Plasma methemoglobin as a potential biomarker of anemic stress in humans

La méthémoglobine plasmatique: un marqueur potentiel du stress anémique chez l'humain

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Abstract

Purpose Transfusion of allogeneic red blood cells (RBCs) is one of the main treatments of acute anemia secondary to blood loss and fluid resuscitation within the operating room. Decisions to transfuse blood are based largely on intermediate biological markers (hemoglobin, arterial oxygen saturation, blood pressure, heart rate) which may not accurately reflect inadequacy of tissue oxygen delivery.

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Based on experimental studies, we hypothesized that anemia-induced tissue hypoxia activates adaptive mechanisms which promote local vascular nitric oxide (NO) production to improve tissue perfusion and survival during acute anemia. Hemoglobin (Hb) oxidation to methemoglobin (MetHb) may be a byproduct of such local NO production. Therefore, we tested the hypothesis that MetHb is a biomarker of hypoxic-anemic stress during acute hemodilution associated with cardiopulmonary bypass.

Methods With institutional ethics approval, routine laboratory arterial blood gas and co-oximetry values were obtained from 295 patients undergoing heart surgery during February 1 to September 30, 2010, and the values were assessed retrospectively. All samples with an arterial oxygen saturation value $\geq 90\%$ were included ($n = 1,421$). The maximal change in Hb associated with hemodilution on cardiopulmonary bypass was determined within 48 hr of surgery ($n = 180$). A chart review was performed to determine the incidence of RBC transfusion and exogenous nitrate administration. All anonymous data were analyzed by linear regression to determine the relationship between

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Hb and MetHb. A Wilcoxon Signed Rank Test and Student's *t* test were used to determine changes in Hb, MetHb, and carboxyhemoglobin (CarboxHb) levels. All data are presented as mean and significance was assigned at $P < 0.05$.

Results A significant decrease in Hb [$118 (20) \text{ g}\cdot\text{L}^{-1}$ vs $94 (18) \text{ g}\cdot\text{L}^{-1}$] was associated with an increase in MetHb [$0.88 (0.22)\%$ vs $0.95 (0.24)\%$] ($P < 0.001$ for both), but not CarboxHb [$1.08 (0.47)\%$ vs $1.08 (0.49)\%$]. Regression analysis revealed a significant relationship between the change in Hb and MetHb ($F = 40.3$; $P < 0.001$) but not between the change in Hb and CarboxHb ($F = 0.2$; $P = 0.694$). This correlation was not influenced by RBC transfusion or exogenous nitrate use.

Conclusions A negative correlation was observed between the change in Hb and MetHb in patients undergoing cardiac surgery and cardiopulmonary bypass. These data support the previously unreported hypothesis that MetHb may be a marker of anemic stress associated with reduced tissue perfusion during acute hemodilution in humans. Further prospective studies are needed to determine if these changes in MetHb are linked to adverse outcomes in patients undergoing cardiac surgery.

Résumé

Objectif La transfusion de globules rouges allogéniques est l'un des principaux traitements de l'anémie aiguë secondaire à une perte sanguine et au remplissage liquidien en salle d'opération. La décision de transfuser du sang repose essentiellement sur des marqueurs biologiques intermédiaires (hémoglobine, saturation en oxygène du sang artériel, pression artérielle, fréquence cardiaque) qui pourraient ne pas refléter avec précision l'insuffisance d'apport d'oxygène aux tissus. À partir d'études expérimentales nous avons fait l'hypothèse qu'une hypoxie tissulaire induite par l'anémie active des mécanismes adaptatifs qui encouragent la production locale d'oxyde nitrique (NO) vasculaire pour améliorer la perfusion tissulaire et la survie pendant l'anémie aiguë. La méthémoglobine (MétHb), résultant de l'oxydation de l'hémoglobine (Hb), peut être un sous-produit de cette production locale de NO. Nous avons donc testé l'hypothèse que la MétHb est un biomarqueur du stress anémique/hypoxique survenant au cours de l'hémodilution aiguë associée à la circulation extracorporelle.

Méthodes Avec l'approbation du comité d'éthique de l'établissement, les résultats de tests de laboratoire de routine des gaz artériels du sang et d'une cooxymétrie ont été collectés auprès de 295 patients subissant une chirurgie cardiaque entre le 1^{er} février et le 30 septembre 2010; les valeurs ont été évaluées de façon rétrospective. Tous les échantillons dont la saturation en oxygène du sang artériel était $\geq 90\%$ ont été inclus ($n = 1\,421$). La

variation maximum du taux d'hémoglobine associée à l'hémodilution au cours de la circulation extracorporelle a été déterminée dans les 48 heures de l'intervention chirurgicale ($n = 180$). Les dossiers ont été analysés pour connaître l'incidence de la transfusion de globules rouges et de l'administration de dérivés nitrés exogènes. Toutes les données anonymes ont été analysées par régression linéaire afin de déterminer la relation existant entre Hb et MétHb. Un test des rangs signés de Wilcoxon et un test *t* de Student ont permis de déterminer les variations d'Hb, de MétHb et de carboxyhémoglobine (CoHb). Toutes les données sont présentées sous forme de moyennes et la signification était atteinte pour $P < 0,05$.

Résultats Une diminution significative de l'Hb [$118 (20) \text{ g}\cdot\text{L}^{-1}$ contre $94 (18) \text{ g}\cdot\text{L}^{-1}$] a été associée à une augmentation de la MétHb [$0,88 (0,22)\%$ contre $0,95 (0,24)\%$] ($P < 0,001$ pour les deux), mais pas de la CoHb [$1,08 (0,47)\%$ contre $1,08 (0,49)\%$]. L'analyse de régression a révélé une relation significative entre les variations d'Hb et de MetHb ($F = 40,3$; $P < 0,001$) mais pas entre les variations d'Hb et de CoHb ($F = 0,2$; $P = 0,694$). Cette corrélation n'a pas été influencée par les transfusions de globules rouges ou l'utilisation de dérivés nitrés exogènes.

Conclusions Une corrélation négative a été observée entre les variations d'Hb et de MétHb chez des patients subissant une chirurgie cardiaque et une circulation extracorporelle. Ces données confortent l'hypothèse antérieure non publiée selon laquelle la méthémoglobine pourrait être un marqueur du stress anémique associé à une perfusion tissulaire réduite au cours d'une hémodilution aiguë chez l'humain. D'autres études prospectives sont nécessaires pour déterminer si ces variations de la MétHb sont liées à des résultats délétères chez des patients subissant une chirurgie cardiaque.

A number of clinical studies have shown that preoperative anemia and acute hemodilution are independent risk factors for increased mortality in cardiac and non-cardiac surgery.¹⁻³ The recent finding that mild and moderate cases of anemia are also associated with increased mortality in patients undergoing cardiac and non-cardiac surgery makes this a critically important area of anesthesia research.^{4,5} Experimental studies have shown that both hypoxic and non-hypoxic mechanisms may contribute to anemia-induced organ injury and mortality.^{3,6,7} The complex nature of the cellular mechanisms involved may explain why anemia-induced mortality is not necessarily improved by therapies which increase blood oxygen content, including red blood cell (RBC) transfusions,^{8,9} use of erythropoiesis stimulating agents (ESAs),¹⁰ or hemoglobin-based oxygen carriers (HBOCs).¹¹ Each of these strategies is capable of increasing blood oxygen content but may not increase tissue oxygen

delivery. Indeed, some of these treatment strategies (transfusion, HBOCs) may limit tissue perfusion by reducing nitric oxide (NO) bioavailability, thereby increasing tissue hypoxia and mortality. These findings underscore the need for a fundamental understanding of the cellular mechanisms involved.

The lack of an early clinical biomarker of “anemic stress” has greatly impaired our ability to develop effective strategies to improve patient outcomes. As recently stated by one of the pioneers of anemia research, Dr. R.B. Weiskopf, “We have no clinical measures that let us know of impending insufficient oxygenation as anemia progresses”.¹² Toward achieving this goal, we have developed experimental models to define the adaptive mechanisms which maintain oxygen homeostasis during acute anemia. Our research has shown that increased NO production by nitric oxide synthases (NOSs) may be an important survival mechanism in acute anemia.^{3,13,14} We have shown that deficiency of neuronal NOS (nNOS) is associated with increased mortality in a model of hemodilutional anemia, such that mice lacking nNOS die at a mean hemoglobin (Hb) value of $35 \text{ g}\cdot\text{L}^{-1}$, a level that is significantly higher than nNOS replete mice or humans ($\sim 25 \text{ g}\cdot\text{L}^{-1}$).^{7,15} In search of a clinical biomarker of NOS activation during anemia, we have hypothesized that increased nNOS-derived NO promotes a tissue-plasma NO gradient that favours the oxidation of Hb to MetHb, as shown by the negative

correlation between increasing MetHb and decreasing Hb in experimental models of anemia (Fig. 1).³

In addition, a number of mechanisms have been proposed to explain how local NO production can regulate vascular tone in “hypoxic” vascular beds (Fig. 2). In one of these proposed mechanisms, MetHb is a reactive intermediate in the production of S-nitrosylated Hb (SNO-Hb); a form of Hb that may subsequently release NO in hypoxic vascular beds.^{16,17} Experimental data in support of another proposed mechanism suggest that the stable NO metabolite nitrite (NO_2^-) may provide a physiological source of bioactive NO by deoxygenated hemoglobin (*deoxyHb*), which can act as a nitrite reductase under allosteric and pH control via the following reaction: $\text{Nitrite } [\text{NO}_2^-] + \text{deoxyHb} + \text{H}^+ \rightarrow \text{NO} + \text{MetHb} + \text{HO}^-$.¹⁸⁻²² In either case, MetHb may be a biomarker of increased NO production and/or decreased oxygen saturation in local vascular beds. These proposed mechanisms may be particularly favoured during anemia since local production of NO would be supported by an increase in tissue oxyHb extraction which is known to occur in anemic vascular beds.^{23,24} For example, in the brain, acute anemia increases Hb O_2 extraction from $\sim 30\%$ ($\text{Hb} \sim 140 \text{ g}\cdot\text{L}^{-1}$) to $\sim 50\%$ ($\text{Hb} \sim 50 \text{ g}\cdot\text{L}^{-1}$),²³ while brain microvascular PO_2 decreases from ~ 70 to $\sim 50 \text{ mmHg}$.^{6,7} As such, methemoglobin (MetHb) production may be favoured in anemic vascular beds.

Fig. 1 Schematic representation of the mechanism by which anemia may lead to up-regulation of perivascular nitric oxide synthase (NOS) and increased nitric oxide (NO) signalling. Anemia increases NOS-derived nitric oxide (NO), which can stabilize hypoxia inducible factor (HIF) in tissues by S-nitrosylation (SNO) of the von Hippel-Lindau protein (pVHL) to activate adaptive cellular mechanisms.⁷

Increased tissue NOS activity generates tissue to blood nitric oxide (NO) gradient, which promotes NO diffusion into the vascular lumen to cause local vasodilation and oxidize hemoglobin (Hb) to methemoglobin (MetHb). In addition, local conversion of nitrite (NO_2^-) to NO by deoxyhemoglobin (DeoxyHb) can promote vasodilation via a blood to tissue NO gradient

Fig. 2 Mechanism by which nitric oxide (NO) may influence cellular biology and methemoglobin formation by: 1) S-nitrosylation (SNO) of globin cysteine (Cys β 93) on hemoglobin⁴²; 2) SNO-modification of circulating proteins⁴³; 3) deoxyhemoglobin (DeoxyHb) reduction of nitrite (NO_2^-) to methemoglobin (MetHb)¹⁸; 4) free radical-mediated formation of MetHb and protein nitration^{44,45}; and 5) redox-mediated protein SNO-modifications⁴⁶

In attempting to identify a link between anemia and MetHb levels in patients, we observed that anemia was a common clinical finding (>84%) in two of the largest reported case series of acquired methemoglobinemia.^{25,26} Therefore, we have hypothesized that increased MetHb may be exploited as a potential biomarker of tissue hypoxia and acute anemic stress. We further assessed this hypothesis in humans by assessing the relationship between changes in Hb concentration and MetHb in patients undergoing acute hemodilution on cardiopulmonary bypass.

Methods

The St. Michael's Hospital Institutional Research Ethics Board approval was obtained to retrieve retrospective data from a clinical database, waiving the need for individual patient consent. All routine arterial blood gas values, co-oximetry data, and relevant clinical data were obtained from patients undergoing cardiac surgery during February 1 to September 30, 2010. All data were collected by automated electronic data collection and stored in a confidential and secure encrypted computer. Data were acquired initially from 6,201 patients. On initial screening of the data, all samples with arterial oxygen saturation (S_aO_2) $\geq 90\%$ were included so as to avoid assessing patients with hypoxemia. This resulted in selection of 1,421 samples from 295 patients. In order to remove duplicate sampling from each patient and to detect the maximal drop in Hb associated with cardiopulmonary bypass, a second data screening was performed. In this screen, only patients with pre-determined initial Hb values $\geq 90 \text{ g}\cdot\text{L}^{-1}$ were included in order to eliminate

patients with pre-existing severe anemia. A second sample was then identified as the maximal change in Hb within the next 48 hr. This strategy was taken to obtain a pair of measurements for each patient that corresponded to a drop in Hb associated with hemodilution on cardiopulmonary bypass and/or surgical blood loss and fluid resuscitation. One hundred and eighty paired samples were detected in 180 patients. A subsequent chart review was used to obtain the incidence of transfusion (*red blood cells, plasma, platelets, cryoprecipitate*) and exogenous nitrate use. Prior to analysis, the data were assigned an independent identification number and any personal or hospital identification was removed.

Hemoglobin species are measured by co-oximeters (Radiometer ABL 800 Flex, Radiometer, Copenhagen, Denmark) which are calibrated every three months against an oxygenated (saturated) whole blood standard prepared by the manufacturer. The Hb value is determined by the manufacturer using a HiCN spectrophotometric reference method using a filter calibrated against the NIST SRM 930D filter (National Institute of Standards and Technology). Quality control material with validated and assigned target values for Hb, MetHb, and carboxyhemoglobin (CarboxyHb) is analyzed three times daily. Methemoglobin and CarboxyHb measurements are calibrated against the total hemoglobin concentration. With respect to MetHb measurements, daily control samples were run at levels of 10%, 5%, and 2%. At the 2% level, the standard deviation is $<0.001\%$.

Statistics

A sample size estimate was based on experimental data in animals which showed that a negative correlation was

observed with 120 paired measurements. Therefore, we estimated that at least this number of paired samples would be needed in humans. We obtained these data from patients undergoing cardiopulmonary bypass in 2010. Data were then assessed in terms of the total and change in hemoglobin, MetHb, and CarboxyHb for each pair. Paired data were assessed by a Wilcoxon Signed Rank Test, and differences in mean nadir MetHb were assessed by Student's *t* test. Linear regression analysis was performed for change in hemoglobin vs MetHb and CarboxyHb, respectively.

Results

All samples were from arterial blood gases as shown by the clinical record and the S_aO_2 cut off of $>90\%$. When all samples were assessed from 295 patients, a significant correlation was observed between the total hemoglobin and MetHb ($n = 1,421$ samples; $F = 75.4$; $P < 0.001$) and between the change in Hb and MetHb ($n = 1,392$ samples; $F = 258.4$;

Fig. 3 Relationships between total and change in hemoglobin (Hb) and methemoglobin (MetHb) (upper panel) and changes in Hb and MetHb (lower panel) in patients undergoing heart surgery with cardiopulmonary bypass ($P < 0.001$ for both)

$P < 0.001$) (Fig. 3). With subsequent screening by hemoglobin $>90 \text{ g}\cdot\text{L}^{-1}$ (1st Sample) and identifying the greatest change in hemoglobin within 48 hr (2nd Sample), 180 paired data points were obtained. In this data set, a significant decrease in hemoglobin [$118 (20) \text{ g}\cdot\text{L}^{-1}$ vs $94 (18) \text{ g}\cdot\text{L}^{-1}$] was associated with an increase in MetHb [$0.88 (0.22)\%$ vs $0.95 (0.24)\%$], but not CarboxyHb [$1.08 (0.47)\%$ vs $1.08 (0.49)\%$] (Fig. 4). Subsequent regression analysis revealed a significant relationship between the change in Hb and MetHb ($n = 180$ samples; $F = 40.3$; $P < 0.001$) (Fig. 5) but not between the change in Hb and CarboxyHb ($F = 0.2$; $P = 0.694$). This relationship was maintained in those patients who received a RBC transfusion ($n = 62$; $F = 16.2$; $P < 0.001$) and in those who did not receive a RBC transfusion ($n = 118$; $F = 28.4$; $P < 0.001$). When assessed in terms of the nadir hemoglobin, only patients with a Hb value < 90 showed a significant increase in MetHb ($P < 0.001$) (Fig. 6).

Fig. 4 Change in hemoglobin, methemoglobin, and carboxyhemoglobin [mean (standard deviation)] between the first preoperative (hemoglobin [Hb] $> 90 \text{ g}\cdot\text{L}^{-1}$) and second hemoglobin sample (largest change in Hb within 48 hr) in patients undergoing heart surgery and cardiopulmonary bypass. Notice that the hemoglobin decreased overall while methemoglobin increased. Carboxyhemoglobin was unchanged

Fig. 5 Relationship between the change in hemoglobin and methemoglobin in 180 paired samples from 180 patients from the first (hemoglobin [Hb] > 90 g·L⁻¹) to the second hemoglobin sample (largest change in Hb within 48 hr)

Fig. 6 Relationship between methemoglobin levels when the nadir hemoglobin was above or below the threshold of 90 g·L⁻¹

Of the 180 patients selected for final assessment, the surgical procedures that were performed included: 1) isolated coronary artery bypass grafting (CABG) ($n = 115$); single or double valve replacement ($n = 39$); aortic aneurism repair ($n = 7$); combined CABG-valve replacement ($n = 13$); and other procedures, including septal defect repairs, pericardiectomy, and atrial myxoma excision ($n = 6$). Data showing the frequency of transfusion of blood products in the 180 patient cohort are presented in the Table. Sixty-two (34%) of these 180 patients received a RBC transfusion, most of which were given after the nadir Hb had been reached. Most of these patients received the RBC transfusion after leaving the operating room (36 of 62). Only 26 patients received a RBC transfusion in the operating room. Preoperative use of long-acting nitrates was recorded in 13 (7%) of 180 patients. None of these patients received intraoperative nitroglycerine and 16 (9%)

Table Record of blood product transfusion

Product transfused (180 patients)	Number of patients transfused, n (%)	Number of units transfused	Number of units per transfused patient
Red blood cells	62 (34)	176	2.8
Plasma	19 (11)	63	3.3
Platelets	18 (10)	24	1.3
Cryoprecipitate	11 (6)	95	8.6

of 180 patients received postoperative nitrate therapy, primarily for treatment of hypertension.

Discussion

These retrospective data support the hypothesis that MetHb may be a useful biomarker of anemic stress in humans. We showed that a reduction in hemoglobin concentration in patients undergoing cardiac surgery and cardiopulmonary bypass is associated with an increase in MetHb levels. This relationship was not altered by RBC transfusion or exogenous nitrate use. A similar relationship did not occur for CarboxHb which served as an internal negative control. The proposed mechanism for increased MetHb may include NO-mediated Hb oxidation or reduction of nitrite (NO₂⁻) by DeoxyHb, as suggested by experimental and clinical studies.^{3,18-22} It will be important to assess these changes in subsequent studies to determine if the increase in MetHb is reflective of activated adaptive mechanisms (nNOS)⁷ and/or increased OxyHb desaturation to DeoxyHb and tissue hypoxia¹⁸⁻²² during acute hemodilutional anemia.

Future prospective studies are planned to establish whether the observed increase in MetHb is predictive of adverse clinical outcomes. These studies will be needed to assess the clinical impact of: 1) postponing surgery to treat anemia and prevent adverse outcomes; or 2) utilizing the rise in MetHb as an appropriate trigger for transfusion to reduce adverse outcomes in cardiac and non-cardiac surgery. In a recently published Phase 1 trial (TRICS 1), the feasibility of completing a transfusion threshold study (80 g·L⁻¹ vs 100 g·L⁻¹) showed twice the number of ischemia-related events in the low Hb threshold group.²⁷ Although the study was not powered to assess differences in patient outcomes, the data raise the frequently posed clinical question regarding whether cardiac surgical patients require a higher Hb threshold. Assessment of MetHb level is planned as a component of the ongoing Phase 2 study (TRICS 2). These future studies are required to establish the importance of MetHb as a functional biomarker for anemic stress, potentially marking the threshold of increased risk of hypoxic tissue injury during acute hemodilution and anemia.

With respect to the selection of appropriate transfusion triggers, the authors do not regard any one measurement as a sole marker for transfusion (Hb, MetHb etc). The clinical decision to transfuse RBCs is always based on a number of parameters, including patient co-morbidities, changes in physiological parameters, type of surgery, patient consent for treatment, and hemoglobin concentration. The purpose of the current study was to assess whether increased MetHb levels could reflect activation of adaptive physiological mechanisms indicative of tissue hypoxia during anemia as suggested by our basic science research.^{3,4,6} The potential biological significance of this approach is emphasized by two possible mechanisms by which increased MetHb production is favoured during anemia. First, the adaptive increase in nNOS during anemia promotes organism survival in part by activating hypoxic cellular mechanisms in anemic mice.⁷ In this case, MetHb is a byproduct of nNOS-derived NO (Fig. 1). Second, the increase in DeoxyHb in local vascular beds, secondary to increased oxygen extraction,^{23,24} would favour the production of NO from nitrite with MetHb as the byproduct (Fig. 2).¹⁸⁻²² In both cases, the increase in MetHb may signify activation of adaptive mechanisms, likely triggered by inadequate oxygen delivery and tissue hypoxia.

The importance of NOS-derived NO as a signalling mechanism for adaptive cardiovascular responses has been extensively reviewed.²⁸⁻³¹ Nitric oxide signalling is known to regulate cardiac output and systemic vascular resistance via direct action at the cardiac myocyte and vascular smooth muscle and by regulation of centrally mediated changes in autonomic function.²⁸⁻³¹ In a model of acute anemia, nNOS protects against mortality,⁷ providing strong rationale to pursue the current line of clinical research.

The importance of maintaining NO signalling at times of hemodynamic stress has been shown indirectly by experimental and clinical studies which have shown adverse outcomes in association with therapy that limits NO availability. For example, NOS inhibition impairs cerebral blood flow responses in anemic animals and those undergoing hemodilution during cardiopulmonary bypass.^{32,33} When systemic NOS inhibitors were administered to increase mean arterial pressure and improve clinical outcomes in septic patients, a surprising increase in mortality was observed.³⁴ Finally, the use of HBOCs to improve oxygen delivery has resulted in the paradoxical finding of increased incidence of myocardial injury and mortality. These adverse outcomes may have been due to reduced NO bioavailability.^{11,35} Thus, generalized NOS inhibition or NO scavenging has been associated with adverse cardiovascular outcomes and increased mortality.

Experimental models of acute hemodilution have shown that anemia results in an increase in both neuronal and inducible NOS (nNOS and iNOS), possibly in response to

anemia-induced tissue hypoxia.^{3,13,14} These tissue responses to hypoxia have been identified since early metazoan development³⁶ and have been shown to support survival during acute anemia.⁷ The link between increased NOS and MetHb has been derived by our analysis of experimental studies assessing acute changes in hemoglobin and MetHb during hemodilution anemia.³ In these models, a characteristic and proportional increase in plasma MetHb was observed with decreasing hemoglobin levels. We chose to assess whether a similar Hb/MetHb relationship occurred in humans. To do this, we chose to study patients undergoing cardiopulmonary bypass, as all of these patients experience a consistent and sudden drop in hemoglobin by hemodilution. The current data are encouraging and support future studies to assess the Hb/MetHb relationship in prospective studies in cardiac and non-cardiac surgery.

Under physiological conditions, most healthy individuals and patients undergoing cardiovascular surgery have baseline blood MetHb levels near 1%, as shown by measurements in the current study. While the increase in MetHb levels in response to a drop in Hb is quite small, it occurred in proportion to the drop in Hb, suggesting that there may be a relationship between the two changes. These levels are not clinically significant from the perspective of absolute oxygen carrying capacity of the blood. However, they may represent a true physiological signal in response to biological adaptation and hypoxic stresses associated with anemia, as described above.^{3,4,6} In addition to being biologically relevant, these small changes are well within the detection range of our co-oximeter, which can detect changes within a standard deviation of 0.001% (please see methods). For example, for all samples in which the MetHb increased by more than 0.001% ($n = 63$ of 180), the mean MetHb increased from 0.007 (0.002)% to 0.011 (0.002)%. This represents a 57% increase ($P < 0.001$). Therefore, the increase in MetHb may be small, but it has the potential of being both biologically and statistically relevant. We did not observe a relationship between changes in Hb and CarboxyHb, possibly because changes in CarboxyHb depend more specifically on blood carbon monoxide levels and are less influenced by redox reactions.

Although the incidence of acquired methemoglobinemia is very low, it frequently occurs in response to the use of topical anesthetic agents.^{25,26,37} The mechanism of anesthetic-induced methemoglobinemia is thought to occur as a result of direct oxidation of hemoglobin by amine metabolites derived from the amide-anesthetics.³⁷ Benzocaine use appears to have the highest incidence of methemoglobinemia.³⁷ However, many other drugs which possess oxidizing capacity can overwhelm the endogenous MetHb reductase and cause methemoglobinemia.²⁵ In addition, direct exposure to inhaled NO can have significant impact on the systemic circulation and remote tissue perfusion via

the action of stable NO metabolites.³⁸ Inhaled NO can also cause elevated MetHb levels in specific clinical settings.³⁹ Despite the rarity of clinically significant increases in MetHb, two of the largest series of case reports have shown a high incidence of anemia in 84% and 94% of cases.^{25,26} In one case series, methemoglobinemia was diagnosed at blood sampling by anesthesiologists during cases in which acute blood loss prompted the measurement of the hemoglobin concentration.²⁵ This suggests that anemia (and acute blood loss) may have been a risk factor for methemoglobinemia in a previously reported case series. This background supports the findings of the current study and supports our hypothesis that MetHb may be a biomarker of anemic stress in humans.

Methylene blue is used to treat methemoglobinemia by its ability to reduce MetHb via non-enzymatic donation of electrons⁴⁰ and by its ability to impair NO-mediated oxidation of hemoglobin.³ Methylene blue is also known for its NO binding capacity and treatment of vasoplegia associated with cardiopulmonary bypass.^{40,41} Taken together, these data suggest that methylene blue is a potent inhibitor of NO production/action and is also associated with a profound vasoconstricting effect. This may maintain blood pressure in the setting of profound vasoplegia associated with cardiac surgery. However, under more physiological conditions (*anemia*) in which systemic NO may be regarded as adaptive, the impact of NO binding by methylene blue may be detrimental.

The current study has some limitations. The retrospective nature and small sample size are identified weaknesses of this study. These data may relate only to cardiac surgical patients undergoing cardiopulmonary bypass and may not be generalized to non-cardiac surgery. The time of Hb sampling was not controlled in this study. The relatively small changes in MetHb levels may make this a relatively insensitive means of detecting anemia-induced changes in cardiovascular physiology. However, these measurements were within the detection limits of the co-oximeters used. The potential impact of extrinsic nitrates was assessed and found not to influence the outcome as few patients received these medications. Although RBC transfusion may influence MetHb levels, we did not observe this effect. In our study, only 26 (14%) of 180 patients received RBCs at the time of surgery when the maximal change in Hb was recorded. The negative correlation between Hb and MetHb was maintained despite the presence or absence of a RBC transfusion. Furthermore, in 34 paired samples in which the Hb increased by an average of 15 (17) g·L⁻¹, corresponding MetHb levels did not change [− 0.001 (0.003)]. Thus, no relationship between RBC transfusion and increased MetHb was observed.

In conclusion, these human data provide support for the hypothesis that increased MetHb levels may provide an

early biomarker of anemic stress in our patients. In future studies, these changes in Hb/MetHb need to be correlated with clinical outcomes, such as renal failure, myocardial infarction, stroke, and death. If a positive correlation is observed between changes in Hb/MetHb and clinical outcomes, then assessment of MetHb levels may provide an early biomarker for the detection of anemia-related adverse outcomes. In addition, new methods of assessing Hb and MetHb in real time may offer a noninvasive means for assessing changes in Hb/MetHb in our patients.²⁸

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Competing interests None declared.

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