In Reply:

We would like to thank Mikhael *et al.* for their interest and comments related to our article[1] and appreciate the opportunity to respond to the raised questions. Our study was by no means designed to prove the usefulness of cutaneous mitochondrial oxygen tension (mito-Po_2) as physiologic transfusion trigger, but had the more modest goal of investigating the effects of hemodilution on this parameter. Because of the surprisingly useful behavior of mito-Po_2 in our model, *i.e.*, a steep decline when the individual critical hemoglobin threshold during hemodilution is neared, mito-Po_2 seems a very promising parameter for use in transfusion medicine. Obviously, as also pointed out in the accompanying editorial,[2] both technical and physiologic questions remain and the work has only just begun. In their letter, Mikhael *et al.* raise some of such remaining questions.

For example, the influence of anesthetic agents on mitochondrial function and their effect on mito-Po_2 remains to be investigated. Mito-Po_2 reflects the balance between cellular oxygen supply and demand,[3] and any drug that alters mitochondrial respiration could therefore have an effect on this parameter. On the supply side, drugs inducing vasodilation or vasoconstriction will alter cellular oxygen supply and might therefore also affect mito-Po_2. So, the authors are correct in suggesting that the use of specific anesthetic agents might influence mito-Po_2 measurements. However, our choice for intravenous agents was not because of any potential advantageous hemodynamic profile, but because it is the standard anesthesia regimen in our laboratory.[4] The animal ventilator used for the protocol was not equipped for the administration of inhalation agents.

As the authors state, heating the measuring site to 38°C might improve local regional blood flow, which potentially counteracts adaptive peripheral vasoconstriction. For us, the primary reason for heating the skin was not because of such vasoactive effects. The calibration constants needed for conversion of a measured triplet state lifetime to oxygen tension,[5] and thus for the mito-Po_2 measurements, are temperature dependent, as is cellular metabolism. Therefore, we chose to standardize the skin temperature in our protocol. It would definitely be interesting to alternatively measure skin temperature and correct the mito-Po_2 measurement accordingly. Such an algorithm is currently under development and evaluation.

For our study design, we followed the concept of critical hemoglobin level (Hb_{crit}) to be defined as the Hb below which total body oxygen consumption starts depending on oxygen delivery.[6] Surely this definition of Hb_{crit} does not guarantee that certain tissues do not become hypoxic at an earlier stage during hemodilution. Key in the idea to use mito-Po_2 in the skin as the parameter in transfusion-related decision making is the "canary" hypothesis. As pointed out by O'Brien and Schmidt,[2] "This 'canary' hypothesis—that cutaneous mitochondrial PO_2 changes foretell changes in other vital organs—while plausible, remains unproven." Therefore, as the authors rightfully suggest, efforts to gather information about the oxygenation status of other organs (like the brain) in relation to cutaneous mito-Po_2 under various pathophysiologic circumstances are important to take into account in future studies.

The authors state that inclusion of mixed venous oxygen saturation and arterial oxygen tension in the original publication could have been valuable and more informative. We understand that these data might be of help for some readers and therefore provide the values in this reply. For the control group at the nine time points from baseline to hemodilution step 8, the values for arterial oxygen tension were (mean ± SD) 199 ± 34, 199 ± 33, 199 ± 34, 199 ± 34, 196 ± 33, 196 ± 34, 196 ± 34, 194 ± 34, 196 ± 33 mmHg and for mixed venous oxygen saturation 72 ± 14%, 72 ± 17%, 68 ± 17%, 69 ± 18%, 66 ± 18%, 67 ± 17%, 66 ± 17%, 66 ± 15%, 66 ± 14%. For the experimental group, these values were (mean ± SD) 207 ± 8, 207 ± 10, 206 ± 5, 206 ± 7, 203 ± 11, 199 ± 12, 196 ± 24, 196 ± 14, 168 ± 56 mmHg and 72 ± 10%, 72 ± 7%, 73 ± 7%, 70 ± 8%, 65 ± 12%, 59 ± 16%, 55 ± 18%, 59 ± 17%, and 53 ± 15%, respectively.

We believe that autonomic metabolic reprogramming and mitochondrial dysfunction are important contributors to the pathophysiology of critical illness. Therefore, we aim at developing our technology to enable measurement of both mitochondrial oxygenation and respiration *in vivo*.[7–10] Since the ultimate goal of erythrocyte transfusion is maintaining a sound oxygen balance and preventing cell death by sustaining cellular respiration, mitochondrial respiration itself might prove to be an important transfusion threshold. In any case, the proof of the pudding will be in the eating, and more experimental and clinical studies are needed to fully understand the potential of mito-Po_2 measurements. Very recently a Conformité Européenne-marked clinical monitoring system enabling mito-Po_2 measurements at the bedside became available.[11] This COMET monitor, with COMET being an acronym for Cellular Oxygen METabolism, paves the way for clinical research and firm testing of the proposed concepts.

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Competing Interests

Dr. Mik is founder and shareholder of Photonics Healthcare B.V. (Utrecht, The Netherlands), a company aimed at developing a clinical monitoring device based on the delayed fluorescence lifetime technology for measuring mitochondrial oxygen. Photonics Healthcare B.V. holds the exclusive licenses to several patents related to this technology, filed and owned by the Academic Medical Center in Amsterdam, The Netherlands, and the Erasmus Medical Center in Rotterdam, The Netherlands. The other authors declare no competing interests.

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Flumazenil Modulation of the γ-Aminobutyric Acid Type A Receptor: Competitive *versus* Noncompetitive Antagonism at the Agonist-binding Site

To the Editor:

A variety of therapeutic drugs administered by anesthesiologists are commonly and effectively pharmacologically reversed. Unfortunately, and somewhat ironically, general anesthetics are not among them. It was, therefore, with great interest that I read the article "*Effects of γ-Aminobutyric Acid Type A Receptor Modulation by Flumazenil on Emergence from General Anesthesia.*"[1] Among the many interesting findings, this study demonstrated that flumazenil can reduce isoflurane-mediated potentiation of the γ-aminobutyric acid (GABA) type A receptor ($GABA_AR$) in the presence of GABA, weakly activate $GABA_ARs$ in the absence of GABA, and shorten the time required for the electroencephalogram to return to an awake-like state after isoflurane administration.

Based on flumazenil's ability to reduce maximal peak currents evoked by GABA without shifting the GABA concentration–response relationship (fig. 7[1]), the authors concluded that the drug binds to the GABA-binding site, where it acts as a competitive antagonist. However, I wish to point out that such behavior is not indicative of a competitive antagonist. Rather, it is the hallmark of a noncompetitive antagonist. Had flumazenil acted as a competitive antagonist of GABA, the expected result would have been quite different: to shift the GABA concentration–response relationship rightward toward higher concentrations without reducing the peak currents evoked by maximal GABA concentrations. More broadly, I believe that this fact calls into question the authors' mechanistic conclusion that the various actions of flumazenil revealed by their *in vitro* studies and the variable results of clinical studies reported by other groups can be explained by flumazenil's interactions with the GABA-binding site.

There are multiple binding sites for benzodiazepines and benzodiazepine-like drugs on the $GABA_AR$ that are known to positively and/or negatively allosterically modulate receptor function. There is the classical high-affinity benzodiazepine site located in the extracellular domain at the α^+–γ^- subunit interface, which is distinct from the GABA-binding site located in the extracellular domain at the β^+–α^- subunit interface.[2,3] There are also low-affinity sites located in the extracellular domain at the α^+–β^- subunit interface and at subunit interfaces in the transmembrane domain where several classes of general anesthetics bind.[4–6] As flumazenil is a benzodiazepine analog that the authors' data show acts noncompetitively with GABA on the $GABA_AR$, it seems more likely that flumazenil produces its inhibitory and enhancing receptor actions by binding to the multitude of sites that are known to bind benzodiazepines and benzodiazepine-like drugs rather than by directly binding to the agonist (*i.e.*, GABA)–binding site.

Competing Interests

The author declares no competing interests.

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