that temporary phrenic nerve palsy is inconsequential in the vast majority of healthy patients and, second, that relatively simple modifications such as minimizing local anesthetic doses and injection volumes (to less than 10 ml), as well as performing injection further distal to the C5–C6 nerve roots (*e.g.*, at the level of the superior trunk or supraclavicular brachial plexus), will significantly reduce the incidence of phrenic nerve palsy. Combined suprascapular and axillary nerve blocks are another alternative to consider in scenarios in which avoiding phrenic nerve palsy is critical, particularly in arthroscopic shoulder surgery. We encourage practitioners to use the principles and methods outlined in this article to refine and tailor their regional anesthetic strategy to each patient in their care, taking into account all the medical and surgical considerations pertinent to that individual.

Acknowledgments

The authors thank Maria Fernanda Rojas Gomez, M.D., Staff Anesthesiologist, Sociedad Especializada de Anestesiologia SEA S.A. Fundacion Oftalmologica de Santander, Clinica Carlos Ardila Lulle, Floridablanca, Santander, Colombia, and Danilo Jankovic, M.D., Chief of Staff, Pain Management Center, St. Elisabeth University Hospital, Cologne, Germany, for their invaluable assistance in the production of the illustrations.

Research Support

Support was provided solely from institutional and/or departmental sources.

Competing Interests

Dr. Chan receives honoraria from SonoSite, Inc. (Bothell, Washington) and is on the medical advisory board of Smiths Medical (Ashford, United Kingdom) and Aspen Pharmacare (Durban, South Africa). The other authors declare no competing interests.

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ANESTHESIOLOGY REFLECTIONS FROM THE WOOD LIBRARY-MUSEUM

William B. Quinn, M.D., Eclectic Physician and Occasional "Anaesthetist"

In Springfield, Ohio, on St. Patrick's Day of 1920, a female patient paid $15 to Clayton W. Russell, M.D. (1866 to 1922). Her receipt from the surgeon (*above*) allocated $5 to his surgical assistant, Howard H. Austin, M.D. (1880 to 1915). The remaining $10 was designated for "Dr. Quinn, Anaesthe[ti]st." So who was Doctor Quinn? A Kentucky native, William Babbit Quinn, M.D. (1892 to 1970), was raised by his Eclectic-physician mother after she was widowed during William's first week of life. Young William followed in his mother's footsteps, graduating in 1913 from her alma mater, the Eclectic Medical Institute of Cincinnati, Ohio. He trained and practiced in Springfield, Ohio, Blackwell's Island, New York, and then Hollywood, California, before settling back in Springfield. The depicted receipt was likely issued from the surgeon's office in the Fairbanks Building, where all three of these Eclectic alumni, Drs. Russell, Austin, and Quinn, maintained professional offices. In this time period, surgeons could charge the equivalent of 5 to 10% of their surgeon's fee from patients to pay for services rendered by the anesthetist.

George S. Bause, M.D., M.P.H., Honorary Curator and Laureate of the History of Anesthesia, Wood Library-Museum of Anesthesiology, Schaumburg, Illinois, and Clinical Associate Professor, Case Western Reserve University, Cleveland, Ohio. UJYC@aol.com.

MIND TO MIND
Creative writing that explores the abstract side of our profession and our lives

Stephen T. Harvey, M.D., Editor

Texting under Anesthesia

David Guardiola, Michael Guardiola, Scott D. Cook-Sather, M.D.

The Patient (age 16)

The morning of my surgery. Oh God. Time for people I have never met to go digging around in my back to pull out a tumor that has been there for God knows how long. I am stressed but don't show it. I use my phone to find a gift for my girlfriend. I am happily absorbed in the search, then sad: I will miss her birthday while recovering in the hospital.

My nervousness returns. And over there are Mom and Dad, tense and uneasy. How bad must they feel right now? Suddenly, an amusing idea. I take my phone and schedule a text message to be sent to my parents well after surgery is underway. This will lighten the mood!

> *Okay so u may be creeped out by me texting you and not being conscious. Weird things happen. Get over it. Just remember that I love you and I'll be out soon.*

The Father

The day before our 20th wedding anniversary we rise before dawn to take our oldest son to the children's hospital. A week ago we received news of a tumor in his spine. Life stops. And now we are in the holding area, about

Carol Wiley Cassella, M.D., served as Handling Editor for this submission.

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Accepted for publication September 22, 2016.

Anesthesiology 2017; 127:192-3

to say "I'll see you later …" He seems distracted, texting away before having to relinquish his phone. Probably writing his friends.

Now he is off. We wait. We take a walk and get breakfast. More waiting. I'm worried–about David, about my wife, about what is to come. More waiting. Another walk to get lunch. Just as we start to eat, we both get a text from our son. I look again at my phone. I look up at my wife. Really, he's texting us? But I can see his phone in my wife's purse. What is going on? I read his message and the tears well up. Seconds later another message confirms he did this to crack us up. He figured out how to delay sending us a text message until mid-surgery. When I saw him texting and seemingly ignoring us earlier, he was crafting a message to reassure us. For an instant, I had thought he was talking to us from beyond. I want to kill him and kiss him at the same time. We tell the nurse what he did and she gets such a kick out of it that she goes to share the story in the OR.

The Anesthesiologist

I meet a quiet patient and family in the preoperative area. In the moment before anything is said, I am taken back some twenty-five years when I was the patient about to undergo surgery for a large, worrisome mass. This quiet is the fear I know well, but I can't tell my story now. I got reprieve; he may not. I am concerned and supportive. I hope that in providing some procedural details, I also offer comfort.

He is safely off to sleep, the intravascular catheters and breathing tube are in, and he is turned prone. The neurophysiologist places electrodes to monitor the integrity of his spinal cord and those nerves in the vicinity of the tumor. The surgeons are operating and we watch for progress. We prepare for potential untoward events. The patient becomes the typical complex array of data projected onto screens. He is here, but he is not here. He is under anesthesia.

Elsewhere the pathologist evaluates the biopsy specimen. We wait in the OR while the beep, beep, beep of the pulse oximeter marks time. The parent nurse liaison enters and reports the texting mischief. The news fills the OR with grins and laughter; the data, metrics and the even the tumor itself all recede. David the prankster emerges! A colleague declares, "hire this guy!" David's creativity and love move us all.

The initial pathology is favorable, and the final–benign.

The Patient

As I recover, I am sore and I am happy. My parents are relieved and proud. I learn that they shared our texting story with some of the nurses and doctors caring for me. I tell myself, "Great now everyone thinks I'm a weirdo." But they don't. They think I am way cool.

Should Neuromuscular Blocking Agents Always Be Reversed?

To the Editor:

We read with great interest the Bulka *et al.*[1] article associating intraoperative neuromuscular blockade administration with postoperative pneumonia. We found the conclusion that not "reversing" neuromuscular blockade was associated with an increased risk of postoperative pneumonia to be particularly important. However, we find the title of the accompanying editorial ("To Reverse or Not to Reverse?" The Answer Is Clear![2]) to be misleading, and the second part of the text in the editorial's figure ("reversal of neuromuscular blocking agents should be routine") to differ from what we would consider safe and patient-centered practice.

We believe that reversal of neuromuscular blockade should only occur when guided by neuromuscular transmission monitoring, preferably quantitative. The authors also believe this, as it is written in their editorial: "Unless there is quantitative evidence that the TOF [train-of-four] ratio at the adductor pollicis has returned to a value of more than or equal to 0.9, an appropriate dose of an anticholinesterase agent or sugammedex should be administered at the end of surgery."[2] However, we find this statement to be incongruent with their conclusion that "reversal of neuromuscular blocking agents should be routine."

"Routine reversal" of neuromuscular blockade with neostigmine is not settled science. It has been shown to improve clinical outcomes,[1,3] have little effect on clinical outcome,[4,5] and even cause harm at high doses,[6] all dependent upon clinical context. Neostigmine should not be used in a patient with deep neuromuscular blockade.[7] Patients who have already recovered their strength (TOF greater than 0.9) may be weakened through the administration of neostigmine, as demonstrated in a healthy volunteer study.[8] The only way to prevent these two dangerous situations is to monitor the patient depth of neuromuscular blockade and reverse appropriately.[9]

Furthermore, we would like to clarify an additional point from the editorial regarding our prospective, observational study of 3,000 postoperative patients who were intraoperatively administered intermediate-acting neuromuscular blocking agents.[10] Quantitative neuromuscular transmission monitoring within 10 min of postanesthesia care unit arrival was included in the statistical model. In our study, neostigmine usage was associated with increased diagnoses of atelectasis, and, in *post hoc* analysis, unwarranted neostigmine usage was independently associated with pulmonary edema and reintubation.

We offer the strong suggestion that the administration of neuromuscular blocking agents and reversal agents be guided by frequent neuromuscular transmission monitoring, preferably quantitatively. No reversal should be administered until there are at least two twitches, and no reversal should be administered if there is a TOF greater than 0.9.[11]

Competing Interests

Dr. Eikermann received research funding from Merck & Co. (Kenilworth, New Jersey) and holds equity stakes in Calabash Bioscience, Inc. (College Park, Maryland). The other author declares no competing interests.

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(Accepted for publication April 13, 2017.)

Neuromuscular Blockade and Risk of Postoperative Pneumonia

To the Editor:

I read with interest the recent article by Bulka *et al.*,[1] which highlighted the association between perioperative use of neuromuscular blocking drugs and risk of postoperative pneumonia. It would have been useful to know which airway devices were used for the patients studied, because endotracheal intubation itself is known to be a risk factor for postoperative pneumonia and could therefore be a confounding factor. Of course, in the majority of cases, neuromuscular blockade is a prerequisite for endotracheal intubation, but not infrequently in the United Kingdom neuromuscular blockade is used in combination with a supraglottic airway device; this is generally restricted to cases where muscle relaxation is required to facilitate surgery and there is no requirement for a definitive airway. It would be telling if the strong association between the use of neuromuscular blocking drugs and postoperative pneumonia persisted irrespective of whether the trachea was intubated.

Competing Interests

The author declares no competing interests.

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(Accepted for publication April 13, 2017.)

Accounting for Planned Postoperative Intubation

To the Editor:

We read with interest the article by Bulka *et al.*[1] regarding the use of intraoperative nondepolarizing muscle relaxants (NDMRs) and their association with postoperative pneumonia. We commend them for increasing knowledge in an area that is exceedingly important. In this article, postoperative pneumonia occurred more frequently in patients who received an NDMR *versus* propensity-matched patients who were not administered an NDMR. Furthermore, within the NDMR subset, lack of neostigmine administration was associated with a greater than twofold higher incidence of postoperative pneumonia than their propensity-matched counterparts.

Although not explicitly stated in the article, we wonder why these patients were not routinely reversed at the end of their procedure. As described in the accompanying editorial,[2] this may have resulted from concerns of paradoxical muscle weakness and/or other side effects of acetylcholinesterase inhibitors. However, another plausible explanation may be that some of these patients were being transported to the intensive care unit for postoperative mechanical ventilation, thus not requiring NDMR reversal. In our experience, the overwhelming reason for nonreversal is predetermined postoperative intubation regardless of patient demographics, attending anesthesiologist, surgeon, or surgical procedure. Because endotracheal intubation and intensive care unit residence are both strongly associated with nosocomial pneumonia,[3] there is a high likelihood that the effect of nonreversal on this outcome is confounded by continued postoperative intubation. To determine whether this manner of confounding exists, separate analyses should be performed that only include patients who were extubated at the end of the surgical procedure before leaving the operating room. Although tedious, these additional investigations would strengthen the argument about the importance of NDMR reversal.

There are also separate issues with the propensity match, in particular with the match for the NDMR/no-NMDR analysis. It can be argued that the biggest determinant of NDMR use is the particular surgical procedure itself, and surgeries that are associated with postoperative pneumonia (thoracotomies, laparotomies, *etc.*)[4–7] are routinely not performed without NMDR. To control for surgical procedure, the authors used Clinical Classifications Software (CCS; Agency for Healthcare Research and Quality; Rockville, Maryland) groupers in the propensity match. Although there are more than 230 single-level CCS procedure categories, there is still too much variability within certain groupings to provide an adequate representation of the surgical procedure variable for the context of the study. As an example, CCS category 96 (fifth most common CCS code in study), "other OR lower gastrointestinal therapeutic procedures," includes more than 80 Current Procedural Terminology codes with both laparoscopic and open colorectal procedures. Thus, a laparoscopic case may have been paired with a laparotomy despite the dissimilar incidence of postoperative pneumonia attributable to these procedures.[4] This is also true for a number of other CCS groupers including category 40, "other diagnostic procedures of respiratory tract and mediastinum," which includes both thoracoscopic surgeries and thoracotomies with differing inherent rates of postoperative pneumonia.[5] Although the CCS classifier is inadequately broad in this respect, the authors still were unable to produce a propensity match with an unbiased (standardized difference less than 10%)[8] surgical procedure variable for the NDMR/no-NMDR analysis. To better separate the effects of the surgical procedure from NMDR use with regard to the incidence of postoperative pneumonia, a balanced match with an adequate procedural variable (*e.g.*, hard-matched Current Procedural Terminology code) must be performed.

Although the use of a more procedurally specific type of matching would most likely lead to a decrease in statistical power within a given data set, selection bias with regard to surgical procedure cannot be properly controlled for without doing so.

In addition, variables that are known to be correlated with postoperative pneumonia need to be accounted for in the analysis to better elucidate the real impact of NMDR and neostigmine reversal on this outcome. These include patient functional status, smoking history, and presence of chronic obstructive pulmonary disease.[3,6,7] Although these variables were indirectly accounted for in this study through the American Society of Anesthesiologists classification, a previous investigation revealed that each of these aforementioned factors were still associated with postoperative pneumonia even after controlling for American Society of Anesthesiologists class.[7] Also, this analysis does not account for the beneficial effects of optimum postoperative analgesia, specifically epidural analgesia,[9] on the occurrence of postoperative pneumonia. Lastly, several references in this article are erroneous. In fact, all four citations in the second paragraph of page 649 do not confirm the ideas expressed in their respective sentences.

Competing Interests

The authors declare no competing interests.

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(Accepted for publication April 13, 2017.)

Risk of Postoperative Pneumonia with Neuromuscular Blockade: Keep It Simple!

To the Editor:

We read with interest the article by Bulka *et al.*[1] regarding the relationship between the management of intraoperative neuromuscular blockade and postoperative pneumonia. The use of large databases to address rare outcomes has increased in recent years. The value in using these databases is the large number of patients who can be assessed. Such large numbers would be extremely challenging to achieve in a randomized controlled study. However, a major limitation and concern with database studies like this one is subsequent confusion between correlation and causation. With regard to residual paralysis, we believe that these challenges can be bypassed with one simple technique—the objective monitoring of the effects of a neuromuscular blocking agent. Although the incidence of residual neuromuscular blockade at extubation is significant,[2] currently, monitoring of neuromuscular blockade is still not an explicitly articulated American Society of Anesthesiologists basic monitoring standard.[3] Whereas many practitioners use such monitoring in their practice, others rely on clinical signs of strength or other outdated measures, such as the 5-s head lift or 50-Hz sustained tetanus to determine adequate recovery from neuromuscular blockade before extubation. Still others simply rely on time from reversal agents being given.[4]

Perhaps the reluctance to consistently monitor the effects of neuromuscular blocking agent and, most importantly, the adequacy of recovery before extubation, represents a peculiar psychologic phenomenon. The practice of anesthesiology is replete with situations in which parameters are monitored at baseline and for the effects of any intervention. In addition, many of our routine practices could be deemed unnecessary in the majority of patients, yet are performed to prevent devastating outcomes in the remaining small percentage of patients. Examples include preoxygenation before the induction of anesthesia, maintenance of blood pressure within certain parameters to prevent stroke or myocardial ischemia, and maintenance of normothermia to prevent wound infection and cardiovascular complications. These practices have become routine or standard because they protect patients from rare but serious complications. As Perrow[5] points out, Murphy's law is wrong: everything that can go wrong usually goes right, and then we draw the wrong conclusion. The ability to adequately ventilate 1,000 successive patients could

lead one to disregard the need for preoxygenation, but this would lead to the trap that Perrow[5] warns against. Similarly, we believe that the low frequency of complications from residual paralysis (reintubation, respiratory distress, and pneumonia) leads to a sense of complacency, because we either do not see or do not recognize these complications, especially if, as with pneumonia, they manifest later. Finally, when we see something rarely, it is easy to equate low risk with no risk, to the point that when the adverse outcome does occur, we are convinced it must be from some other cause. However, when common causes are ruled out, uncommon causes become very likely. Although twitch monitors are not without their own limitations, we believe the routine confirmation of adequate strength before extubation, using a quantitative train-of-four ratio greater than 0.9 or sustained 5-s tetanus at 100 Hz, can reduce the risk of adverse events from residual neuromuscular blockade and should become a standard of care.

Competing Interests

The authors declare no competing interests.

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(Accepted for publication April 13, 2017.)

Science or Fiction? Risk of Postoperative Pneumonia with Neuromuscular Blockade

To the Editor:

We read with interest the study by Bulka *et al.*,[1] which suggested a higher risk of postoperative pneumonia (POP) after the use of neuromuscular blocking agents (NMBAs). We believe that there are inconsistencies and calculation errors that significantly change the results of their study. In the NMBA analysis, there were 38 POP cases among 1,455 patients from the 10,594 patients who received an NMBA during surgery, yielding a POP incidence rate of 2.6%. However, in the NMBA reversal analysis, these same 10,594 patients were split into two subgroups: 1,623 patients who did not receive reversal and 8,971 patients who were given neostigmine. To our surprise, the POP incidence rates are significantly higher in both subgroups, with 149 POP cases in the 1,320 patients (11.5%) who received NMBA without reversal and 70 POP cases in the 1,320 patients (5.3%) who received NMBA and were reversed with neostigmine. Because these two subgroups are from the same 10,594 patients in the NMBA group, we do not understand why the POP rates are so much higher in the two subgroups.

The authors are silent on this apparent discrepancy in POP incidences. We believe that this is due to calculation errors. In Table 2 of the article,[1] the POP incidence rates are presented as "Incidence per 10,000 person-days at risk" because each patient was followed for up to 30 days. There are four such values, 9.00, 5.22, 4.22, and 1.88, representing patients who received NMBA, those who did not receive any NMBA, those who received NMBA without reversal, and those who received NMBA with reversal, respectively. The last two numbers appear to be incorrect; we believe that they should be 42.2 (not 4.22) and 18.8 (not 1.88). Thus, the actual POP incidence rates are much higher in the two NMBA subgroups than that of the total NMBA group (42.2 and 18.8 compared with 9.00). These errors in data collection and calculation lead to invalid conclusions.

We also wonder about the study design, with its 30-day observation period. Although it has been suggested that many postoperative complications require a 30-day follow-up,[2] we do not think this applies to NMBA complications. Any postoperative residual neuromuscular blockade in these patients would be clinically insignificant in a matter of hours, and a POP related to that should easily be evident within 1 week. It would seem to be erroneous to attribute any POP cases that occurred several weeks after surgery to the use of NMBA.

Competing Interests

The authors declare no competing interests.

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(Accepted for publication April 13, 2017.)

In Reply:

Thank you for providing an opportunity to respond to the interesting letters written by Drs. Cumberworth, Meyer and Eikermann, Austin and Lam, Caruso *et al.*, and Zhang *et al.* In our study, "Nondepolarizing Neuromuscular Blocking Agents, Reversal, and Risk of Postoperative Pneumonia,"[1] a small minority of patients (approximately 4%) had only a supraglottic airway device used during the case. Approximately 6% of patients included in our analysis were admitted postoperatively to the intensive care unit with an endotracheal tube in place. We did not formally adjust for these groups of patients in our analyses but agree that doing so may have strengthened our findings. Regardless of this potential improvement, based on our results, we agree with the sentiment that reversal of neuromuscular blocking agents should be both routine and guided by neuromuscular transmission monitoring (preferably quantitative). We appreciate that current national practices around neuromuscular monitoring are evolving and not uniform. National practice guidelines would help, as would additional refinements to the monitoring technology itself, given its immaturity. Our research group recently published an article outlining existing barriers and calling for the development of more robust, user-friendly neuromuscular monitoring technology.[2] Finally, we appreciate the comment regarding residual confounding in our propensity analysis. Although not included in table 1 of our article (Patient Demographics and Clinical Characteristics before and after Matching),[1] the rates of smoking and chronic obstructive pulmonary disease were similar between groups. We appreciate very much the interest in our work and hope that our findings will help raise attention to the importance of developing strategies to reduce postoperative pneumonia.

Competing Interests

The authors declare no competing interests.

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(Accepted for publication April 13, 2017.)

In Reply:

After a careful rereading of the letter from Drs. Meyer and Eikermann, we remain confused as to their objection to our editorial.[1] Their concern seems to be semantic in nature. In particular, they seem uneasy with the term *routine*. We think that they have ignored the basic message that we were attempting to make.

As stated in our editorial, neostigmine administration is not required once it has been determined that the train-of-four (TOF) ratio at the adductor pollicis has returned to a value of 0.90 or greater. This information can only be ascertained by using a quantitative neuromuscular monitor. Unfortunately, we suspect that the great majority of anesthesia practitioners still do not have access to these devices. What then is a clinician who only possesses a conventional peripheral nerve stimulator to do at the end of surgery when tactile or visual fade on TOF stimulation can no longer be detected?

It is our contention, in these circumstances, that the risk of respiratory complications from failure to reverse residual block far outweighs any theoretical adverse effects of neostigmine-induced "paradoxical paralysis." We are unaware of any documented clinical morbidity associated with the use of low-dose neostigmine (less than or equal to 0.04 mg/kg) even when administered at TOF values of 0.90 or greater.

Competing Interests

Dr. Murphy has served as a consultant for Merck & Co. (Kenilworth, New Jersey). The other author declares no competing interests.

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(Accepted for publication April 13, 2017.)

Assessing Success of Rescue Intubation Techniques after Failed Direct Laryngoscopy

To the Editor:

In a multicentered, observational study comparing the success rate of commonly used rescue intubation techniques after a failed direct laryngoscopy, Aziz *et al.*[1] showed that video laryngoscopy was associated with a higher success rate of rescue intubation and was more commonly used than other tools, including a fiberoptic bronchoscope, a supraglottic airway device, an optical stylet, and a lighted stylet. In addition to the limitations described in the discussion, however, there are several questions in this study that must be clarified.

First, the majority of rescue intubations (1,023 of 1,511 cases, 68%) were defined after one failed direct laryngoscopy attempt. This practice is not in agreement with the definition of difficult or failed laryngoscopy in the current practice guidelines for difficult airway management by the American Society of Anesthesiologists.[2] Because the authors did not provide the detailed causes of failed direct laryngoscopy, it was unclear why the anesthetists abandoned direct laryngoscopy after the first attempt. In fact, difficulty in performing laryngoscopy depends on the anesthetists' level of skill, the patient's features, and procedure circumstances. In this study, the authors did not specify whether an optimal-best laryngoscopy attempt was executed when a failed direct laryngoscopy was defined. The components of an optimal-best laryngoscopy attempt include a reasonably experienced (at least 3 full recent years) anesthetist, use of an optimal sniffing position, change of length or type of blade one time, and use of external laryngeal manipulation.[3] Only when an optimal-best laryngoscopy attempt is performed may difficult or failed laryngoscopy be readily obvious to an experienced anesthetist on the first attempt and thus is independent of both number of laryngoscopy attempts and time. According to the data provided by the authors, we cannot determine whether a definitively failed direct laryngoscopy occurs in each patient receiving rescue intubation.

Second, in this study, the failed direct laryngoscopy included the use of a device without a tube passage attempt, although the goal of direct laryngoscopy is to carry out tracheal intubation. It must be emphasized that the laryngeal view obtained by direct laryngoscopy is often used as an important variable for difficult or failed intubation, but they are not synonymous in most patients.[3] Successful intubation is dependent more on the skill level of the anesthetists than on the laryngeal view obtained by direct laryngoscopy, and thus the degrees of difficulty with direct laryngoscopy and tracheal intubation may be incompatible. For example, some patients with a class 3 or 4 laryngeal view may be successfully intubated by an experienced anesthetist on the first or second attempt if the distal end of the tracheal tube is suitably curved by a malleable stylet or an intubating introducer (*e.g.*, a gum-elastic bougie).[4] Actually, 61 of 1,619 patients with failed direct laryngoscopy in this study had a return to direct laryngoscopy again for airway rescue. Thus, when defining a failed direct laryngoscopy, the exclusion of a tube passage attempt is unreasonable.

Third, the success rate of rescue intubation with the supraglottic airway device was described as the final endpoint of performance. The final goal of airway management is maintenance of oxygenation rather than performing tracheal intubation. After a failed initial intubation attempt, restitution of ventilation by either a noninvasive (*i.e.*, supraglottic airway device) or an invasive intervention is the priority.[5] Thus, use of the supraglottic airway device as a rescue tool of failed direct laryngoscopy can not only provide a conduit to intubate the trachea but also is an effective ventilatory measure with a high success rate.[6] If the rescue intubation *via* the supraglottic airway device is unsuccessful, the existence of an effective airway can evidently be lifesaving. Thus, we argue that only comparing the success rate of rescue intubation using video laryngoscopy and a supraglottic airway device after failed direct laryngoscopy in this study is not a complete comparison.

Finally, video laryngoscopy provided a high success rate of rescue intubation after failed direct laryngoscopy but did not give a 100% success rate. This suggests that when attempting to rescue a failed direct laryngoscopy, no single device can address all issues. Furthermore, we agree with Hagberg *et al.*[5] that no one tool is better than others in all conditions, because each tool has individual properties that may be advantageous in some conditions but disadvantageous in others. The use of video laryngoscopy as the first rescue choice at the early stage of failed direct laryngoscopy seems rational,[7] but an important problem we are facing is what anesthetists should do if difficult video laryngoscopy occurs. In fact, recent work shows that first attempt failure at intubation using video laryngoscopy is also associated with increased complications.[8] Thus, we believe that to rescue a failed direct laryngoscopy expeditiously and safely, anesthetists must master the several different airway devices and should use the techniques with which they have the most experience and competence, with strict adherence to the current practice guidelines for difficult airway management.

Competing Interests

The authors declare no competing interests.

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(Accepted for publication April 9, 2017.)

Is Airway Management Better?

To the Editor:

The article by Aziz *et al.*[1] describes difficult airway management over a 8- to 9-yr period and analyzes the use and success of different airway devices for rescue after failed direct laryngoscopy. The authors found that video laryngoscopy was used most often and had the highest rate of success as a rescue tool (92%) compared to fiberoptic bronchoscopy, lighted stylets, optical stylets, and supraglottic airways (67 to 78% success rate). They speculate that the results may "reflect . . . widespread availability of video laryngoscopy, an anticipated high success rate, and growing comfort and familiarity with this technique." The authors state that the growing use of the video laryngoscopes, of which the GlideScope was used 83% of the time, is a "practice improvement." The attractiveness of video laryngoscopy is understandable as it is technically similar to direct laryngoscopy and, compared to other rescue techniques, may be easier to teach, learn, and master, perhaps fueling the increased use as highlighted in this article.

However, based on these data, we wonder whether there is an improvement in airway management or just a change in clinical practice and training. Moreover, we are concerned that this change in practice and training has resulted in a decriment in clinical skills. Despite its increasing use, the reported rate of failure of video laryngoscopy consistently ranges from 5 to 20%,[1–5] despite reports of improved view of the glottis.[5,6] The current investigation reports an 8% failure of video laryngoscopy as a rescue tool, at which time the practitioner used either fiberoptic bronchoscopy or direct laryngoscopy with or without bougie to rescue the rescue.[1] There are significant limitations to video laryngoscopy seen with small mouth opening, tongue and/or soft-tissue swelling (*e.g.*, infection, angioedema), altered neck anatomy (radiation, surgery, airway displacement, presence of a halo), and/or any airway obstruction.[1,4]

Despite reporting significant *P* values, the authors recognize the retrospective and unmatched nature of the study.[1] Important unknown variables include the reasoning for selection of a particular rescue airway device, which was at the practitioner's discretion. The equivalency of the patient's airways between the groups is not known. We do not know, for example, how many patients rescued with fiberoptic bronchoscopy had known predictors of failed video laryngoscopy. With regard to general conclusions of difficult airway management, the success of video laryngoscopy may have been artificially high if practitioners did not attempt to use video laryngoscopy if predictors of failure were present.

The authors did not discuss the 81% of the initial 7,259 cases that were excluded. Because the airway was ultimately secured with direct laryngoscopy, 40% of cases were excluded. In the other 41% (2,951 cases), another primary technique was used (*i.e.*, not direct laryngoscopy). There are no further data describing what technique was used nor how they were rescued. If consistent with the practice trends, then these initial "nondirect laryngoscopy attempts" would more commonly have included video laryngoscopy. If this were the case, then the success of video laryngoscopy is not accurately represented. Perhaps the failure rate of video laryngoscopy is significantly greater than 8%.

Airway trauma was reflected by the number of attempts made before the rescue attempt. The retrospective nature of the study precludes any conclusions regarding which technique was superior because there is no explanation as to how practitioners decided when "enough was enough." Furthermore, the only pharygeal and airway injuries (1% of total) reported in the present study occurred during use of video laryngoscopy. Finally, the present investigation reports an incidence of failed intubation of 2% (7,259 of 346,861), which is significantly higher than the 0.9%[7] or 0.1%[8] previously reported.

We do not refute the value of video laryngoscopy but want to emphasize the benefit of maintaining expertise with multiple airway management techniques. If teaching video laryngoscopy is overemphasized, then other skills will deteriorate. Prior investigations report success rates with fiberoptic bronchoscopy to be greater than 95%.[9,10] In another study of 100 cases of "unanticipated difficult airway," the practitioners reported a rescue success of 98% using a specific airway management algorithm that included adjustments in direct laryngoscopy, laryngeal mask airway, and a gum-elastic bougie.[7] These studies allude to the importance and impact of training.

There are limitations for each airway technique, and a failure to appreciate them will have adverse consequences. Aside from the video laryngoscope, no other device or class of devices were used in more than 9% of the study group.[1] Instead of showing a practice improvement, we are concerned that airway management, training, and education has declined as a result of reduced emphasis on becoming expert with multiple techniques to allow greater versatility in managing any airway.

Competing Interests

The authors declare no competing interests.

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(Accepted for publication April 9, 2017.)

Apneic Intubation: Video Laryngoscopy Lacks the Continuous Ventilation Offered by Other Airway Management Techniques

To the Editor:

The article by Aziz *et al.*[1] significantly contributes toward understanding the response of anesthesiologists to failed intubation attempts with conventional direct laryngoscopy. We are concerned, however, that one unwise message that may be drawn from this paper is that video laryngoscopy is the *sine qua non* for management of an unexpected difficult direct laryngoscopy. Indeed, Aziz *et al.* found an 8% failure rate with video laryngoscopy (90 of 1,122), underscoring the fact that anesthesiologists must have other trusted responses to failed conventional direct laryngoscopy. Additionally, it must be recognized that video laryngoscopy is an apneic intubation technique; oxygenation and ventilation are not maintained during laryngoscopy and intubation.

Aziz *et al.* reported inferior success rates with both intubation using a supraglottic airway as a conduit and intubation using a flexible fiberoptic bronchoscope (78% for both *vs.* 92% with video laryngoscopy). However, there are two important considerations to weigh when evaluating intubations using a supraglottic airway and/or fiberoptic bronchoscopy in these situations. First, because this was a multicenter study and no data were reported regarding the practitioners' prior training and experience with any of these techniques, it is impossible to know whether practitioners had equal competence with all three techniques. In general, most practitioners have more experience with video laryngoscopy. It is entirely possible that in experienced hands the success rates for intubation using a supraglottic airway as a conduit and intubation using a flexible fiberoptic bronchoscope would be higher. Second, and most importantly, many intubation techniques using a supraglottic airway and/or fiberoptic bronchoscopy allow for continuous ventilation during airway management and intubation, an advantage that video laryngoscopy does not offer and one that can be critical when a difficult intubation occurs in the setting of difficult or impossible mask ventilation. Previously described techniques for intubation using a supraglottic airway as a conduit and intubation using flexible fiberoptic bronchoscopy *while maintaining continuous ventilation* involve placing a supraglottic airway or an intubating oral airway with a mask and connecting the supraglottic airway or the mask to the ventilator using a bronchoscopy elbow.[2–4] An Aintree catheter can then be loaded onto a fiberoptic bronchoscope and advanced through the bronchoscopy elbow, through the supraglottic airway or mask and intubating oral airway combination and into the trachea, all while continuously oxygenating and ventilating the patient. An endotracheal tube is then threaded over the intratracheal Aintree catheter, and the Aintree catheter is removed.[2] Alternatively, an endotracheal tube can be placed within an *in situ* intubating supraglottic airway and the ventilator connected to a bronchoscopy elbow placed on the endotracheal tube. Again, continuous oxygenation and ventilation are maintained as a fiberoptic bronchoscope is passed through the bronchoscopy elbow, through the endotracheal tube placed within the supraglottic airway, and into the trachea. The endotracheal tube is then advanced over the fiberoptic bronchoscope and into the trachea.[3,4]

Effective fiberoptic-guided intubation is a skill that, although infrequently necessary, is critical in its ability to continuously oxygenate and ventilate the patient when a difficult laryngoscopy occurs in the setting of difficult or impossible mask ventilation. This critical advantage over video laryngoscopy should not be underestimated, and

indeed, the American Society of Anesthesiologists Difficult Airway Algorithm encourages practitioners to "actively pursue opportunities to deliver supplemental oxygen throughout the process of difficult airway management."[5]

It is imperative that the anesthesiology community continue to teach residents techniques for airway management beyond direct and video laryngoscopy with a focus on those techniques that allow for continuous oxygenation and ventilation during airway management. Equally as important, once these skills are attained, anesthesiologists must make efforts to maintain these skills through their practical application. We hope that, rather than highlighting the efficacy of video laryngoscopy over other techniques, the article by Aziz *et al.* will serve to underscore the importance of the competent practitioner having an arsenal of techniques, with which they are well versed, to secure the difficult airway.

Competing Interests

The authors declare no competing interests.

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(Accepted for publication April 9, 2017.)

In Reply:

We thank Drs. Xue *et al.*, Drs. Herway and Benumof, and Drs. Maslow and Panaro for their interest and thoughtful comments regarding our recent publication.[1] They offer several interesting insights and questions regarding our article that we wish to address.

All three letters point out that video laryngoscopy was not universally successful as a rescue technique and that other approaches to intubation and oxygenation should be considered. Furthermore, training and competency with other primary or rescue tools should be maintained. We absolutely agree. The practical application of our findings provides a framework for prioritizing how to best invest time and training in rescue techniques. The supraglottic airway in particular offers advantages to maintain oxygenation and ventilation as a definitive airway or as a conduit for final tracheal intubation. Indeed, many patients in this data set were effectively temporized in this fashion. However, when used to guide tracheal intubation with or without the use of a flexible bronchoscope, the supraglottic airway was not as successful as video laryngoscopy. Nor was the flexible bronchoscope as successful. Does this mean that these well-established techniques should be abandoned? Certainly not! They have a clear role when video laryngoscopy is not feasible or when used by providers more experienced with these techniques. That said, if a higher risk of failure is anticipated or when preparing for an unanticipated difficult direct laryngoscopy, our data support the immediate availability of video laryngoscopy.

It is likely true that performance with the supraglottic airway and flexible bronchoscopic intubation would have been improved with better training. However, this data set represents the experience of 353 distinct attending anesthesiologists in large tertiary care academic medical centers. While they all may have experienced different performance with different training, we believe this sample represents the reality of clinical practice in academic medicine in the United States. Similar discussions occurred in the United Kingdom regarding rescue surgical airway approaches after publication of the fourth national audit project.[2] The study observed higher success rates with the scalpel approach compared to percutaneous techniques, and national guidelines soon called for only the scalpel technique.[3] Appropriate cautionary editorials were provided that discussed the importance of training and human factors when selecting rescue techniques.[4] We believe both of these rescue situations represent opportunities for improvements in training, but it is as important to recognize why certain techniques may have failed and why one performed better than the other. We believe the high success rate with video laryngoscopy relates to ease of use and experience in both urgent and nonurgent situations. Furthermore, we recognize that competence at the highest level may not be feasible with all available devices, and it is useful to understand what may work most frequently in most providers' hands. We need to understand better why such a large group of anesthesia providers may have not performed as well with flexible bronchoscope techniques and intubating supraglottic airways. We also hope that our article will encourage others to research these questions.

Xue *et al.* had a particular question about the definition of a failed direct laryngoscopy attempt. They are correct that we cannot confirm that the initial direct laryngoscopy attempt was optimized through patient positioning or laryngeal manipulation. However, all intubations were supervised or performed by anesthesiologists with sufficient experience. Furthermore, we did describe alternation of direct laryngoscopy blade types (see table 4 of our article[1]). We agree that often an inadequate laryngeal view with direct laryngoscopy can be overcome with optimization maneuvers or when utilizing a gum-elastic bougie. These cases were *a priori* excluded from analysis as we were interested in the mechanisms of rescue after direct laryngoscopy has failed by whatever means. We cannot determine why direct laryngoscopy was abandoned after one attempt and/or if tube placement was actually attempted along with that failed direct laryngoscopy. Certainly, the providers who performed direct laryngoscopy first aimed to intubate the patient but simply could not, even though such appropriate adjuncts were available and/or used. So, we did not describe *failed intubation via* direct laryngoscopy *per se*, but we do believe we appropriately described *failed direct laryngoscopy*.

Maslow and Panaro had some questions about the validity of the data set that we believe represent a misunderstanding that should be clarified. They question the high exclusion rate from the primary query. The automated query identified 7,259 cases that involved multiple laryngoscopy attempts and notations of device(s) of interest in an effort to "screen" the electronic record for potential cases as only the narrative could describe the actual sequence of events. These were not necessarily failed direct laryngoscopy attempts but a trigger to further evaluate the record. The final analysis included 1,427 failed direct laryngoscopy cases from 346,861 intubation records (0.4%). Also, our data do not address the primary success rate of either direct laryngoscopy or video laryngoscopy. The data set only speaks to the success rate of various techniques after direct laryngoscopy has failed. So, the primary success rate of video laryngoscopy is not reported. However, we did publish such findings in a different study and observed a 98% success rate with video laryngoscopy as the primary technique despite early clinical experience with the device.[5]

Competing Interests

The authors declare no competing interests.

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(Accepted for publication April 9, 2017.)

Calculating Ideal Body Weight: Keep It Simple

To the Editor:

We read with much interest the editorial on protective ventilation by Hedenstierna and Edmark in the December issue of Anesthesiology.[1] We agree with most of the ideas put forward. However, as thoracic anesthesiologists, we strongly believe in the importance, during one-lung ventilation, of low tidal volume based on ideal body weight.[2,3]

Many authors still recommend using the gender-specific Acute Respiratory Distress Syndrome Network (ARDSnet) formulas to calculate ideal body weight.[4] Ideal body weight is computed in men as 50 + (0.91 × [height in centimeters – 152.4]) and in women as 45.5 + (0.91 × [height in centimeters – 152.4]). A simple alternative would be to compute ideal body weight as the weight corresponding to an ideal body mass index of 22 kg/m^2. Ideal body weight is then simply calculated as 22 × ([the actual patient's height in meters]^2) or by using body mass index charts available on our anesthesia cart.[5] We chose 22 kg/m^2 as the ideal body mass index after comparing the ideal body weight corresponding to body mass indices ranging from 20 to 25 to ideal body weight calculated from ADRSnet formulas. For example, a 1.75-m man would have an ideal body weight of 67 kg (22 × [1.75^2]) compared to 71 kg if using ARDSnet; a 1.60-m woman would have an ideal body weight of 56 kg (22 × [1.60^2]) compared to 52 kg if using ARDSnet.

The method we propose is simple and easy to remember. The same computation applies for both men and women and involves simple arithmetic.

Competing Interests

The authors declare no competing interests.

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(Accepted for publication April 8, 2017.)

In Reply:

We appreciate the important comment by Moreault *et al.* on our article, "Protective Ventilation during Anesthesia: Is It Meaningful?"[1] We agree fully with the opinion that a low tidal volume should be based on ideal body weight to avoid harmful stress and strain to the lungs during anesthesia. This is even more important during one-lung ventilation. Ideally, the tidal volume should be adjusted to the size of the ventilated lung, but without a simple recording of lung volume, ideal body weight is a reasonable alternative. However, we also believe that an appropriate positive end-expiratory pressure is a prerequisite when using a low tidal volume, whatever the calculation method of ideal body weight. We find the method proposed by the authors commendable and indeed easy to remember as most anesthesiologists already are familiar with the method for calculating body mass index.

Competing Interests

The authors declare no competing interests.

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(Accepted for publication April 8, 2017.)

Evaluation of Nitrous Oxide in the Gas Mixture for Anesthesia II (ENIGMA II) Revisited: Patients Still Vomiting

To the Editor:

We read the secondary analysis of the Evaluation of Nitrous Oxide in the Gas Mixture for Anesthesia II (ENIGMA II) trial for severe postoperative nausea and vomiting (PONV) with great interest.[1] Because PONV remains an often-cited risk in using nitrous oxide,[2] the investigation of methods to mitigate PONV using existing data generated from randomized controlled trials is an important undertaking. We wish to respond to this thorough reanalysis.

The authors used a retrospective propensity score approach to investigate the effects of antiemetic prophylaxis on the nitrous oxide and non-nitrous oxide arms. The well-recognized limitations of this approach were openly acknowledged in the publication, including the inability to control for hidden covariates and the need to truncate available data.[3] In the abstract, the authors conclude that the emetogenic effects of nitrous oxide are near eliminated by the addition of antiemetics. However, the results from the propensity score-matched analysis do not seem to support this conclusion, as the nitrous/antiemetic group had statistically higher odds of PONV compared with the nonnitrous/nonantiemetic group. In addition, administration of antiemetic prophylaxis among participants who did not receive nitrous oxide counterintuitively increased the odds of PONV. Although various clinical and scientific reasons may be hypothesized to explain this phenomenon, perhaps the simplest hypothesis is the presence of hidden covariates. Therefore, it is our opinion that the conclusion of negating PONV with antiemetics when nitrous is used is not supported by the results of this retrospective analysis, and the use of propensity score matching in this instance may not have resulted in a balanced comparison.

In light of the aforementioned results, another statistic (risk ratio, 0.74 [95% CI, 0.63 to 0.84]; $P < 0.001$) is quoted in the report[1] to support the conclusion that PONV is not increased when antiemetics are used in conjunction with nitrous oxide. This risk ratio does not appear among the results generated by propensity score matching but appears to be the result of a subgroup analysis for the PONV outcome in the original ENIGMA II report for patients who received antiemetic prophylaxis.[4] However, the lack of blinding of attending anesthesiologists to treatment allocation may have introduced selection bias into antiemetic prophylaxis, a possibility supported by the statistically significant difference in antiemetic administration between the nitrous and non-nitrous arms. If selection bias were present in antiemetic administration, the efficacy of this originally randomized subgroup analysis to equalize hidden covariates may have been compromised.[5]

Although this secondary analysis[1] of antiemetic prophylaxis on PONV has important limitations, we believe that

the authors' dose-effect analysis of nitrous oxide on PONV is very clinically relevant, although not emphasized in the abstract or report. The authors note in the report that nitrous oxide, when used for less than 2 h, did not seem to result in added PONV compared with the non-nitrous arm. This observation is congruent with existing literature,[6] is a randomized comparison that carries with it the methodologic robustness of the original ENIGMA II trial, and has applicability in a wide variety of clinical settings. In closing, we thank the authors for their thorough reanalysis and presentation of the ENIGMA II data for the PONV outcome. This secondary analysis is revealing, but the conclusion that prophylaxis nearly eliminates PONV seems untenable.

Research Support

Support provided by the University of Saskatchewan, Saskatoon, Canada, for the salaries of Dr. Li, Dr. Balbuena, and Dr. Gamble.

Competing Interests

The authors declare no competing interests.

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(Accepted for publication March 13, 2017.)

In Reply:

We thank Li *et al.* for these perspectives. We agree that nonrandomized studies have greater risk of bias and confounding, and the results may therefore be misleading. This certainly applies to studies using propensity-based methods. We would first like to point out that in their letter Li *et al.* state we used propensity score matching. In fact, we actually used inverse probability of treatment weighting. These are distinct methods (although both based on propensity scores) and estimate different quantities (effect of treatment overall *vs.* effect of treatment in the treated).

Table 1. The Incidence (%) and Relative Risk of Postoperative Nausea and Vomiting in Patients Receiving Nitrous Oxide for Major Surgery in the ENIGMA II Trial[1]

	Nitrous Oxide (n = 3,483)	No Nitrous Oxide (n = 3,509)	Relative Risk (95% CI)	*P* Value
Overall	14.5%	10.8%	1.35 (1.19–1.53)	<0.001
Antiemetic prophylaxis				
No	16.6%	9.6%	1.75 (1.43–2.13)	<0.001
Yes	13.1%	11.7%	1.12 (0.95–1.32)	0.18

The risk estimate differed according to use of antiemetic prophylaxis; interaction *P* value 0.001.

ENIGMA II = Evaluation of Nitrous Oxide in the Gas Mixture for Anesthesia II.

More importantly however, our comments regarding the risk mitigation associated with antiemetic prophylaxis in patients exposed to nitrous oxide were based not on the secondary analysis referred to by Li *et al.* but in a preplanned secondary analysis of the original large randomized trial.[1] Relevant, expanded details are provided in table 1. The emetogenic effect of nitrous oxide was less apparent in those who received prophylactic antiemetics before the end of surgery compared with those who did not. The interaction *P* value was 0.001, indicating that there was a statistically significant differential effect between these two subgroups. We acknowledge that use of antiemetic prophylaxis was left to the discretion of the attending anesthesiologist, but such use was more likely in those with more risk factors for postoperative nausea and vomiting (PONV; as we reported in our publication).[2] That is, there was a selection bias, but it would underestimate the protective effect of antiemetic prophylaxis because such use was higher in those with greater risk of PONV. We therefore stand by our conclusion that PONV prophylaxis near-eliminates the risk of nitrous oxide-induced severe PONV after major surgery.

Competing Interests

The authors declare no competing interests.

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(Accepted for publication March 13, 2017.)

Promoting Sustainable Practices *via* Art

To the Editor:

A few plastic caps from medication vials used for an individual anesthetic may seem insignificant; however, these items accumulate. Using five vials per case for 30,000 cases annually, we waste 150,000 caps per year. At the University of Wisconsin–Madison, we identified an opportunity to divert this commonly discarded material from landfills. Although too small for comingled recycling, caps can be recycled successfully when collected separately. Recycling rates of 20 to 25% are achievable in the operating room without compromising infection control or creating financial constraints.[1]

Forming a multidisciplinary green team is an effective means for promoting sustainable practices.[2,3] To raise provider awareness of the amount of waste that can be generated in a healthcare setting, our green team initiated a vial cap collection (fig. 1). In addition to recycling caps, we collaborated with our hospital art coordinator to create mosaic artwork from this colorful material (fig. 2). Interest in the art project was greater than anticipated, creating dialogue between staff in all areas of the hospital. Staff have joined together for several art-making events in which participants sort the caps by color and participate in gluing the caps to a large art piece. Educational information about green efforts in the healthcare setting was on display for participants to learn more. Seeing the large collection of small plastics conveys the impact of medical waste. Holding these plastics in their hands to create artwork inspires healthcare providers to look at the bigger picture of the environmental impact of our practice.

Research Support

Support was provided solely from institutional and/or departmental sources.

Competing Interests

Dr. Zuegge receives nonclinical time for her role as Medical Director of Sustainability for University of Wisconsin Health. The Department of Planning, Design, and Construction funded the printing, materials, and supplies for, and framing of, the artwork. The other authors declare no competing interests.

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Fig. 2. One of the completed artworks now on display in our hospital.

Fig. 1. Hospital staff participate in a vial cap sorting event.

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(Accepted for publication March 31, 2017.)

ERRATUM

Nebulization of Antiinfective Agents in Invasively Mechanically Ventilated Adults: A Systematic Review and Meta-analysis: Erratum

In the article beginning on page 890 in the May 2017 issue, the first sentence of the Competing Interests section is incorrect due to a publisher error. The correct sentence is "Dr. Rello received research grants and consulting fees from Bayer (Leverkusen, Germany) and Genentech (San Francisco, California)." This error has been corrected in the online version of the article.

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New Diplomates, American Board of Anesthesiology®, Spring 2017

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Anesthesiologists – University of Kentucky

The Department of Anesthesiology at the University of Kentucky seeks Board-certified or Board-eligible anesthesiologists in the areas of cardiothoracic anesthesia, critical care medicine, and pediatric anesthesia, Fellowship training and eligibility for ABA subspecialty certification or TEE certification are required. These positions are open to all ranks. UK HealthCare has undergone a sustained and remarkable growth trajectory for a decade. Clinical volumes have doubled in the past 6 years and the acuity of patient care is high. UK HealthCare's Albert B. Chandler Hospital was named No. 1 in Kentucky in the *U.S. News & World Report's* Best Hospitals rankings. The Department of Anesthesiology is a collegial group that is well-respected in the medical center. The residency program is strong and nationally recognized. The enterprise financially supports the academic and the clinical mission of the Department of Anesthesiology.

Cardiothoracic Anesthesia - UK Healthcare has an exceptionally active, rapidly expanding Cardiac Surgery Service. Procedures include heart transplants, lung transplants aortic root replacement, TAVR, and minimally invasive thoracic surgery including pulmonary resection and esophagectomy.

Critical Care Medicine - The anesthesiology critical care medicine service provides coverage in the Neurocritical Care unit and manages cardiac surgery and thoracic surgery patients in the cardiovascular ICU as well as solid organ transplant patients in the Surgery ICU.

Pediatric Anesthesia - UK Healthcare has a Level IV Neonatal ICU. All types of pediatric surgical subspecialties are represented. Willingness to care for adults as well as children is essential.

Salary and benefits of this position are commensurate with experience and rank.

The University of Kentucky is an Affirmative Action Equal Opportunity Employer and actively seeks the candidacy of minorities and women. Upon offer of employment, successful applicants must pass a pre-employment drug screen and undergo a national background check as required by University of Kentucky Human Resources.

This position may be applied to at this link: **http://ukjobs.uky.edu/postings/113065**

Interested candidates should contact:
Robert Gaiser, MD, Professor and Chair, Department of Anesthesiology
800 Rose Street, N202, UKMC, Lexington, KY 40536-0293
Phone: 859-323-5956 Fax: 859-323-1080
Robert.gaiser@uky.edu **eahesl2@uky.edu**

College of Medicine
Anesthesiology

UNIVERSITY of MARYLAND
SCHOOL OF MEDICINE

Department of Anesthesiology

Chief, Division of Obstetric Anesthesiology

We are actively seeking a Chief for our Division of Obstetric Anesthesiology (OBA). The University of Maryland School of Medicine and the University of Maryland Medical Center are nationally-respected leaders in the care of high-risk pregnancies. Over 90% of our 1800 deliveries are considered either maternal and/or fetal high-risk. A program in Complex Obstetric Surgery receives regular region-wide referrals for delivery of pregnancies complicated by super-morbid obesity, placental implantation abnormalities and coexisting medical and trauma-related conditions. A busy Center for Advanced Fetal Care performs evaluation and treatment, including fetal (EXIT) surgery. A multidisciplinary, intensive-care, consulting service is always available. A new Labor and Delivery Suite project has been funded and is being planned.

The Division consists of 10 anesthesiologists who provide dedicated 24 x 7 coverage. All anesthesiology residents rotate through the OBA service. There is 1 ACGME-approved fellow. A MOCA-certified simulation center is used for multi-professional training. The Department has well-established mentoring and faculty development programs. Anesthesia and integrated hospital-wide information management systems provide large data sets for outcomes research in perinatal medicine. Robust departmental clinical and translational research programs include: human factors research; neuronal bioenergetics; neuroinflammation; traumatic brain and spinal cord injury; sepsis-related mechanisms of cardiac dysfunction; outcomes after anesthesia and surgery; and, problems in coagulation.

The successful candidate must be a superb clinician and educator with at least seven years of experience.

In addition, this individual must:

- Qualify for appointment in the School of Medicine as an Associate- or Full- Professor (Tenure or Non-Tenure Track);
- Be certified by the American Board of Anesthesiology;
- Be fellowship-trained in Obstetric Anesthesiology;
- Be actively involved in clinical or translational research;
- Be recognized for their contributions to Obstetric Anesthesiology; and
- Be eligible for an unrestricted license to practice Medicine in Maryland as well as Federal and Maryland controlled substances licenses

The University of Maryland and the Department provide competitive salary and benefits.

Interested individuals should send a letter or email (preferred) expressing interest and include a CV and names of three references to: Andrew M. Malinow, MD, Professor and Vice-Chair for Academic Affairs and Faculty Development, Department of Anesthesiology, University of Maryland School of Medicine, 22 S. Greene St., S11C00, Baltimore, Maryland 21201, Email: **amalinow@anes.umm.edu**

Please visit **www.medschool.umaryland.edu/anesthesiology** to learn more about our department.

The University of Maryland, Baltimore is an Equal Opportunity, Affirmative Action employer. Minorities, women, individuals with disabilities, and protected veterans are encouraged to apply

BE/BC Anesthesiologists

UNIVERSITY of MARYLAND SCHOOL OF MEDICINE

Department of Anesthesiology University of Maryland

The **University of Maryland Department of Anesthesiology** is growing. We are seeking BE/BC Anesthesiologists of all academic ranks who possess a strong commitment to academic anesthesiology, especially those with interests in joining the Divisions of Pediatric, Critical Care Medicine, Cardiothoracic Anesthesiology, Adult Multi-Specialty Anesthesiology. Responsibilities include clinical care and teaching of fellows, residents, medical students, student nurse anesthetists, and paramedics. The Department offers competitive compensation, excellent benefits, and a generous retirement plan. Academic time is available for academic, scholarly and administrative activities. Candidates must be ABA board eligible/certified.

Pediatric Anesthesiology - Subspecialty-board certified pediatric anesthesiologists provide dedicated 24 x 7 anesthesia coverage for over 3000 cases per year, representing the complete scope of pediatric anesthesiology, including: open-heart, kidney transplantation and neonatal emergent surgery as well as more routine procedures involving children presenting for ENT, proton-beam irradiation and acute pain management procedures. *Preference will be given to those qualified candidates with interest in and/or experience staffing open heart surgeries for congenital heart disease.* Candidates must be certified (or eligible) for added ABA subspecialty qualifications in pediatrics.

Critical Care Medicine - Faculty members in the Division of Critical Care Medicine provide care for patients in the surgical intensive care unit, neurosciences intensive care unit, cardiac surgical intensive care unit, and trauma/neurotrauma intensive care units at the University of Maryland Medical Center. Division faculty serve as site investigators for investigator-initiated multi-center trials and are involved in unique clinical, translational and basic science research with collaborators from the Shock Trauma and Anesthesiology Organized Research Center. Faculty members oversee an ACGME-approved fellowship. Candidates must be certified (or eligible) for added ABA subspecialty qualifications in critical care medicine or in neurocritical care.

Cardiothoracic - CT division faculty members staff cases at two hospitals, providing anesthesia for over 1600 patients (50 heart-lung transplants; 1000 on-pump cases) and performing over 1400 TEE studies. The division's research is focused on blood management and disorders of coagulation and there are numerous opportunities for clinical, translational, and basic research. Faculty members oversee an ACGME-approved fellowship. Candidates must be fellowship-trained and certified (or eligible) in Perioperative TEE.

Adult Multi-Specialty - Faculty members in the AMS division provide anesthesia for more than 22,000 cases per year including orthopedic surgery, endoscopy, vascular, neurosurgery, ophthalmology, ENT, gynecology, plastics, podiatry, urology, transplantation, oncology and general surgery. Anesthetic care is provided in 27 modern surgical suites, two endoscopy suites and off-floor locations throughout the University of Maryland Medical Center. Candidates with fellowship training are preferred.

Community Anesthesiology – Department members will either individually provide anesthesia or direct an anesthesia care team in both community hospital and ambulatory surgical center settings. Typical cases are drawn from most surgical specialties excluding open-heart, obstetrics, and complex neurosurgical and vascular cases. Proficiency in ultrasound-directed nerve blocks is preferred.

For immediate consideration, please send a letter of interest and curriculum vitae to:

The University of Maryland, Baltimore is an Equal Opportunity, Affirmative Action employer. Minorities, women, individuals with disabilities, and protected veterans are encouraged to apply.

Peter Rock, M.D., M.B.A., F.C.C.M.
Chair, Department of Anesthesiology
University of Maryland School of Medicine
22 South Greene Street, S11C00
Baltimore, MD 21201
Or e-mail: **prock@anes.umm.edu**

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Volume 127, Number 1, July 2017

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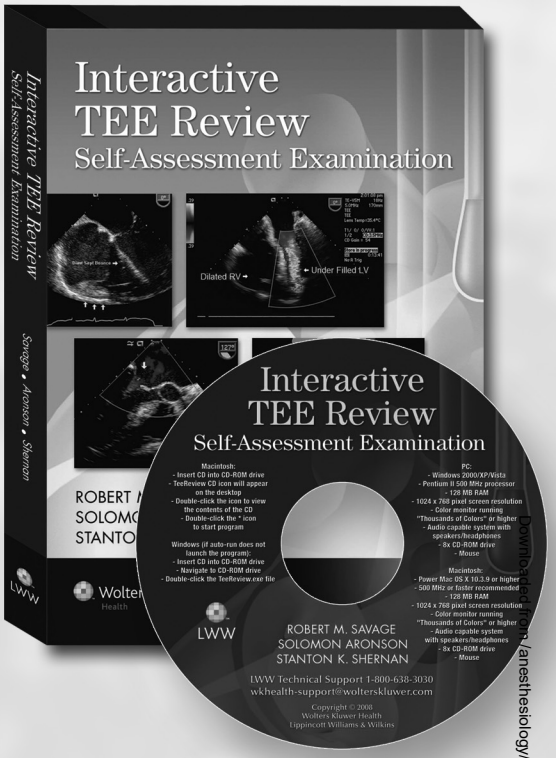

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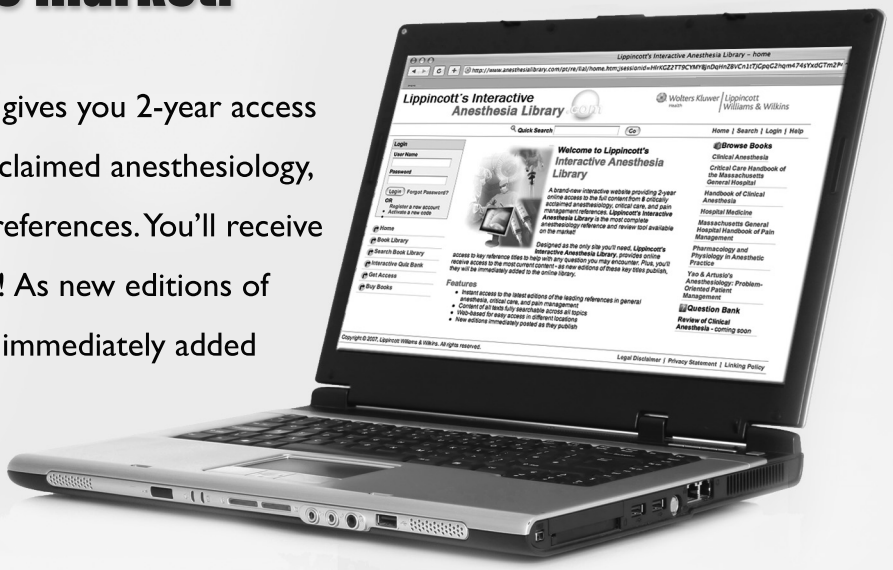

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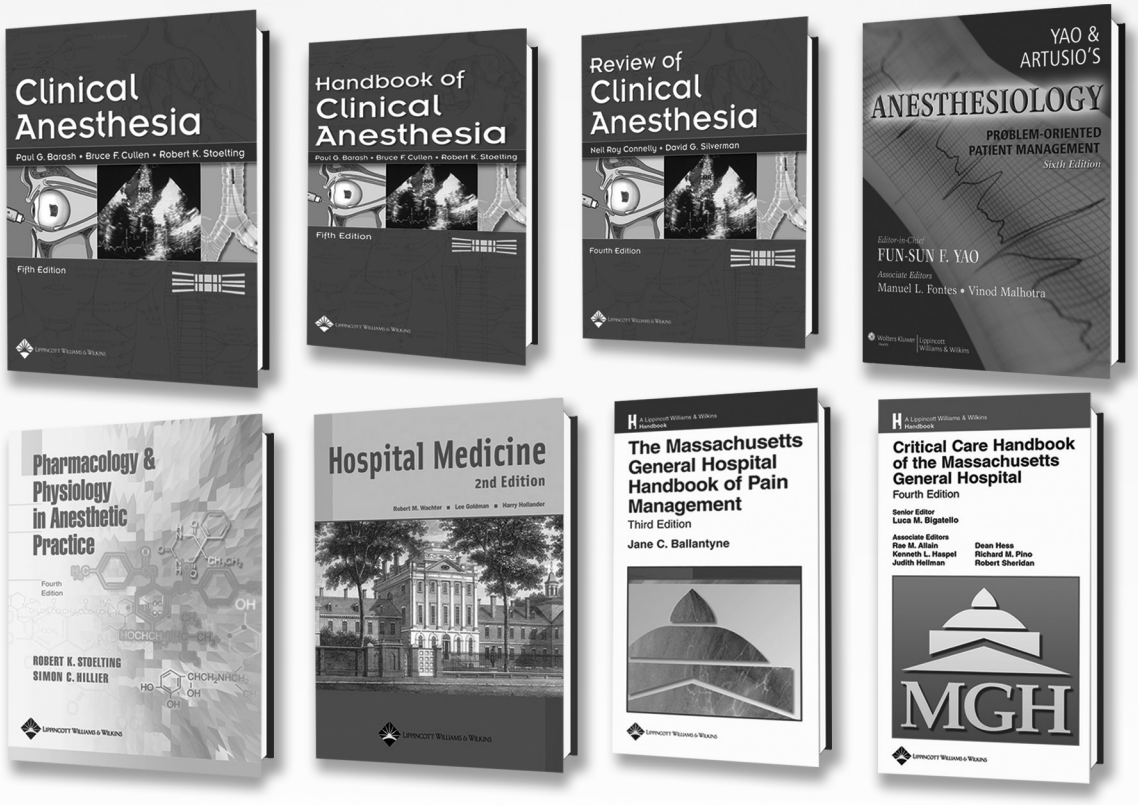

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International Journal of Anaesthesia
Volume 118 Number 6 June 2017