Original Article

Increased Circulating Levels of Neutrophil Extracellular Traps During Cardiopulmonary Bypass

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Abstract

Background: The intensity of inflammatory response triggered by cardiopulmonary bypass (CPB) during cardiac surgery has been associated with adverse outcomes. Neutrophils might contribute to organ injury through the liberation of DNA histone-based structures named “neutrophil extracellular traps” (NETs). Our objective was to assess circulating NETs levels before and after cardiac surgery in low-risk and high-risk patients.

Methods: This prospective cohort study included 2 groups of patients undergoing elective cardiac surgery with the use of CPB. The first group consisted of low-risk patients (European System for Cardiac Operative Risk Evaluation II ≤ 1%), and the second group included high-risk patients (European System for Cardiac Operative Risk Evaluation II ≥ 5%). Blood samples were drawn pre-CPB and 3 hours post-CPB separation. Measurements of circulating NETs, interleukin-6, C-reactive protein, and myeloperoxidase were performed.

Results: This study reports the first prospective assessment of NETs in low-risk and high-risk patients undergoing CPB. Circulating NET levels were significantly increased in the high-risk group compared to the low-risk group. Interleukin-6 and C-reactive protein levels were also significantly higher in the high-risk group.

Conclusion: The results of this study indicate that NET levels are increased in high-risk patients undergoing CPB and suggest that NETs may contribute to the inflammatory response associated with CPB.

Résumé

Contexte : L’intensité de la réponse inflammatoire déclenchée par la circulation extracorporelle en cours de chirurgie cardiaque a été associée à des résultats défavorables. Les neutrophiles pourraient contribuer à des lésions organiques par la libération de structures d’ADN liées à des histones appelées « pièges extracellulaires des neutrophiles » (ou NETs, de l’anglais neutrophil extracellular traps). Nous sommes donc donné pour objectif d’évaluer les taux de ces pièges extracellulaires dans la circulation avant et après la chirurgie cardiaque chez des patients exposés à des risques faibles et à des risques élevés.

Méthodologie : Cette étude prospective de cohortes a été menée chez deux groupes de patients subissant une chirurgie cardiaque non urgente au cours de laquelle nous avons eu recours à la circulation extracorporelle. Le premier groupe était composé de patients exposés à un faible risque et le deuxième groupe était composé de patients exposés à un risque élevé.

Conclusion : Les résultats de cette étude indiquent que les taux de pièges extracellulaires des neutrophiles sont augmentés chez les patients à risque élevé subissant la circulation extracorporelle et suggèrent que les pièges extracellulaires contribuent à la réponse inflammatoire associée à la circulation extracorporelle.

Surgery involving cardiopulmonary bypass (CPB) results in a general inflammatory response of varying intensity. The innate immune system is the main culprit of the adverse effects seen with unabated general inflammation response in pathological settings. Neutrophils, which are well known as the principal cells involved in host defense against microbial pathogen infections in the innate immune system, are also playing a key role in the inflammatory response to injury.

In addition to phagocytosis and degranulation, the production of neutrophil extracellular traps (NETs) was identified as another distinct neutrophil function. NETs are web-like structures composed of decondensed chromatin and antimicrobial proteins. Although NETs contribute to defense against infection, they have also been implicated in the pathophysiology of multiple diseases, such as sepsis, transfusion-associated lung injury, thrombotic microangiopathy, pre eclampsia, cancer metastasis, acute respiratory distress syndrome, sepsis, and acute kidney injury (AKI). Local liberation of histones, such as citrullinated histone 3 (H3Cit), and myeloperoxidase (MPO) are thought to cause endothelial injury and exacerbate inflammation-induced organ injury. Recent evidence also implicates a potential

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Ethics Statement: The research reported has adhered to the relevant ethical guidelines.

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myeloperoxidase, citrullinated histone 3, and pentraxin-related protein 3 levels were performed at each time point.

**Results:** Twenty-four patients, 12 high-risk and 12 low-risk patients, were included. Circulating NETs measurements changed from a median of 0.054 before CPB to 0.084 at 3 hours post-CPB separation, with a median increase of 0.037 ($P < 0.001$) per patient. No difference was noted between the high-risk and low-risk groups. A linear relationship was found between the circulating NETs measurements 3 hours post-CPB and CPB duration ($R = 0.047$; confidence interval, 0.012-0.081; $P = 0.01$ $R^2 = 0.27$). A correlation was found between the change in NETs with citrullinated histone 3 and myeloperoxidase levels, but not between NETs and other inflammatory biomarkers.

**Conclusions:** Circulating NETs measurements increases during cardiac surgery with postsurgical levels proportional to CPB duration. The clinical significance of NETs production during cardiac surgery should be further investigated.

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role of NETs in linking sterile inflammation with thrombosis. The formation and deposition of NETs could impair perfusion at the microcirculation level because it has been demonstrated in some models of organ dysfunction in which microcirculatory compromise is well described, such as the myocardial no-reflow phenomenon and septic AKI.

In the context of cardiac surgery, production of NETs could have pathophysiological implications. Patients with vascular disease and other comorbidities such as diabetes may have chronic inflammation, resulting in a priming of circulating neutrophils before surgery. The general inflammatory reaction triggered by CPB may consequently result in a burst of NETs production in the perioperative period. Occlusion and injury of the pulmonary and systemic microcirculation by an intense NETs formation triggered by CPB may be implicated in the pathogenesis of adverse patient outcomes by inducing organ injury in multiple systems.

The objective of this study was to perform circulating NETs quantification in patients undergoing cardiac surgery. We hypothesized that circulating NETs levels are increased 3 hours after surgery compared with baseline measurements before CPB. Furthermore, we hypothesized that this increase might be more pronounced in high-risk diabetic patients. We also investigated whether NETs production may be associated with the duration of CPB and the release of other markers known to be associated with NETosis (MPO, H3Cit), neutrophil activation (pentraxin-related protein 3 [PTX3]), and inflammatory mediators such as interleukin-6 (IL-6) and C-reactive protein (CRP).

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**Material and Methods**

**Participants**

This prospective cohort study (NET1; ICM#2016-2060) included patients undergoing elective cardiac surgery with the use of CPB. Adult participants (aged ≥ 18 years) able to provide informed consent were admissible. Patients were screened and approached the day before scheduled surgery. Two groups of equal size were formed on the basis of preoperative risk assessment. The low-risk group included patients with a European System for Cardiac Operative Risk Evaluation (EuroSCORE) II $\leq 1\%$ without diabetes, and the high-risk group included participants with a EuroSCORE II $\geq 5\%$ and with known diabetes mellitus. We excluded patients undergoing urgent surgery or unable to provide consent, and patients with active infection, malignancy, autoimmune diseases (arthritis, vasculitis, inflammatory bowel disease), acute ischemic event in the last 14 days, or severe chronic kidney disease (estimated glomerular filtration rate $< 15$ mL/min/1.73 m$^2$ based on the Modified Diet in Renal Disease equation). The project was approved by the local ethics board. Written informed consent was obtained for all participants. Patient recruitment was performed from November 2016 to July 2017. Serum were also collected from 12 healthy volunteers who were enrolled in the NEUTRO study (6) (ICM #2012-1374) and the SAUNA FMD study (6) (ICM #2017-2179).

**Patient assessment**

Before surgery, demographic information and medical history were recorded from the participants’ records, including prior diagnosis of chronic hypertension, tobacco use, and...
peripheral artery disease. Other information included functional status using the New York Heart Association (NYHA) classificationleft ventricular ejection fraction based on preoperative transthoracic echocardiography, planned procedure(s), baseline renal function based on the most recent available serum creatinine measurement, and recent diagnosis of myocardial infarct (< 90 days). The euroSCORE II was calculated from the gathered information using an online calculator. Finally, the following laboratory parameters were also collected the day before surgery: leucocyte, neutrophil, and lymphocyte count.

During the perioperative period, two 5 mL blood samples were collected in the operating room after the induction of general anaesthesia before CPB initiation. A second series of samples were collected in the intensive care unit after surgery precisely 3 hours after CPB separation.

The following information was gathered during the perioperative period: duration of the procedure, intraoperative fluid balance, complications during or shortly after CPB separation, duration of intensive care unit and hospital stay after surgery, complications after surgery including AKI, stroke, pneumonia, arrhythmias, delirium, major bleeding, and urgent reinterventions for any indications. Hemodynamic parameters were also recorded at CPB separation. A difficult CPB separation was defined as the need for at least 1 vasopressor (norepinephrine, vasopressin) and 1 inotropic medication (epinephrine, dobutamine, intravenous milrinone) during CPB separation or the use of mechanical circulatory support after the first CPB weaning attempt.

AKI was defined as an increase of > 26 µmol/L or > 50% in serum creatinine as per the Kidney Diseases: Improving Global Outcome group criteria.

Quantification of NETs and biomarkers

NETs-associated MPO-DNA complexes were quantified as previously described. Briefly, 5 µg/mL of mouse anti-human MPO antibody (Bio-Rad Antibodies, Hercules, CA) was coated to 96-well microtier plates. After a 1-hour blocking with 1% bovine serum albumin, serum samples were added together with a peroxidase-labeled anti-DNA monoclonal antibody (component 2 of the Cell Death Detection ELISA PLUS kit, Millipore Sigma/Roche, Oakville, ON). After a 2-hour incubation on the shaker at 320 rpm, the peroxidase substrate was added according to the manufacturer’s instructions. The optical absorbance was measured at 405 nm. CRP in serum samples was quantified by nephelometry at the Biochemistry Laboratory at the Montreal Heart Institute. IL-6, MPO, and PTX3 were measured using a 113-175 minutes. The duration of the procedure was significantly longer in high-risk patients (213 [IQR, 175-239] minutes vs 136 [IQR, 113-175] minutes \( P = 0.01 \)). The mean age of all patients was 62.2 ± 14.9 years and statistically not different from the mean age of healthy volunteers used for comparison (63.9 ± 6.3 years; 6 men and 6 women).

Before the initiation of CPB, circulating NETs measurements were higher in the patients compared with healthy controls \((0.054 [IQR, 0.039-0.073] vs 0.026 [IQR, 0.021-0.048], P = 0.02)\). However, no significant difference was noted between the pre-CPB NETs measurements between the high-risk and low-risk groups \((0.046 [IQR, 0.039-0.069] vs 0.064 [IQR, 0.039-0.091], P = 0.56, as shown in Table 2). A significant increase in circulating NETs measurements was observed 3 hours after CPB (median change: +0.037 [0.019-0.063], \( P < 0.0001 \)), as shown in Figure 1A. This increase was observed in both the low-risk and high-risk groups as shown in Figure 1B and C (median change of

Data are presented as frequency (percentages) and mean ± standard deviation or median (interquartile range [IQR], quartile 1; quartile 3) where appropriate. The normality of the data for continuous variables was assessed graphically using histograms and the Shapiro–Wilk test. The difference between NETs measurements before and after CPB was assessed using the Student \( t \) test for paired samples or Wilcoxon rank-sum test for paired sample depending on the distribution of the data. The difference between parameters in the low-risk and high-risk groups was assessed using the Student \( t \) test for independent samples or the Mann–Whitney \( U \) test for independent samples depending on the distribution for continuous variables, and chi-square test for dichotomous variables. The difference among 3 or more groups was assessed using 1-way analysis of variance or Kruskal–Wallis test depending on the distribution. When a \( P < 0.05 \) was observed, the difference between individual groups was further assessed using multiple comparisons with Bonferroni correction.

The relationship between the NETs measurements and the duration of CPB was assessed using linear regression, and the results are presented as \( \beta \) coefficient with 95% confidence intervals and \( R^2 \) for model fit. Outliers were defined as a data point more than 1.5 × IQR lower than the first quartile or higher than the third quartile (Tukey’s rule) and were excluded from the linear regression analysis. A multivariable linear regression analysis was used to determine if the association remained significant after adjustment for baseline NETs measurements. The relationship between NETs measurements and other inflammatory biomarkers was assessed using Spearman correlation, and results are presented as correlation coefficients (\( r \)). Significance level for all tests was set at 95%.

Results

From November 2016 to July 2017, 24 patients were included in the study. The characteristics of the studied subjects are presented in Table 1. Patients in the high-risk group had a lower left ventricular ejection fraction (38%; IQR, 33; 50%) vs 60% (IQR, 55; 65%) \( P < 0.001 \), worse functional class \((P < 0.001)\), greater proportion of peripheral artery disease (1 vs 0 patients, \( P = 0.03 \)), and chronic kidney disease (7 patients [58.3%] vs 1 patient [8.3%], \( P = 0.009 \)). The duration of the procedure was significantly longer in high-risk patients (213 [IQR, 175-239] minutes vs 136 [IQR, 113-175] minutes \( P = 0.01 \)). The mean age of all patients was 62.2 ± 14.9 years and statistically not different from the mean age of healthy volunteers used for comparison (63.9 ± 6.3 years; 6 men and 6 women).

Statistical analysis

For the sample size calculation, it was determined that a total sample size of 24 patients gives a 90% power to detect an effect size (Cohen’s \( d \)) of 0.69 for an increase in the NETs measurements during the perioperative period in a 2-sided paired Student \( t \) test with a 95% confidence level. Additionally, 2 groups of 12 patients give 90% power to detect an effect size (Cohen’s \( d \)) of 1.39 in the difference of NETs measurements between the high-risk and low-risk groups.
### Table 1. Patient characteristics

|                          | All patients (n = 24) | Low-risk (n = 12) | High-risk (n = 12) | P value |
|--------------------------|-----------------------|------------------|-------------------|---------|
| Preoperative characteristics |                       |                  |                   |         |
| Age (y)                  | 62.2 ± 14.9           | 57.4 ± 14.6      | 66.9 ± 14.2       | 0.12    |
| BMI (kg/m²)              | 31.0 ± 7.3            | 29.7 ± 5.0       | 32.3 ± 9.1        | 0.40    |
| euroSCORE II (%)         | 4.0 ± 3.5             | 0.8 ± 0.2        | 7.2 ± 1.9         | < 0.001 |
| Left ventricular ejection fraction (%) | 55 (38-60)          | 60 (55-65)       | 38 (33-50)        | < 0.001 |
| Functional class (NYHA)  |                       |                  |                   |         |
| Class I                  | 5 (20.8%)             | 5 (41.7%)        | 0 (0%)            |         |
| Class II                 | 7 (29.2%)             | 5 (41.7%)        | 2 (16.7%)         |         |
| Class III                | 12 (50.0%)            | 2 (16.7%)        | 10 (83.3%)        |         |
| Class IV                 | 0 (0%)                | 0 (0%)           | 0 (0%)            |         |
| Hypertension             | 16 (66.7%)            | 7 (58.3%)        | 9 (75%)           | 0.39    |
| Smoker                   | 5 (20.8%)             | 1 (8.3%)         | 4 (33.3%)         | 0.13    |
| Peripheral artery disease| 1 (4.2%)              | 0 (0%)           | 1 (8.3%)          | 0.03    |
| Recent myocardial infarct (< 90 d) | 2 (8.3%)   | 1 (8.3%)         | 1 (8.3%)          | 1.0     |
| Chronic kidney disease (eGFR < 60 mL/m/1.73 m²) | 8 (33.3%)   | 1 (8.3%)         | 7 (58.3%)         | 0.009   |
| Type of surgery          |                       |                  |                   |         |
| - Isolated coronary artery | 7 (29.2%)            | 4 (33.3%)        | 3 (25.0%)         | 0.07    |
| - Bypass                 | 10 (41.7%)            | 7 (58.3%)        | 6 (50.0%)         |         |
| - Isolated valvular procedure | 7 (29.2%)        | 1 (8.3%)         | 6 (50.0%)         |         |
| Leukocyte count before surgery | 8.1 ± 2.1            | 7.7 ± 1.2        | 8.5 ± 2.8         | 0.38    |
| Neutrophil count before surgery | 5.2 ± 1.4            | 4.9 ± 1.1        | 5.5 ± 1.7         | 0.34    |
| Lymphocyte count before surgery | 1.9 ± 0.8            | 1.8 ± 0.7        | 1.9 ± 1.0         | 0.73    |
| Intraoperative characteristics |                   |                  |                   |         |
| Duration of surgery (min) | 175 (136-223)         | 136 (113-175)    | 213 (175-239)     | 0.01    |
| Duration of CPB (min)    | 89 (50-113)           | 61 (46-103)      | 93 (80-121)       | 0.13    |
| Duration of aortic clamping (min) | 63 (40-77)   | 49 (28-69)       | 72 (62-80)        | 0.07    |
| Intraoperative fluid balance (L) | 1.3 (0.8-1.9)       | 1.3 (0.7-1.9)    | 1.3 (0.8-2.0)     | 1.00    |

Data are presented as mean ± standard deviation or median (IQR) where appropriate.

BMI, body mass index; CPB, cardiopulmonary bypass; eGFR, estimated glomerular filtration rate; euroSCORE, European System for Cardiac Operative Risk Evaluation; NYHA, New York Heart Association.

0.043 [IQR, 0.026-0.103] P = 0.010 and 0.027 [IQR, 0.014-0.045] P = 0.008, respectively). However, there was no difference between the high-risk and low-risk groups in regard to the median change or NETs measurement 3 hours after CPB (P = 0.19) (Table 2).

One patient in the low-risk group was identified as an outlier because he had elevated NETs measurements before CPB (outlier: 0.316, median in the group: 0.054 [IQR, 0.039-0.073]) and 3 hours after CPB (outlier: 0.458, median in the group: 0.084 [IQR, 0.066-0.144]). A brief clinical description of this outlier is presented in Supplemental Appendix S1. After exclusion of this outlier, a positive linear association was observed between the duration of CPB and the NETs measurement 3 hours after CPB (ß = 0.047 [CI, 0.012-0.081] P = 0.01 R² = 0.27) (Fig. 2). This association remained significant after adjustment for baseline NETs measurements (ß = 0.039 [CI, 0.004-0.074] P = 0.03) (Table 3).

Baseline measurements of IL-6 and CRP were higher in patients compared with healthy controls (3.05 pg/mL vs 1.26 pg/mL [1.17-2.39] P = 0.002; and 1.68 mg/L [0.91-3.28] vs 0.66 mg/L [0.43-1.13] P = 0.002, respectively), whereas no significant baseline differences were found in MPO, H3Cit, and PTX3. Patients in the high-risk group had higher IL-6 measurement at baseline compared with patients in the low-risk group (5.38 pg/mL [3.82-7.77] vs 2.30 pg/mL [1.78-2.47] P = 0.004) (Supplemental Table S1), but there was no difference in baseline CRP measurements as shown in Supplemental Table S1. No significant difference was observed in MPO, H3Cit, and PTX3 levels.

During the perioperative period, a significant elevation in IL-6, CRP, MPO, H3Cit, and PTX3 was observed in the studied patients (median change: 245 pg/mL [IQR, 153-577] P < 0.001 and 0.28 mg/L [IQR, 0.04-0.55], P = 0.011) (Fig. 3). No differences between the changes or the postoperative levels of all biomarkers were observed between the low-risk and high-risk groups (Supplemental Tables S2-S6). Significant correlations were found between the change in NETs measurements and the change in MPO, H3Cit during

### Table 2. Measurements of circulating NETs

|                          | All patients (n = 24) | Low-risk (n = 12) | High-risk (n = 12) | P value |
|--------------------------|-----------------------|------------------|-------------------|---------|
| NETs measurements before CPB | 0.054 (0.039-0.073) | 0.064 (0.039-0.091) | 0.046 (0.039-0.069) | 0.56    |
| NETs measurements 3 h after CPB | 0.084 (0.066-0.144) | 0.113 (0.061-0.193) | 0.081 (0.067-0.101) | 0.40    |
| Median change in NETs measurements | 0.037 (0.019-0.063) | 0.043 (0.026-0.103) | 0.027 (0.014-0.045) | 0.19    |
| Median fold-change in NETs measurements | 1.60 (1.34-2.37) | 1.83 (1.35-2.48) | 1.51 (1.28-2.30) | 0.55    |

For comparative purpose, the median NETs measurement in 12 healthy volunteers was 0.026 (IQR, 0.021-0.048). Results are presented as median (IQR). CPB, cardiopulmonary bypass; NETs, neutrophil extracellular traps.
the perioperative period ($r = 0.58, P = 0.003$ and $r = 0.67, P < 0.001$, respectively), and the absolute measurements of these markers $3$ hours after CPB ($r = 0.62, P = 0.001$ and $r = 0.52, P = 0.01$, respectively). The baseline measurement of NETs before CPB was also correlated with MPO ($r = 0.44, P = 0.03$) but not with H3Cit. No correlation was found between NETs measurements and IL-6, CRP, or PTX3 at any time point. Furthermore, no correlation was present between the change in IL-6, CRP, or PTX3 and the change in NETs during the perioperative period (Supplemental Table S7).

Figure 1. Circulating neutrophil extracellular traps (NETs) in the perioperative period. (A) Measurements in all patients ($n = 24$), (B) in low-risk patients ($n = 12$), and (C) in high-risk patients ($n = 12$). Significant differences were present between measurement before cardiopulmonary bypass (CPB) and after CPB (Wilcoxon signed-rank test, $P < 0.05$). OD, optical density.

Figure 2. Relationship between circulating NETs measurement $3$ hours after surgery and the duration of CPB. Linear regression line is shown with $95\%$ confidence intervals (CIs) (blue line) ($b = 0.047; 95\%$ CI, 0.012-0.081; $P = 0.01$. $R^2 = 0.27$). The triangle marker denotes the outlier that was excluded for the analysis. CPB, cardiopulmonary bypass; NETs, neutrophil extracellular traps; OD, optical density.
After cardiac surgery, 50% of patients developed complications, including difficult separation for CPB, AKI, stroke, delirium, pneumonia, arrhythmia (atrial fibrillation, atrioventricular block), haemorrhage, and urgent reoperation (Table 4). Patients in the high-risk group had a longer hospital stay (8.5 [5.5-9.5] days vs 5.0 [4.5-5.5] days $P = 0.020$). Circulating NETs measurements before CPB and 3 hours after CPB, and the difference between baseline and 3 hours after CPB were not associated with individual complications or with a composite end point of major complications (Supplemental Table S8). There was no significant difference in NETs measurements in participants who received blood transfusions during surgery compared with those who did not (Supplemental Table S9).

**Discussion**

In this study, we observed an increase in circulating NETs measurements in patients undergoing on-pump cardiac surgery. Before surgery, the subjects had a median NETs measurement 2-fold higher than healthy controls, which increased significantly after surgery, attaining a median level more than 3-fold higher than in healthy controls. No difference between circulating NETs measurements was observed between the low-risk and high-risk patients. The post-CPB NETs measurement was proportional to the duration of CPB. We observed a correlation between NETs quantification and biomarkers known to be associated with NETs production, such as MPO and H3Cit. However, no association was observed with the other inflammatory biomarkers studied or with clinical complications after surgery.

We are the first to demonstrate that cardiac surgery with the use of CPB is associated with release of NETs. Paunel-Gorgulu et al. demonstrated an increase in cell-free DNA in the perioperative period, with prolonged CPB being associated with greater elevations. However, cell-free DNA may be produced by apoptosis, necrosis, and cell damage induced by the contact of blood to the extracorporeal circuit, and cell-free DNA measurements may not be proportional to NETs release as it was demonstrated in patients undergoing liver transplantation. Therefore, cell-free DNA is not a direct evidence of NETs production. We used an assay to detect MPO-associated DNA to specifically assess cell-free DNA originating from neutrophils, therefore providing evidence of circulating NETs increase during the CPB procedure. Furthermore, we supported these results by demonstrating a parallel increase in circulating MPO and H3Cit, which are known to be associated with NETs release.

The triggers leading to NETs formation can be of multiple origins, including in response to microorganisms, from the contact with activated platelets or activated endothelial cells, and during the general inflammation response via proinflammatory cytokines. In the specific context of “on-pump” cardiac surgery, contact with the CPB circuit is known to cause activation of the coagulation cascade and complement system activation resulting in production of C3a and C5a, which are potent proinflammatory chemokines. Ischemia–reperfusion injury and operative trauma promote endothelial cell activation and expression of adhesion molecules, which promote migration of neutrophils to the site of injury. Unabated NETs production may be a major pathway leading to injury in critically ill patients (Fig. 4). Recent evidence has shown that NETs formation by neutrophils collected from a cohort of critically ill patients independently predicts mortality and disseminated intravascular coagulation. Consequently, inhibiting NETs formation by the use of peptidyl arginine deiminase inhibitors or inducing degradation of circulating NETs by using exogenous DNase I may represent promising therapeutic avenues.

Our original hypothesis was that NETs formation would be correlated with production of IL-6, an early inflammation marker contributing to neutrophil activation. However, we found that measurement of NETs post-CPB was positively associated with CPB duration but not with IL-6 production. Consequently, it is possible that NETs formation in the perioperative period is triggered by direct contact with the extracorporeal circuit or with other pathways, such as complement activation, platelet activation, and endothelial cell activation. On the basis of our in vitro study, in which the peak of NETs synthesis was observed within 3 hours poststimulation with proinflammatory mediators, we elected to measure circulating NETs 3 hours postprocedure. Additionally, it is possible that measuring NETs at this time point after CPB separation does not allow enough time for the triggering of NETs formation by the general inflammatory response associated with CPB. In support of this, CRP increase was minimal in the studied period. CRP production by the hepatocytes is stimulated by IL-6, but the increase in serum is delayed, usually peaking 24 hours after the initiation of the inflammatory response. Therefore, this marker is associated with the late inflammatory response of CPB and thus may contribute to NETs formation later than the immediate postoperative period. In support of this, Qin et al. reported that IL-6 levels increase early after cardiac surgery, but other mediators of inflammation, such as mitochondrial DNA, have
a more delayed response. However, they found that the peak concentration of mitochondrial DNA correlates with the intensity of early IL-6 release. Another possibility is that NETs measured in the circulation could be an underestimation of the total amount of NETs being produced because NETs could be deposited in the capillary bed or bound to primed neutrophils sequestered in systemic and pulmonary microvascular beds.

PTX3 is an acute-phase biomarker that increases dramatically in the context of sepsis or other inflammatory disorders. It is hypothesized that PTX3 may act to protect against the adverse consequences of NETs by binding circulating histone, thereby preventing endothelial injury and acting as an anti-inflammatory mediator in patients undergoing cardiac surgery. We observed that the PTX3 level increased during the perioperative period and reached abnormal levels.

Figure 3. Measurement of (A) interleukin-6 (IL-6; pg/mL), (B) C-reactive protein (CRP; mg/L), (C) myeloperoxidase (MPO; ng/mL), (D) citrullinated histone 3 (H3Cit; ng/mL), and (E) pentraxin-related protein (PTX3; pg/mL) during the perioperative period. Significant differences were present between measurement before CPB for all biomarkers (Wilcoxon signed-rank test, \( P < 0.05 \)). CPB, cardiopulmonary bypass.
(> 2000 pg/mL) after CPB, but did not reach high levels commonly seen in sepsis (> 14,000 pg/mL). Additionally, we found no correlations between PTX3 and NETs measurements during the perioperative period.

**Study limitations**

This study has several limitations. First, the small sample size was not sufficient to assess the clinical implications of circulating NETs measurements. Furthermore, what represents a clinically significant increase in circulating NETs measurements in the perioperative period of a major surgery is unknown. In addition, only 2 time points were studied, which preclude us to precisely determine when the increase in NETs formation occurs and if NETs production continues beyond 3 hours after cardiac surgery. Regarding the outlier patient, we were not able to explain the observed outlier NETs value, considering that all the other biomarkers values were within the normal range observed for the other patients before CPB. Finally, all patients underwent cardiac surgery with the use of CPB. A control group undergoing off-pump cardiac surgery would be needed to confirm that exposure to CPB is the primary factor leading to production of NETs.

**Conclusions**

In this exploratory study, we demonstrate for the first time that circulating NETs increase during cardiac surgery. The exposure of blood to the extracorporeal circuit during CPB may trigger production of NETs. Whether the phenomenon may predispose patients to complications after cardiac surgery should be further investigated to determine if circulating NETs represent a potential treatment target.

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**Disclosures**

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Supplementary Material

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