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ORIGINAL RESEARCH

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Exploring the association of erythrocytic NO-ferroheme, a surrogate marker of endothelial function with perioperative cardiovascular events in low/intermediate risk patients undergoing elective non-cardiac surgery

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Abstract

Purpose Pre-operative assessment of cardiovascular risk currently relies on scores, such as the American Society of Anesthesiologists (ASA) score, biased towards high-risk, but neglecting middle/lower risk patients. Endothelial dysfunction is a precursor to cardiovascular events (CVEs), due to impaired nitric oxide (NO) bioavailability. We previously showed that the erythrocytic NO-ferroheme including the 5-coordinated NO-heme-α-hemoglobin (HbNO), a complex between NO and deoxyhemoglobin correlates with endothelial function assessed by digital tonometry. The aim of this study was to evaluate if HbNO is associated with the different cardiovascular risk factors and to explore its association with CVE in patients undergoing elective non-cardiac surgery.

Methods We conducted a prospective, monocentric study in adult patients scheduled for elective non-cardiac surgery. At preoperative visit, blood samples were collected, and erythrocytes isolated to measure baseline HbNO levels, along with other biomarkers routinely used to evaluate pre-operative risk factors. NO-ferroheme signals were quantified using electron paramagnetic resonance spectroscopy. Follow-up visits and data analysis using electronic health records were conducted at 1-, 3-, 6- and 12- months postoperatively. The primary endpoint was the occurrence of a composite of CVE, including arrhythmias, chest pain/unstable angina, myocardial infarction/ischemia, pulmonary edema, pulmonary embolism, stroke, deep venous thrombosis, cardiac failure and death of any cause.

Results Between November 2019 and June 2022, 2,500 patients were screened and 1,066 patients underwent an elective non-cardiac surgery. Among the 1,066 patients kept for the final analysis, 23 subjects developed a perioperative CVE up to 30 days after surgery (*p*-30d CVE). Linear regression analysis revealed several independent factors significantly correlated with HbNO levels, including hemoglobin, anticoagulant usage, and smoking status.

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Patients who developed *p*-30d CVE exhibited lower mean HbNO levels (124.2 ± 96.6 nM) compared to those who did not (154.8 ± 104.1 nM; $p = 0.028$). Using a threshold of 124 nM for HbNO, levels below this cutoff (HbNO < 124 nM) were associated with an increased risk of *p*-30d CVE (OR [95% CI] = 4.21 [1.55–11.41]), as did classification in ASA III or higher (OR [95% CI] = 3.23 [1.38–7.59]). However, after excluding patients at high risk of CVE a priori, HbNO < 124 nM remained associated to *p*-30d CVE (OR [95% CI] = 5.52 [1.57–19.33]) while the association to ASA-score was no longer significant (OR [95% CI] = 0.89 [0.20–3.97]).

Conclusion In patients scheduled for non-cardiac surgery, known cardiovascular risk factors, such as active smoking independently and negatively correlates with erythrocytic NO-ferroheme including HbNO. In patients without severe comorbidities, despite the limited number of CVEs observed, HbNO levels under 124 nM were independently and positively associated with *p*-30d CVE up to 30 days after surgery, while the ASA score was no longer correlated to *p*-30d CVE. HbNO measurements could help to improve the preoperative evaluation of low/intermediate risk patients.

Trial registration Registered at ClinicalTrials.gov on June 19, 2019 (NCT03994900).

Keywords NO-ferroheme, Erythrocytic Nitrosylated hemoglobin (HbNO), Peri-operative cardiovascular events, Endothelial function, Nitric oxide bioavailability, Non-cardiac surgery

1 Introduction

At preoperative anesthesiology visit, the assessment of cardiovascular risk is important for the prevention of cardiovascular events (CVEs). Indeed, the surgical stress induces inflammation and a neuroendocrine response leading to overexpression of inflammatory mediators and immune suppression that may increase the risk of perioperative complications in specific patients [1]. In 2014, a report of the European Society of Cardiology (ESC) showed that worldwide, 3–5% of the patients undergoing non-cardiac surgery develop a CVE and in Europe at least 167,000 per- or post-operative cardiovascular complications are recorded per year [2]. In order to assess the risk of developing complications, anesthesiologists rely on clinical scoring systems such as the American Society of Anesthesiologists (ASA) Physical Status Classification System (ASA score), the Revised Cardiac Risk Index (RCRI) and Systematic Coronary Risk Evaluation (SCORE).

The first two scoring systems identify patients with a documented cardiac history. However, they may underestimate the risk in patients who have never experienced a cardiac event, despite being more susceptible to developing CVE in the immediate postoperative period simply due to the surgical stress [3].

Some cardiac biomarkers have shown an added value for the long-term follow up of patients after surgery, but their measurement in the pre-operative period is still restricted to patients at high risk, including patients with clinical signs such as murmurs, chest pain, dyspnea, or peripheral edema [4]. Nevertheless, the peri- and postoperative cardiovascular risk assessment of patients without cardiovascular signs or symptoms remains difficult.

The occurrence of virtually all CVE is preceded by the silent development of endothelial dysfunction (ED). ED is characterized by an impaired homeostatic balance in the endothelium with excessive thrombogenicity, vasoconstriction and endothelial cell activation leading to sub-intimal inflammation, smooth muscle cells proliferation and atherogenesis, with plaque rupture eventually resulting in vessel occlusion [5]. Among pleiotropic factors, the decreased endothelial production of nitric oxide (NO) and/or its excessive degradation are at the core of ED. Classical risk factors of cardiovascular diseases, including age, smoking habits, hyperlipidemia, diabetes, or arterial hypertension are well known to contribute to ED in part by decreasing endothelial NO.

Our group previously demonstrated that the concentration of erythrocytic NO-ferroheme including the 5-coordinated NO-heme-α-hemoglobin (HbNO), correlates with endothelial function. This was assessed using digital tonometry, a non-invasive technique that measures endothelium-dependent changes in peripheral arterial tone with sensors placed on the fingertips. This technique registers the changes in arterial tone produced by a post-occlusion hyperemic response that involves, in part, the shear-stress mediated production of NO [6]. We devised a method to measure NO-ferroheme, a complex between NO and Fe^{2+}-heme in erythrocytes collected from venous blood by electronic paramagnetic resonance (EPR) spectroscopy upon the revelation of its specific hyperfine structure after digital subtraction of spectral components of protein-free radicals [6, 7]. Recent studies have implicated the mobile NO-ferroheme as a novel signaling species in the vasculature [8–10], which may shed from the vascular bed and associate with erythrocyte membranes, likely contributing part of the erythrocyte HbNO signal in addition to HbNO. Building on these premises, we tested the hypothesis that cardiovascular risk factors are independently associated with

preoperative HbNO. Additionally, as part of an exploratory analysis, we investigated the potential association between preoperative HbNO levels and perioperative CVE in patients undergoing elective non-cardiac surgery.

2 Material and methods

2.1 Design of the study

This prospective and monocentric study was carried out in collaboration with the Department of Anesthesiology of the Cliniques Universitaires Saint-Luc (CUSL) in Brussels. The study protocol was approved by the ethical committee (Comité d'Ethique Hospitalo-Facultaire, CUSL), chaired by Prof. J-M Maloteaux, on July 16, 2019 (Approval Number: 2019/16JUL/323) and was registered in ClinicalTrials.gov (ID: NCT03994900). The study results are presented according to the STROBE cohort reporting guidelines [11].

2.2 Patients

Patients were recruited during their pre-intervention visit. All patients involved signed an informed consent form.

Patients aged 18 years or older, with at least one cardiovascular risk factor and scheduled for elective non-cardiac surgery were included. In a subsequent amendment (approved on May 10, 2021), the inclusion criteria were extended to patients with or without cardiovascular risk factors (> 18 years) in order to extend the identification of clinical and biological factors correlated to HbNO. Patients requiring emergency surgery were excluded due to the potential impact of urgent medical conditions on biological biomarkers and clinical parameters, as well as their higher likelihood of developing complications compared to those undergoing elective surgeries. The full list of inclusion and exclusion criteria is described in Supplementary Table 1.

2.3 Schedule of the study

A total of 2,500 patients were recruited at CUSL between November 2019 and June 2022. The recruitment was interrupted during the COVID-19 pandemic for a total of 3 months from March 2020 to June 2020.

Following enrollment, patients completed a questionnaire detailing their health profile, while data were extracted in parallel from their medical records to fill the electronic case report form (eCRF). Subsequently, patients were directed to the venipuncture center of the CUSL for blood collection. Outcomes were monitored through medical records at 1-, 3-, 6- and 12- months post-surgery. Additionally, patients received online questionnaires and/or phone calls to verify any occurrence of CVE not documented in their medical files.

2.4 Electronic data capture

To minimize interviewer and data collection bias, eCRFs were designed and validated during the protocol development phase and subsequently utilized throughout the study. Study data were collected and managed using the REDCap (Research Electronic Data Capture) tool hosted at UCLouvain. REDCap is a secure, web-based software platform designed to support data capture for research studies, facilitating data validation, tracking encoded data, sending surveys to patients and downloading the database to common statistical software [12, 13].

2.5 Sample processing and HbNO measurement

Following the collection of blood samples and the addition of 200 µL of an antioxidant mixture, the samples underwent centrifugation for 10 min at 800 g. Subsequently, 400 µL of erythrocytes were extracted and promptly frozen in liquid nitrogen and stored in a −80°C freezer. In order to maintain the stability of the NO-ferroheme including HbNO complex, samples needed to be processed and erythrocytes frozen within 30 min after venipuncture, as delayed freezing might have elicited hemoglobin oxidation and prevented proper quantification.

Low-temperature EPR spectra from the frozen samples were recorded using an EPR quartz finger Dewar filled with liquid nitrogen at 77 K, on a Bruker EMX100 X-band spectrometer with the following setting: microwave frequency, 9.35 GHz; modulation frequency, 100 kHz; microwave power, 20 mW; modulation amplitude, 7 mT; 7 scans [7].

Additional blood analyses were performed at baseline to assess patients' global risk, including hemoglobin, glomerular filtration rate (GFR), creatinine, urea, aspartate aminotransferase, alanine aminotransferase, gamma-glutamyl transferase, C-reactive protein (CRP), total cholesterol and triglycerides.

2.6 Assessment of cardiovascular risk factors at preoperative visit

High blood pressure was defined as baseline blood pressure ≥ 135/85 mmHg with or without antihypertensive drugs (angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, calcium channel blockers, diuretics, beta-blockers).

Hypercholesterolemia was defined as baseline total cholesterol level ≥ 250 mg/dL with or without hypolipemiant drugs (fibrate, statin or ezetimibe).

Anemia was diagnosed when hemoglobin was ≤ 13 g/L or 12 g/L, respectively, in male and female patients.

Renal failure was diagnosed when creatinine was ≥ 1.5 mg/dL and/or GFR < 60 mL/min and/or mention of chronic renal failure in the medical file.

Inflammation was diagnosed when CRP > 5 mg/dL.

2.7 Scores used to assess cardiovascular risks at preoperative visit

ASA score categorizes patients from ASA I to ASA VI based on their medical history and the severity of known diseases, determining their capacity to endure surgery and anesthesia. ASA I represents healthy patients, ASA II patients with moderate comorbidities, ASA III patients with severe comorbidities, ASA IV patient with severe comorbidities that is a constant threat to life, ASA V a moribund patient who is not expected to survive without the operation and ASA VI a declared brain-dead patient whose organs are being removed for donor purposes [14].

RCRI is based on six parameters: (1) Elevated-risk surgery, (2) history of ischemic heart disease, (3) history of congestive heart failure, (4) history of cerebrovascular disease, (5) pre-operative treatment with insulin and (6) pre-operative creatinine > 2 mg/dL. This score is divided into 4 classes: RCRI I (0 point), RCRI II (1 point), RCRI III (2 points) and RCRI IV (≥ 3 points) that are correlated with a risk of CVE during and 30d after surgery of 0.5%, 1.3%, 4%, and 9%, respectively [15, 16].

SCORE based on sex, smoking status, total cholesterol levels, systolic blood pressure and age provides an estimate of an individual's risk of experiencing a fatal CVE within a period of 10 years. In our evaluation, the SCORE table for low-risk regions of Europe was used. Note, however, that SCORE is not specifically designed to assess perioperative risk [17].

2.8 Assessment of cardiovascular events

Following the surgical procedure, patients' medical records were reviewed to identify documented events in both the intraoperative report and during post-operative hospital stay. Subsequently, patients underwent a follow-up visit one-month post-surgery, from which additional information was collected in their medical records.

CVE, the primary endpoint of our study was a composite of:

- Arrhythmia (supraventricular or ventricular tachycardia, atrial fibrillation);
- Myocardial infarction/ischemia;
- Pulmonary edema;
- Pulmonary embolism;
- Chest pain, characterized as angina, including unstable angina;
- Stroke;
- Deep venous thrombosis;
- Congestive heart failure;
- Death of any cause.

3-, 6- and 12- months following the surgery, patients received an online questionnaire to assess self-reported complications. Additionally, a study nurse called the patients and consulted the medical file to verify the self-reported outcome at 6- and 12- months post-surgery.

Events were counted in a way to prevent the duplication of endpoint CVE occurrences at different time points. This approach distinguishes CVE incidents during the perioperative period (up to 30 days) from those observed between 30 days and 3 months, from 3 to 6 months, or 6 months to 12 months post-surgery, thereby avoiding duplication.

2.9 Assessment of surgery grade

The surgeries were classified using the report of the Belgian Federal Center for Healthcare Expertise (KCE): "Routine preoperative testing report 280 B" (Supplementary Table 2) [18].

2.10 Statistical analyses

2.10.1 Sample size

A calculation of the sample size was performed using the software PASS 14 (Power Analysis and Sample Size Software (2015), NCSS LLC: Kaysville, UT, USA). Considering an occurrence of cardiovascular complications of 3%, 1,452 subjects (44 positive subjects with CVE and 1,408 negative subjects without CVE) provide an area under the curve equal to 0.800 with a 95% width confidence interval (CI) of 0.160. An interim analysis in February 2021 showed a dropout rate of 18%. This high rate was due to the technical difficulty to process the samples within the 30 min limit. As a consequence, recruitment was extended to 2,500 patients.

2.11 Statistical tests

Due to the non-normal distribution of HbNO (Shapiro–Wilk test; $p < 0.001$), a logarithmic transformation was used and all subsequent statistical analyses were performed using Log_{10} (HbNO). HbNO values were expressed in nanomolar (nM) units.

Patient baseline characteristics were presented as counts and percentages. Median HbNO [minimum–maximum] and mean HbNO ± SD (standard deviation) were reported for each patient category, followed by student t-tests for comparison. Univariate regression analyses

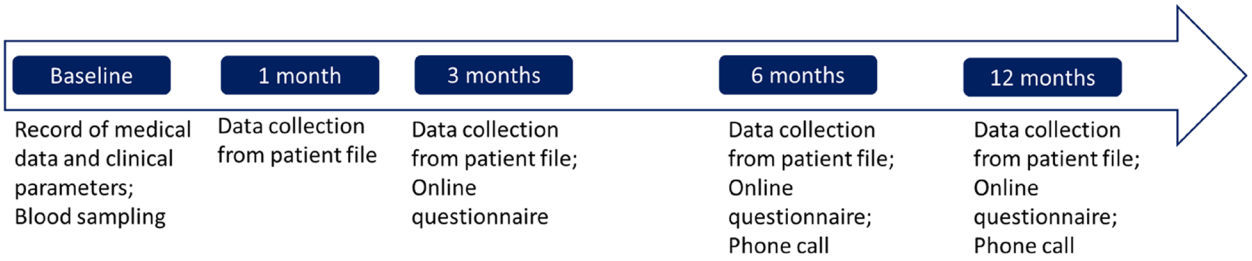

Fig. 1 Schedule of the study

were performed to examine associations between HbNO and clinical and biological factors at baseline. Statistically significant univariate correlates ($p < 0.05$) were included in a subsequent multivariate linear regression to identify independent factors correlating with HbNO.

To investigate the association with CVE, odds ratios (ORs) were calculated. HbNO levels were also compared between patients who experienced CVE and those who did not using a T-test.

The association with the occurrence of CVE was also compared between pre-operative HbNO and the clinical scores used by anesthesiologists, both in the peri-operative up to 30 days period, and at 3-, 6- and 12-months post-surgery. For this comparison, a threshold value of HbNO to discriminate patients at risk/or not of CVE was derived from the mean HbNO level of patients who experienced CVE.

Missing values were not restricted to primary outcomes and were assumed to be missing at random. To address this, pairwise deletion was employed as a method to manage these missing data points. Additionally, patients lost to follow-up at 3-, 6-, and 12-month post-surgery visits were managed using the same approach.

Data analysis was performed using Stata: Release 17 (Statistical Software, StataCorp LLC: College Station, TX, USA) and graphs were performed by GraphPad Prism version 10.0.0 for Windows (GraphPad Software, Boston, MA, USA).

All statistical tests were two-tailed and a significance was set at $p < 0.05$.

3 Results

3.1 Study flowchart

A total of 2,500 patients were enrolled (Figs. 1 and 2). 1,015 samples were processed in more than 30 min because of technical delays in the routine clinic and were excluded from analysis. 360 samples were processed within 30 min but had an EPR spectrum that was either unanalyzable, due to overlapped methemoglobin EPR signal and/or protein free radicals signal. A total of 1,375 samples were then excluded from the statistical analysis. 1,125 patients had valid measurements of HbNO which were used to assess correlations with baseline cardiovascular risk factors. 59 patients (and their HbNO values) were excluded because they needed a cardiac surgery and/or their surgery was cancelled and/or they needed an emergency surgery, resulting in residual 1,066 patients and HbNO values for associations with CVE.

3.2 Is HbNO correlated to cardiovascular risk factors?

We first compared the values of HbNO at baseline in patients with or without cardiovascular risk factors, as reported in Table 1. Due to the non-normal distribution of HbNO, its representation took two forms: median with range, and mean accompanied by standard deviation, both described for each category in Table 1. To compile this table, we used the total number of patients with a valid HbNO value (i.e., 1,125 patients; see Fig. 2).

The analysis presented in Table 1 revealed several differences in pre-operative HbNO among some categories of patients. Active smokers had a lower HbNO level compared to non-smokers ($HbNO_{Active\ smoker} = 127.1 \pm 78.4$ nM vs $HbNO_{non\text{-}smoker} = 160.0 \pm 107.1$ nM; $p < 0.001$). Conversely individuals with anemia had a higher HbNO levels ($HbNO_{\_with\_anemia} = 178.9 \pm 134.4$ nM) than those without anemia ($HbNO_{\_without\_anemia} = 149.2 \pm 94.7$; $p = 0.009$). Patients diagnosed with chronic obstructive pulmonary disease (COPD) had lower HbNO levels ($HbNO_{\_with\_COPD} = 115.4 \pm 54.7$ nM) compared to those without COPD ($HbNO_{\_without\_COPD} = 154.3 \pm 103.5$ nM; $p = 0.025$). There were no significant differences in HbNO levels between patients with a history of cardiovascular diseases and those without.

The proportions of patients taking major classes of cardiovascular and metabolic medications and their respective HbNO values are detailed in Supplementary Table 3. A higher HbNO level was observed only in patients taking anticoagulants compared to non-users ($HbNO_{\_anticoagulant} = 192.4 \pm 145.4$ nM *vs* $HbNO_{\_no\_anticoagulant} = 151.1 \pm 99.3$ nM; $p = 0.002$).

Univariate regression results, depicted in Table 2, include the regression coefficient (β) and standard error (SE). These analyses revealed a positive main effect of age, as well as anticoagulant use on HbNO levels.

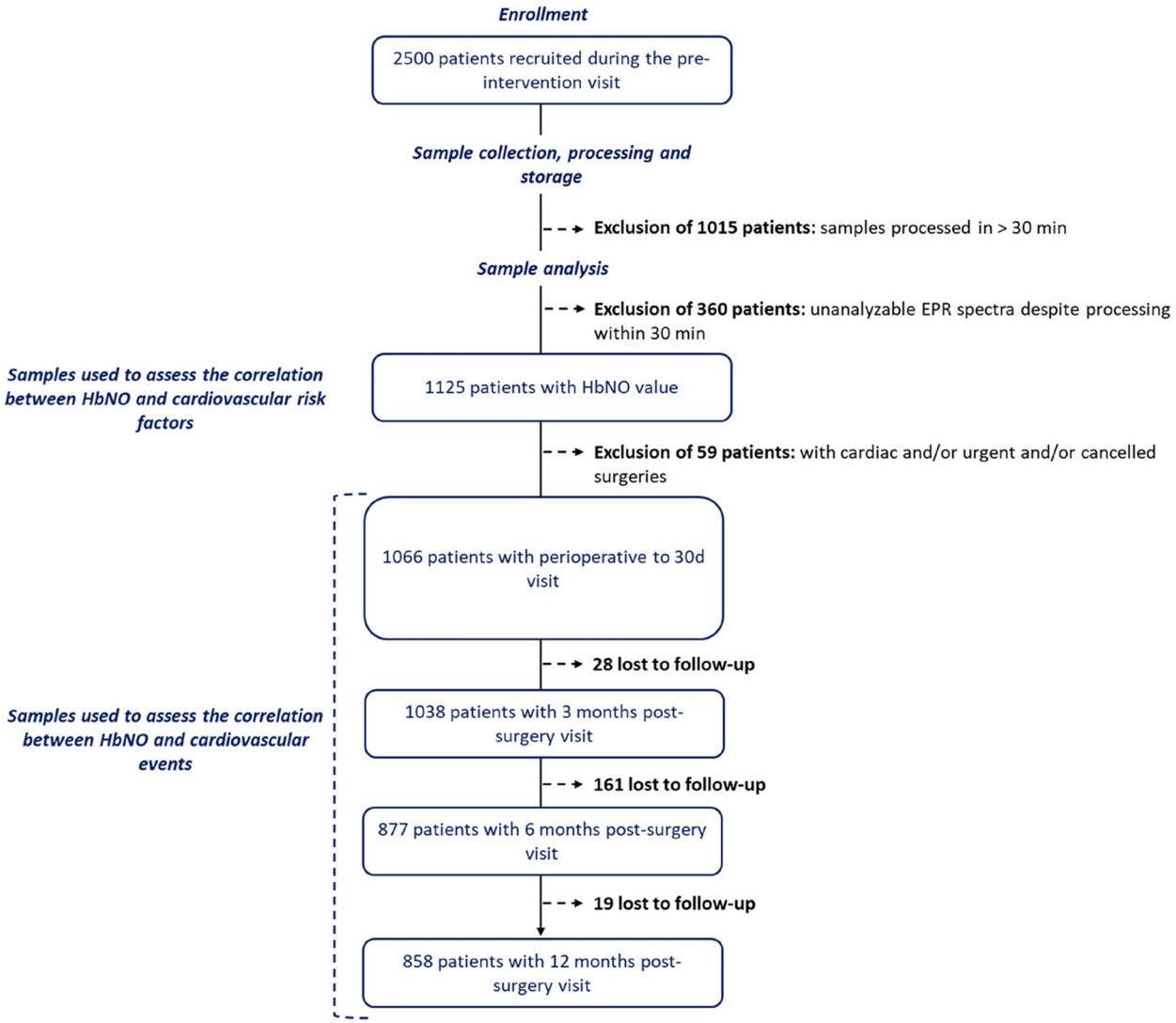

Fig. 2 Study flow chart. *HbNO* 5-coordinated NO-heme-α-hemoglobin, *EPR* electronic paramagnetic resonance

Conversely, smoking and hemoglobin levels negatively impacted HbNO. Variables that correlated significantly with HbNO levels in univariate regression analyses were integrated into a multivariate regression model (Table 2). This multivariate analysis echoed the significant effects observed in univariate regression, with the exception of age.

As HbNO results, in part, from a complex between hemoglobin and NO, the inverse correlation between HbNO and hemoglobin values seemed counter-intuitive. In search of potential explanations, we further investigated clinical factors associated with anemia (Supplementary Table 4). Notably, anemia was more prevalent in patients with chronic renal failure and inflammation (CRP > 5 mg/dL), respectively. Upon exclusion of patients with inflammation and chronic renal failure, the regression between HbNO and hemoglobin was no longer significant ($p = 0.117$; Supplementary Table 5). This suggests an ancillary effect of both inflammation and renal failure on the biology of NO that may influence its propensity to form HbNO.

3.3 Is HbNO associated to peri-operative to 30d cardiovascular events (*p*-30d CVE)?

The exploratory objective of this study was to evaluate the association between pre-operative HbNO and *p*-30d CVE. In this cohort, among the 1066 patients that had elective non-cardiac surgery (Fig. 2), we recorded 10 cases of arrhythmia, 2 of deep venous thrombosis (DVP), 8 cases of myocardial infarction/ischemia (with elevated troponin, creatine kinase-myocardial band (CK-MB) with or without N-terminal pro-B type natriuretic

Table 1 Association of preoperative HbNO levels and baseline characteristics of the patients

	N (%)	HbNO (nM)		T-tests
		Median (min–max)	Mean ± SD	*p*-values
Cardiovascular risk factors				
Sex				0.173
Male	459 (41.0)	127.1 (22.5–766.2)	146.7 ± 86.6	
Female	666 (59.0)	132.1 (21.8–1034)	158.1 ± 112.5	
Age				0.065
< 60y	599 (53.3)	127.1 (21.8–1034)	146.8 ± 95.1	
> 60y	526 (46.7)	132.4 (22.5–879.9)	161.1 ± 110.5	
Active smoking				**< 0.001**
Yes	224 (19.9)	111.1 (21.8–766.2)	127.1 ± 78.4	
No	900 (80.1)	134.9 (23.4–1034)	160.0 ± 107.1	
Hypercholesterolemia (total cholesterol ≥ 250 mg/dL)				0.893
Yes	101 (10.5)	138.0 (39.0–779.4)	154.6 ± 110.6	
No	859 (89.5)	130.1 (22.5–1034.0)	154.9 ± 101.7	
Diabetes				0.768
Yes	171 (15.2)	133.4 (31.0–770.2)	152.2 ± 98.4	
No	954 (84.8)	129.6 (21.8–1034)	153.7 ± 103.6	
High blood pressure (≥ 135/85 mmHg)				0.227
Yes	395 (45.2)	129.2 (22.5–1034)	152.7 ± 110.2	
No	479 (54.8)	133.4 (21.8–952.7)	159.2 ± 105.7	
Obesity (≥ 30 kg/m^2)				0.695
Yes	317 (28.2)	129.7 (28.1–770.2)	149.0 ± 90.3	
No	808 (71.8)	129.6 (21.8–1034)	155.2 ± 107.3	
Cardiovascular history				
History of stroke				0.072
Yes	45 (4.0)	121.7 (26.3–591.7)	138.8 ± 107.2	
No	1,080 (96.0)	129.7 (21.8–1034)	154.1 ± 102.6	
History of heart failure				0.856
Yes	27 (2.4)	122.5 (49.4–880.0)	161.6 ± 160.4	
No	1098 (97.6)	129.7 (21.8–1034)	153.2 ± 101.1	
History of myocardial infarction				0.800
Yes	64 (5.7)	124.6 (22.5–880.0)	161.5 ± 141.4	
No	1061 (94.3)	129.7 (21.8–1034)	153.0 ± 100.1	
Other comorbidities				
Anemia (< 13 g/dL in male or 12 g/dL in female)				**0.009**
Yes	174 (16.6)	136.6 (29.5–952.7)	178.9 ± 134.4	
No	873 (83.4)	129.7 (21.8–1034)	149.2 ± 94.7	
Chronic obstructive pulmonary disease				**0.025**
Yes	24 (2.1)	117.9 (22.5–265.2)	115.4 ± 54.7	
No	1,101 (97.9)	129.9 (21.8–1034)	154.3 ± 103.5	
Chronic renal failure				0.759
Yes	51 (4.5)	118.6 (29.5–530.3)	157.5 ± 109.5	
No	1074 (95.5)	129.7 (21.8—1034)	153.3 ± 102.5	
Active cancer				0.280
Yes	274 (24.4)	134.8 (26.3–879.9)	157.9 ± 98.9	
No	851 (75.6)	127.3 (21.8–1034)	152.0 ± 104.0	

N Number of patients, *SD* Standard deviation, *HbNO* 5-coordinated NO-heme-α-hemoglobin

Table 2 Univariate and multivariate linear regression to assess the independent factors correlating with HbNO

Dependent variable: Log_{10} (HbNO)	**Univariate analysis**		**Multivariate analysis**	
Independent factors:	**β ± SE**	***p***	**β ± SE**	***p***
Male	−0.024 ± 0.016	0.134		
Age (year)	0.001 ± 0.000	**0.023**	-	-
Active smoking	−0.098 ± 0.021	**< 0.001**	−0.081 ± 0.020	**< 0.001**
Hypercholesterolemia (total cholesterol > = 250 mg/dL	−0.003 ± 0.025	0.893		
Hemoglobin (g/dL)	−0.018 ± 0.005	**< 0.001**	−0.015 ± 0.005	**0.002**
BMI (kg/m^2)	−0.001 ± 0.001	0.423		
Diabetes	−0.001 ± 0.022	0.964		
High blood pressure ≥ 135/85 mmHg	0.015 ± 0.016	0.353		
History of heart failure	0.047 ± 0.053	0.383		
History of stroke	−0.049 ± 0.039	0.213		
History of myocardial infarction	0.001 ± 0.034	0.975		
Chronic obstructive pulmonary disease	−0.100 ± 0.052	0.052		
Chronic renal failure	−0.001 ± 0.038	0.969		
Active cancer	0.025 ± 0.018	0.169		
Beta blockers	0.038 ± 0.021	0.063		
Angiotensin receptor blockers	0.034 ± 0.025	0.178		
Angiotensin-converting enzyme	0.017 ± 0.025	0.593		
Anticoagulant drugs	0.118 ± 0.033	**< 0.001**	0.112 ± 0.033	**0.001**
Statin	−0.014 ± 0.020	0.477		
Fibrate	−0.052 ± 0.066	0.432		
Ezetimibe	−0.056 ± 0.051	0.268		
Insulin	0.059 ± 0.041	0.156		
Metformin	−0.021 ± 0.027	0.429		

β Regression coefficient, *SE* Standard error

Table 3 List of cardiovascular events (primary endpoints)

	Peri-operative to 30d CVE (n = 23)	**Late CVE (n = 53)**			**Total (n = 76)**
Post-operative time	**≤ 30 days**	**3 months**	**6 months**	**12 months**	
CVE	**n/N**				
Arrhythmia	10/1066	2/1038	2/877	3/858	17
Congestive heart failure	0/1066	0/1038	0/877	0/858	0
Deep venous thrombosis	2/1066	1/1038	2/877	4/858	9
Myocardial infarction/ischemia	8/1066	0/1038	0/877	1/858	9
Pulmonary edema	0/1066	0/1038	1/877	0/858	1
Pulmonary embolism	0/1066	0/1038	1/877	1/858	2
Stroke	0/1066	1/1038	0/877	2/858	3
Unstable angina	3/1066	2/1038	2/877	1/858	8
Death of any cause	0/1066	5/1038	5/877	17/858	27
Total (n)	23	11	13	29	76

CVE Cardiovascular event

peptide (NT-proBNP), and 3 cases of unstable angina, for a total of 23 *p*-30d CVE (Table 3). Note that in our sample no death occurred up to 30 days postoperatively.

Supplementary Fig. 1 illustrates a forest plot describing the association of clinical factors with *p*-30d CVE. These univariate analyses revealed significant associations

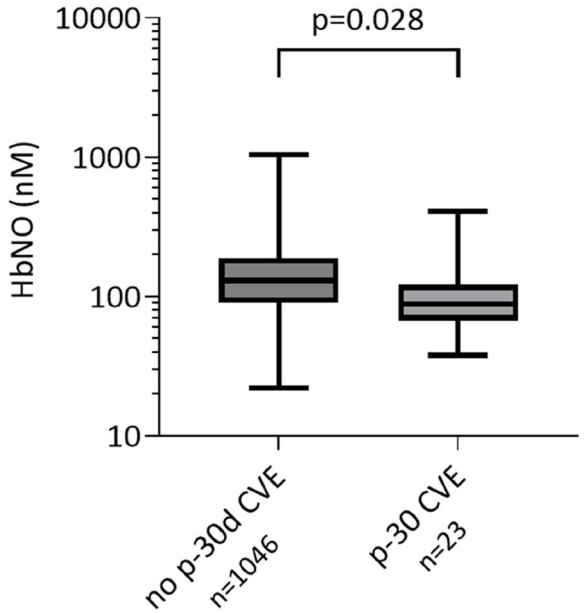

Fig. 3 Box plot comparing HbNO levels in patients with and without peri-operative to 30d cardiovascular events (*p*-30d CVE). These box plots illustrate the comparison of pre-operative HbNO levels between patients who experienced *p*-30d CVE and those who did not. A T-test was employed to assess the differences in HbNO levels between these two groups. The Y-axis is presented on a logarithmic scale. For patients with *p*-30d CVE, the mean HbNO level was 124.2 ± 96.6 nM, whereas for those without *p*-30d CVE, it was 154.8 ± 104.1 nM. The associated *p*-value is also provided. *CVE* Cardiovascular event

between *p*-30d CVE and various factors. Notably, individuals over the age of 60 exhibited a higher prevalence (OR [95% CI] = 2.67 [1.09–6.55]) of *p*-30d CVE. Furthermore, a history of heart failure (OR [95% CI] = 7.67 [2.11–27.92]) and active cancer (OR [95% CI] = 2.48 [1.07–5.72]) were also significantly linked to *p*-30d CVE. In terms of medications (Supplementary Fig. 2), only the use of angiotensin receptor blockers (ARBs) showed an association with *p*-30d CVE (OR [95% CI] = 2.77 [1.07–7.16]).

We next compared HbNO levels in patients with and without *p*-30d CVE (Fig. 3). Patients who experienced a *p*-30d CVE had a lower level of HbNO compared to patients without *p*-30d CVE ($HbNO_{\_with\_p\_30d\ CVE}$ = 124.2 ± 96.6 nM *vs* $HbNO_{\_without\_p\_30d\ CVE}$ 154.8 ± 104.1 nM; $p = 0.028$).

We next tested whether the mean HbNO level of patients with *p*-30d CVE (Fig. 3), could be used as a threshold to identify individuals at risk of developing *p*-30d CVE, i.e., whether patients with an HbNO level < 124 nM could be classified as high risk, and those with an HbNO level ≥ 124 nM could be classified as low risk individuals.

We then compared the discriminatory value of the HbNO threshold (124 nM) to other scoring systems routinely used by anesthesiologists to assess the risk of peri-operative CVE, as well as the SCORE index conventionally used in cardiovascular prevention in general (Fig. 4). The SCORE and RCRI showed no association with *p*-30d CVE in this cohort. Interestingly, ASA III (or higher) classification (OR [95% CI] = 3.23 [1.38–7.59]) and HbNO lower than 124 nM (OR [95% CI] = 4.21 [1.55–11.41]) demonstrated significant associations with the presence of *p*-30d CVE, i.e., patients who had a level of HbNO < 124 nM had 4.21 times higher odds to develop *p*-30d CVE than those who had a HbNO higher or equal than 124 nM; and those who had an ASA score ≥ III had 3.23 times higher odds to develop *p*-30d CVE than those who had lower ASA score.

We also investigated the influence of the surgery grade on the prevalence of *p*-30d CVE. The level of surgery (minor, intermediate or major, defined according to pre-established criteria [18]) showed no effect on the prevalence of *p*-30d CVE (Supplementary Table 6) in our cohort.

After including all pre-operative clinical and biological parameters in a separate univariate analysis, a multivariate analysis including significant univariate predictors ($p < 0.05$) was used to determine independent factors linked with *p*-30d CVE. Given that ASA ≥ III threshold regroups patients with history of heart failure, active cancer, ARB and old age, we removed this parameter from the multivariate analysis (Table 4).

The multivariate analysis showed that only HbNO below the threshold of 124 nM was independently correlated to *p*-30d CVE (OR [95% CI] = 4.25 [1.55–11.55]), while age, history of stroke, active cancer and ARB were no longer significantly associated (Table 4).

To test the discriminatory value of the HbNO threshold specifically in patients at low/middle risk, the univariate analysis was repeated after excluding patients with a history of heart failure, chronic renal failure or high levels of CRP, resulting in 927 patients with 17 *p*-30d CVE; this univariate analysis showed that HbNO 124 nM threshold could still identify 14 patients who experienced a *p*-30d CVE (with an OR [95% CI] = 5.52 [1.57–19.33], Fig. 5) while ASA II or higher was no longer a significant predictor.

3.4 Is HbNO associated to late cardiovascular events (late CVE)?

Following the *p*-30d CVE analysis, our investigation extended to CVE beyond the initial 30-day period, referred to as late CVE. Table 3 outlines a total of 53 late CVE cases, distributed as follows: 11 events between 30d and 3 months post-surgery, 13 between 3 and 6 months,

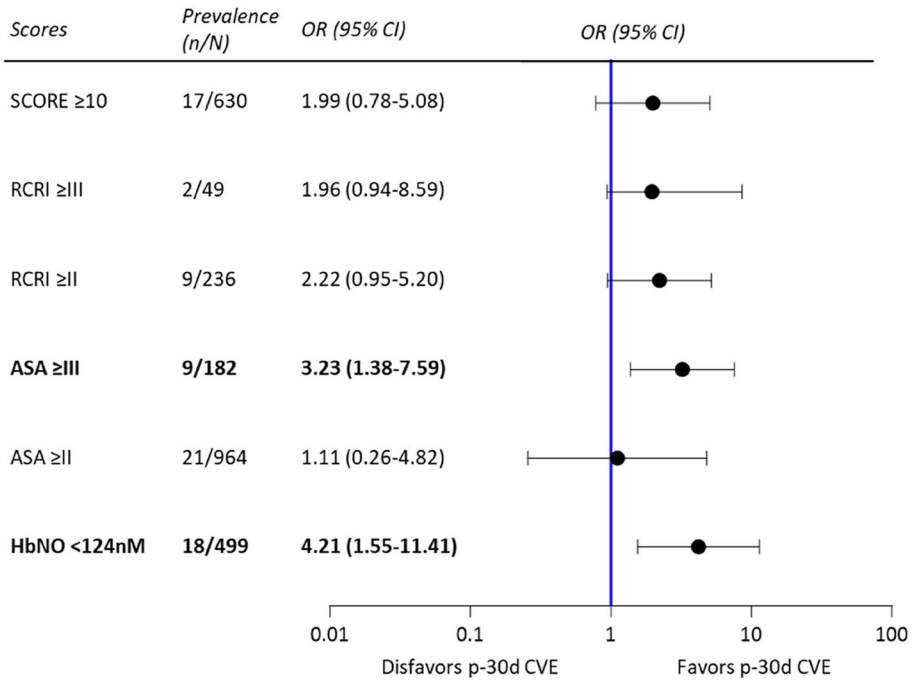

Fig. 4 Forest plot describing the association of pre-operative scores with peri-operative to 30d cardiovascular events (*p*-30d CVE). This forest plot investigates the association between pre-operative scores and the occurrence of *p*-30d CVE. Prevalence is defined as the proportion of patients experiencing the event (*p*-30d CVE) out of the total patients within each subpopulation. Each dot represents the odds ratio (OR), while the horizontal lines denote the lower and upper limits of the 95% confidence interval (95% CI). Factors highlighted in bold indicate statistical significance, $p < 0.05$. *SCORE* Systematic Coronary Risk Evaluation, *RCRI* Revised Cardiac Risk Index, *ASA* American Society of Anesthesiologists, *HbNO* Erythrocytic NO-ferroheme, including nitrosylated hemoglobin

Table 4 Univariate and multivariate logistic regression to assess the independent factors associated with *p*-30d cardiovascular events

Dependent variable: *p*-30d CVE	**Univariate analysis**		**Multivariate analysis**	
Independent factors	**OR (95% CI)**	***p*-value**	**OR (95% CI)**	***p*-value**
Age ≥ 60y	2.67 (1.09–6.55)	**0.032**	2.01 (0.77–5.30)	0.156
History of heart failure	7.67 (2.11–27.92)	**0.002**	3.74 (0.91–15.36)	0.067
Active cancer	2.48 (1.07–5.72)	**0.033**	1.96 (0.80–4.78)	0.139
Angiotensin receptor blockers	2.77 (1.07–7.16)	**0.036**	1.79 (0.63–5.02)	0.272
HbNO < 124 nM	4.21 (1.55–11.41)	**0.005**	4.25 (1.55–11.55)	**0.005**

OR Odds ratio, *CI* Confidence interval

and 29 between 6 months and one year post-surgery. These late CVE included 7 episodes of arrhythmia, 7 episodes of DVP, 1 case of myocardial infarction, 1 case of pulmonary edema, 2 cases of pulmonary embolism, 3 cases of strokes, 5 cases of unstable angina and 27 deaths of any cause (Table 3). Of note, these late CVE cases were distinct from those observed within the initial 30-day postoperative period, as detailed in Table 3.

In contrast to *p*-30d CVE, HbNO levels below 124 nM were not associated to late CVE (OR [95% CI] = 0.94 [0.55–1.63], Fig. 6). Nonetheless, RCRI ≥ II threshold was associated with a significantly increased risk of developing a CVE between 3 and 12 months post-surgery (OR [95% CI] = 5.10 [2.91–8.94], Fig. 6). Similarly, thresholds at ASA ≥ III (OR [95% CI] = 4.53 [2.60–7.91], Fig. 6) and SCORE ≥ 10 (OR [95% CI] = 2.90 [1.48–5.68], Fig. 6) were associated with an increased likelihood of late CVE development.

4 Discussion

This prospective cohort study included patients scheduled for elective non-cardiovascular surgery with a large diversity of clinical profiles, ranging from low to high perioperative risk, with two aims; i. to study the

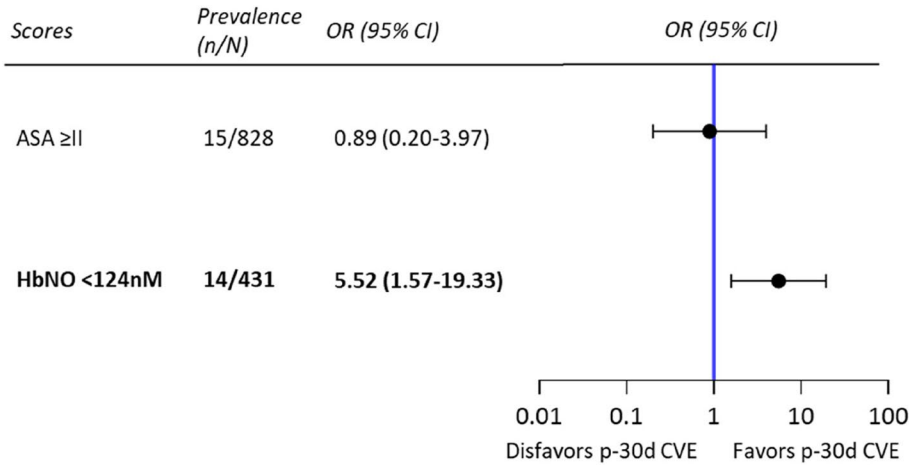

Fig. 5 Forest plot describing the association of pre-operative scores with peri-operative to 30d cardiovascular events (*p*-30d CVE) restricted to low/middle risk patients. This forest plot investigates the association between pre-operative scores and the occurrence of *p*-30d CVE in low/middle risk patients. Prevalence is defined as the proportion of patients experiencing the event (*p*-30d CVE) out of the total patients within each subpopulation. Each dot represents the odds ratio (OR), while the horizontal lines denote the lower and upper limits of the 95% confidence interval (95% CI). Factors highlighted in bold indicate statistical significance, $p < 0.05$. *ASA* American Society of Anesthesiologists, *HbNO* Erythrocytic NO-ferroheme, including nitrosylated hemoglobin

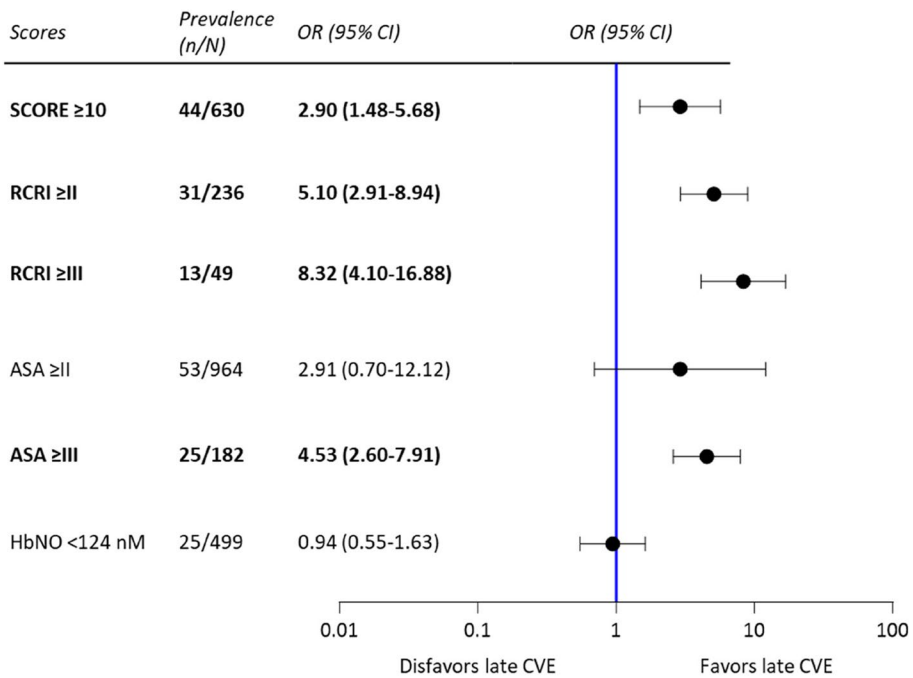

Fig. 6 Forest plot describing the association of pre-operative scores with late cardiovascular events (late CVE). This forest plot investigates the association between pre-operative scores and the occurrence of late CVE. Prevalence is defined as the proportion of patients experiencing the event (late CVE) out of the total patients within each subpopulation. Each dot represents the odds ratio (OR), while the horizontal lines denote the lower and upper limits of the 95% confidence interval (95% CI). Factors highlighted in bold indicate statistical significance, $p < 0.05$. *SCORE* Systematic Coronary Risk Evaluation, *RCRI* Revised Cardiac Risk Index, *ASA* American Society of Anesthesiologists, *HbNO* Erythrocytic NO-ferroheme, including nitrosylated hemoglobin

association between erythrocytic levels of NO-ferroheme including HbNO, a surrogate marker of endothelial function, and cardiovascular risk factors; ii. to evaluate the independent association of pre-operative HbNO with 30-day CVE and late CVE.

HbNO results from the non-covalent binding of NO with the Fe^{2+}-iron-heme of erythrocyte hemoglobin in its de-oxygenated form (T-state). Upon appropriate blood sampling, it is detectable by EPR spectroscopy in isolated erythrocytes from venous human blood, in addition to potential other NO-ferroheme species associated to the erythrocyte membranes. The resulting EPR signal has been quantitatively correlated with human endothelium-dependent microvascular dilatation measured by digital tonometry [6]. Accordingly, lower HbNO levels have been observed in diseases affecting vascular homeostasis, such as endothelitis and cardiovascular complications of COVID-19 infection [19]. The present study allowed a more thorough characterization of the independent factors correlated to HbNO levels, particularly in relation to traditional cardiovascular risk factors, in a large prospective patient cohort.

The independent factors associated to HbNO in the entire cohort are active smoking, the use of anticoagulants and the level of hemoglobin, albeit in different directions.

Indeed, the linear regression model showed that active smoking is inversely correlated with HbNO level. Exposure to cigarette smoke is well known to impair endothelial function, in part by increasing vascular oxidant stress and decreasing NO bioavailability, with a resultant increased endothelial expression of monocyte adhesion molecules [20]. In the long term, this is expected to favor the development of atherosclerosis and vascular ischemic disease.

Given the involvement of factor Xa and thrombin in endothelial dysfunction and atherogenesis, it is perhaps not surprising that HbNO positively correlates with the use of anticoagulant drugs (that block the effects of the above factors) in the present study. In particular, the serum of patients treated with direct oral anticoagulant drugs was shown to promote the expression of vascular endothelium growth factor (a known activator of endothelial nitric oxide synthase [eNOS]) and angiogenesis in cultured HUVECs (Human Umbilical Vein Endothelial Cells), which may promote increased NO bioavailability in vivo [21, 22].

Surprisingly, HbNO levels were found to be inversely correlated with circulating hemoglobin levels, with patients suffering from anemia displaying higher HbNO levels compared to non-anemic patients. This observation may initially seem counter-intuitive given that HbNO is a complex formed between hemoglobin and NO. However, in our cohort, anemia was often associated with chronic renal failure and inflammation. Inflammation can cause anemia and is well known to activate the expression of the inducible nitric oxide synthase that releases large amounts of NO which could increase circulating HbNO levels. Indeed, our group previously showed that patients with severe systemic infection have higher HbNO levels compared to matched-controls [19]. Like inflammation, chronic renal failure is a well-known cause of anemia due to the accumulation of uremic toxins and a decrease of erythropoiesis [23]. The link to increased HbNO here is less obvious, but may involve the facilitated transformation of blood nitrite (derived from dietary nitrate) to NO under conditions of uremic metabolic acidosis [24, 25].

These findings highlight the complex interaction of the different pathologies present in the cohort, providing insight into the intricate mechanisms governing HbNO regulation in pathological conditions. This may explain why HbNO levels remained unaffected by other cardiovascular risk factors such as age, diabetes, hypertension (with or without medications, see Supplementary Table 7), or hypercholesterolemia (with or without medications, see Supplementary Table 8). For the latter, there is only a trend for an inverse correlation, possibly due to the fact that, for most cases, we only had total cholesterol values; a stronger correlation may have been observed with non-high-density lipoprotein cholesterol, which contains most pro-atherogenic lipoproteins.

In terms of HbNO association with *p*-30d CVE our study illustrates two main points: i. In the entire cohort, the pre-operative HbNO levels of patients with *p*-30d CVE were significantly lower compared to those of patients indemn of complications. While numbers (of *p*-30d CVE) are admittedly small, this result is consistent with the hypothesis that endothelial dysfunction, mirrored by lower HbNO, predisposes the patients to cardiovascular complications during or following surgical stress; ii. Using a threshold of 124 nM, pre-operative HbNO levels below 124 nM were significantly associated with a higher incidence of *p*-30d CVE compared to patients with HbNO levels of 124 nM or higher (OR [95% CI] = 4.21 [1.55–11.41]). Additionally, among patients without severe comorbidities, the likelihood of association with *p*-30d CVE was higher for HbNO levels < 124 nM (OR [95% CI] = 5.52 [1.57–19.33]), but the ASA ≥ II score was no longer a significant predictor.

This may not be surprising as ASA classification entails a significant degree of subjectivity and depends on the clinical appraisal by the anesthesiologist, with a rather lax definition of the different ASA classes [26]. Consequently, this can result in the under-classification of patients supposed to have minimal risk.

The current ESC guidelines for the management of perioperative risk recommend to measure cardiac biomarkers only in patients with known cardiovascular risk a priori [4]. These biomarkers include high sensitivity cardiac troponin T (hs-cTnT), CK-MB or NT-proBNP. However, these biomarkers would be elevated and indicate myocardial injury and/or heart failure only after the damage is done, but would have little predictive value in a still healthy patient before the operation. For example, hs-cTnT has a limited predictive value in young, healthy adults [27]. Thus, preoperative measurement of HbNO may be more valuable in these lower-risk patients.

In the context of evaluating surgical risk, a 2014 study demonstrated a correlation between endothelial dysfunction and myocardial injury following intermediate- to high-risk non-cardiac surgery. Endothelial dysfunction was assessed using the reactive hyperemia peripheral arterial tonometry (RH-PAT) index [28]. This multicenter study included 238 patients and defined endothelial dysfunction as an RH-PAT index ≤ 1.22. The RH-PAT index was found to be highly specific (96%) but had low sensitivity (31%). A 2018 study also indicated that RH-PAT could predict adverse events after cardiovascular surgery; however, it identified a different threshold for the RH-PAT index, ≤ 1.64 [29]. The ESC has noted this limitation regarding the lack of a standardized reference value for the RH-PAT index in diagnosing endothelial function [30].

Over the past decade, researchers have attempted to explore endothelial dysfunction by examining eNOS and NO bioavailability through blood sampling. A recent study used a logistic regression model to suggest that asymmetric dimethylarginine (ADMA), an endogenous competitive inhibitor of nitric oxide synthase enzymes, can predict cardiovascular complications [31]. However, the study did not find a significant difference in ADMA levels between patients with and without CVEs and failed to identify a threshold to distinguish between them. Another study found that the L-arginine/ADMA ratio was lower in patients with CVE 4–24 h compared to 72–96 h after major emergency abdominal surgery [32]. Again, there was no significant difference of L-arginine/ADMA ratio level between patients with or without CVE. While corroborating, to some extent, our data in patients with CVE, it should be noted that these biomarkers indirectly reflect NO production by NO synthases, whereas HbNO is a direct, quantitative assay of NO bioavailability. As there is currently no other equipment or biomarker to quantitatively evaluate endothelial function in clinical routine, HbNO may be promising for the identification of patients at risk of perioperative CVE.

However, regarding late CVE, HbNO did not emerge as an independent factor. Instead, RCRI ≥ II, ASA ≥ III, and SCORE ≥ 10 seem to be more valuable. In the case of RCRI ≥ II, this observation is supported by other studies, which have indicated that the cutoff of RCRI ≥ II correlates with an increased risk of myocardial injury and mortality after 30 days in patients undergoing orthopedic surgery [33, 34]. Nevertheless, contrary to findings in the aforementioned studies, our cohort did not demonstrate a positive association between RCRI ≥ II and myocardial injury within 30 days [33, 34]. Furthermore, in one of these previous studies, ASA score ≥ III was not correlated with either early (≤ 30d) or late myocardial injury [34], whereas ASA ≥ III was significantly correlated in our study.

The observed difference in the prognostic effects of classical tools (i.e., ASA, RCRI, SCORE) and HbNO levels in predicting late CVE can be attributed to the distinct nature of these classical scores compared to HbNO. For example, the ASA classification is a comprehensive assessment that includes a wide range of comorbidities and their severity, providing a large evaluation of the physiopathological state of the patient [14, 26]. In contrast, HbNO specifically reflects endothelial function and NO bioavailability, which may be more directly related to acute CVE after surgical stress. Indeed, the ASA classification includes factors such as chronic diseases (hypertension, diabetes, renal dysfunction, history of cardiovascular disease…) that could increase the risk of a CVE independently of the surgery. Thus, ASA provides a general overview of patient health that could impact long-term recovery. Additionally, there is no direct evidence that the late CVE are specifically due to surgery, as late CVE are generally self-reported, which is a limitation of our study.

In the literature, the measurement of biomarkers such as hs-cTnT appears to offer greater accuracy for long-term mortality prediction. Indeed, research has demonstrated that preoperative hs-cTnT concentrations are significantly associated with postoperative myocardial infarction and long-term mortality following non-cardiac surgery in high-risk patients [35, 36]. However, as mentioned before, the measurement of this biomarker is usually restricted to high-risk patients, overlooking low-risk patients. A systematic review investigated the added value of cardiac biomarkers when combined with perioperative scores such as the RCRI. This review highlighted that incorporating cardiac biomarkers, such as hs-cTnT, enhances the predictive accuracy of RCRI. However, it also suggested that some biomarkers alone, such as NT-pro-BNP, exhibit superior predictive performance [37]. Finally, even if SCORE is not initially used to assess per- or post-operative CVE, this tool should be considered for patients undergoing non-cardiac surgery, as it appears in our study that patients with SCORE ≥ 10 are more

associated with late CVE, including death. Furthermore, exploring the potential added value of SCORE on RCRI or ASA warrants consideration to enhance their sensitivity for the prediction of late CVE.

4.1 Limitations

The triplet hyperfine structure observed in our EPR data is not exclusively attributable to erythrocytic HbNO, but can also be ascribed to other NO-ferroheme species circulating in plasma or associated with erythrocyte membranes. While erythrocytic HbNO specifically refers to the nitrosylation of deoxyhemoglobin on its α-subunit, NO-ferroheme refers to a broader class of heme complexes in which NO is coordinated to the iron (Fe^{2+}) center of the heme group. Recent studies have highlighted the signaling roles of these NO-heme species in vascular biology [8–10], with evidence suggesting their potential shedding from the vascular bed and association with erythrocyte membranes. Such interactions may contribute to the erythrocytic HbNO signal detected in our experiments. Although our EPR data cannot distinguish between NO-ferroheme complexes, it is important to note that erythrocytic NO-ferroheme in our study was measured in isolated erythrocytes derived from venous blood, where deoxyhemoglobin in the T state is a main heme-protein amenable for nitrosylation.

The pre-analytical handling of erythrocyte samples for measuring erythrocytic NO-ferroheme including HbNO presents a significant challenge, as more than 1,000 samples were processed beyond the 30-min stability window and were excluded from the analysis, thereby reducing our sample size and potentially affecting representativeness. Additionally, 360 samples had been stored for more than one year before spectra acquisition, resulting in overlapping signals from protein-free radicals and methemoglobin, which made the erythrocytic NO-ferroheme signal unanalyzable. Nevertheless, sample exclusions were only explained by delays in sample transport or processing, independently of the patients' phenotype. To address these technical limitations, future studies will consider implementing protocols to optimize sample processing times and storage conditions to minimize bias.

The large heterogeneity among patients in terms of age, underlying diseases and medications may introduce confounding variables, not all of which may have been controlled for in our regression models. This may influence the adequate HbNO value to distinguish between patients susceptible or not to experience *p*-30d CVE. Despite this limitation, we could define a threshold discriminating such patients in a large cohort reflecting clinical routine in anesthesiology. While we previously validated the correlation between erythrocytic NO-ferroheme, including erythrocytic HbNO and endothelial function, we did not include other biomarkers of NO-dependent signaling, such as nitrite/nitrate; the latter, however, are sensitive to dietary intake, which would have imposed dietary restrictions difficult to implement in the present setting. There was also heterogeneity in terms of type and grade of surgery leading to different degrees of non-cardiovascular complications (infection, sepsis, bleeding, etc.) that affect the surgical stress. Thus, patients were not subjected to equivalent surgical stress, although we demonstrated in our cohort that the degree of surgery had no impact on the prevalence of CVE. Another limitation is the low number of *p*-30d CVE, 23 in total and 17 after the exclusion of patients with a history of severe comorbidities, thereby raising a caveat in the interpretation of our results. Moreover, further studies incorporating more biomarkers (hs-cTnT, CK-MB, NT-proBNP) and clinical parameters are desirable to build a more comprehensive risk prediction model.

The complications reported by patients at 3-, 6- and 12-months, despite a personal phone call and verification by our team, could not all be validated from the medical records, as a large number of patients were not followed up at our clinic at 6 months but returned to their local hospital or family doctor. Therefore, the results of the present study deserve to be tested in an independent validation cohort, from which a larger number of primary endpoints should strengthen our conclusions.

Overall, the exploratory nature of this study mandates a cautionary note and emphasizes the need for further studies in more homogenous populations with standardized pre-analytical conditions, not the least to properly assess metrics reflecting the analytical variability as needed for future biomarker development.

5 Conclusion

Despite the limited number of CVEs observed, preoperative erythrocytic NO-ferroheme including HbNO better independently correlates to perioperative cardiovascular events up to 30 days post-surgery than the ASA classification in patients without severe comorbidities. HbNO could particularly improve the preoperative management of ASA I and ASA II patients, (i.e., clinically healthy patients and patients with mild comorbidities), in whom low HbNO may indicate endothelial dysfunction and higher risk of cardiovascular events. Further studies are needed to refine and validate an appropriate HbNO threshold.

Abbreviations

ADMA	Asymmetric dimethylarginine
ARB	Angiotensin receptor blocker
ASA	American Society of Anesthesiologists
CI	Confidence interval
CK-MB	Creatine kinase-myocardial band

COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
CUSL	Cliniques Universitaires Saint-Luc
CVE	Cardiovascular event
DVP	Deep venous thrombosis
eCRF	Electronic case report form
eNOS	Endothelial nitric oxide synthase
ED	Endothelial dysfunction
EPR	Electronic paramagnetic resonance
ESC	European Society of Cardiology
GFR	Glomerular filtration rate
HbNO	5-coordinated NO-heme-α-hemoglobin
hs-cTnT	High sensitivity cardiac troponin
NO	Nitric oxide
NT-proBNP	N-terminal pro-B type natriuretic peptide
OR	Odds ratio
p-30d CVE	Peri-operative to 30d cardiovascular events
RCRI	Revised Cardiac Risk Index
RH-PAT	Reactive hyperemia peripheral arterial tonometry
SCORE	Systematic Coronary Risk Evaluation
SD	Standard deviation
SE	Standard error

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1007/s44254-025-00096-4.

Supplementary Material 1: Supplementary Tables.

Supplementary Material 2: Supplementary Figure.

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Author's contributions
Hasnae Boughaleb: On-site logistics set-up, recruitment of patients, data collection, statistical data analysis, data formatting (tables and figures), writing first draft. Jean-Luc Balligand and Nancy Van Overstraeten: Conceptualization and Methodology, Study design. Jean-Luc Balligand: Writing-review and editing (provided critical appraisal and further development of the original draft). Nancy Van Overstraeten: on-site logistics set-up, recruitment of patients. Jerome Liden: data collection & and data analysis, manuscript review and editing. Annie Robert: Supervision of data analysis, manuscript review and editing. Arvind Soni & Nathalie Fabian: data collection, analysis of HbNO by electron paramagnetic resonance (EPR) spectroscopy methodology. Irina Lobysheva: Advise on EPR spectroscopy and HbNO measurements; Virginie Montiel: manuscript review and editing. Mona Momeni and Marie-Agnès Docquier: on-site logistics set-up and writing-review and editing (provided critical appraisal and further development of the original draft).

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Data availability
The data that support the findings of this study can be made available upon reasonable request to the corresponding author.

Declarations

Ethics approval and consent to participate
The study protocol was approved by the ethical committee (Comité d'Ethique Hospitalo-Facultaire, CUSL), chaired by Prof. J-M Maloteaux, on July 16, 2019 (Approval Number: 2019/16JUL/323) and was registered in ClinicalTrials.gov (ID: NCT03994900). Each patient signed an informed consent form before starting the study.

Consent for publication
All authors gave their consent for publication.

Competing interests
The creation of Spinovit, a spin-off company of UCLouvain was supported by the Walloon Region, which awarded a First Spin-Off grant and contributed to the PICA proof-of-concept clinical trial. JLB and NVO were stakeholders of Spinovit during the course of the clinical trial.

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