

Basic Research

Neutrophil Extracellular Trap-related Biomarkers Are Increased in the Synovial Fluid of Patients With Periprosthetic Joint Infections

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Abstract

Background The diagnosis of periprosthetic joint infection (PJI) is a major challenge in clinical practice. The role of neutrophils in fighting infection has been increasingly understood, and one mechanism of action of these cells is neutrophil extracellular traps. However, little is known about this process in PJI.

Questions/purposes (1) Are the biomarkers of neutrophil extracellular trap formation (citrullinated histone H3 [H3Cit], cell-free DNA [cf-DNA], and myeloperoxidase

[MPO]) increased in the synovial fluid of patients with PJI? (2) What is the diagnostic accuracy of biomarkers of neutrophil extracellular trap formation for PJI?

Methods Between May 2020 and March 2021, 43 patients who underwent revision THA or TKA were enrolled in this study. Eleven patients were excluded and 32 patients were categorized into the PJI group (n = 16) or non-PJI group (n = 16) according to the 2018 Second International Consensus Meeting on Musculoskeletal Infection criteria.

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Ethical approval for this study was obtained from the institutional review board of the National Institute of Traumatology and Orthopedics Jamil Haddad (protocol number: 21100519.1.0000.5273).

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There were 15 men and 17 women in this study, with a median (range) age of 70 years (60 to 80 years). Twenty-seven patients had TKA and five had THA. We measured cf-DNA, MPO, and H3Cit in synovial fluid. The sensitivity, specificity, and receiver operating characteristic curve were calculated for each biomarker using the Musculoskeletal Infection Society criteria as the gold standard for diagnosis and considering a clinical surveillance of 2 years for patients in the non-PJI group.

Results Patients with PJI had higher levels of synovial fluid cf-DNA (median [range] 130 ng/ μ L [18 to 179] versus 2 ng/ μ L [0 to 6]; $p < 0.001$), MPO (1436 ng/ μ L [55 to 3996] versus 0 ng/ μ L [0 to 393]; $p < 0.001$), and H3Cit (2115 ng/ μ L [5 to 2885] versus 3 ng/ μ L [0 to 87]; $p < 0.001$) than those in the non-PJI group. In receiver operating characteristic curve analyses, we observed near-perfect performance for all biomarkers evaluated, with an area under the curve of 1 (95% CI 0.9 to 1), 0.98 (95% CI 0.9 to 1), and 0.94 (95% CI 0.8 to 0.99) for cf-DNA, MPO, and H3Cit, respectively. The sensitivity for detecting PJI using synovial fluid was 100% for cf-DNA, 94% for MPO, and 88% for H3Cit. The specificity was 100% for cf-DNA and MPO, and 88% for H3Cit.

Conclusion Our results show that neutrophils in the periprosthetic microenvironment release neutrophil extracellular traps as part of the bactericidal arsenal to fight infection. These results allow a better understanding of the cellular and molecular processes that occur in this microenvironment, enabling the design of more assertive strategies for identifying new biomarkers and improving the available ones. Novel studies are needed to define whether and how neutrophil extracellular trap-related biomarkers can be useful for diagnosing PJI.

Level of Evidence Level II, diagnostic study.

Introduction

Periprosthetic joint infection (PJI) is one of the most-feared complications after total joint arthroplasty because it is potentially catastrophic for patients who experience it [8, 16, 2]. PJI is one of the main causes of lower limb arthroplasty revision [9, 10]. Its diagnosis is still a challenge and requires a combination of clinical findings, evaluation of biomarkers in serum and synovial fluid, and histopathologic analyses and microbiological culturing, with no single biomarker having good accuracy [15]. There are often controversies about available biomarkers regarding their diagnostic performance, as observed for alpha-defensin, and a better understanding of the processes that occur in an infected microenvironment might allow PJI biomarkers to be used more rationally [1, 5].

Neutrophils are a hallmark in PJI, and the evaluation of such cells or their byproducts in synovial fluid has been

shown to be more accurate than an evaluation of systemic biomarkers, because they directly reflect the infected periprosthetic microenvironment [11]. In addition to classic bactericidal mechanisms, which include phagocytosis, reactive oxygen species production, and degranulation, neutrophils generate and release decondensed chromatin fibers decorated with granular enzymes, or so-called neutrophil extracellular traps (NETs), through a process called NETosis. NETosis was first described in 2004 and can entrap and kill microorganisms such as fungi and bacteria [3]. In NET formation, neutrophil DNA is released with granule proteins. The citrullination of histone H3 by the enzyme peptidyl arginine deiminase 4 is an important step in this process because it is required for nuclear chromatin decondensation and release [12]. This process is assumed to be neutrophil-specific and independent from other cell death mechanisms, such as apoptosis and necrosis; therefore, citrullinated histone H3 (H3Cit) has been proposed as a target biomarker reflecting NET formation [18]. Other putative biomarkers for NETs are cell-free DNA (cf-DNA) and granule enzymes such as myeloperoxidase (MPO), which may have sources other than NET formation, such as cell death and degranulation [13, 17]. Recently, cf-DNA [4] and MPO [7] have been shown to be increased in the synovial fluid of patients with chronic PJI. However, little is known about the NET release process (because of NETosis) in PJI.

In the present study, we investigated whether the levels of H3Cit, cf-DNA, and MPO are increased in the synovial fluid of patients with PJI. Additionally, we evaluated the diagnostic accuracy of NET formation biomarkers for PJI. We asked: (1) Are the biomarkers of NET formation (H3Cit, cf-DNA, and MPO) increased in the synovial fluid of patients with PJI? (2) What is the diagnostic accuracy of biomarkers of NET formation for PJI?

Patients and Methods

Study Design and Setting

This prospective, comparative study was conducted in a tertiary healthcare center that specializes in high-complexity orthopaedic surgery.

Patients

Patients 18 years or older who underwent revision TKA or THA between May 2020 and March 2021 at the Brazilian National Institute of Traumatology and Orthopaedics were included. All patients provided informed consent for this study. Exclusion criteria included acute PJI, systemic inflammatory disease, insufficient synovial fluid volume, and lack of appropriate clinical and laboratory data. Patients undergoing the second stage of revision arthroplasty were

not eligible, and none of the patients were treated with antibiotics in the 15 days before surgery. Chronic PJI was diagnosed according to the 2018 Second International Consensus Meeting on Musculoskeletal Infection criteria as the gold standard for diagnosis [15]. Participants who met at least one of the major criteria or who had a total score of 6 or more comprised the PJI group. Patients with a score of less than 3 comprised the non-PJI group. Patients with an inconclusive diagnosis, that is, a score of 3 to 5 points, were excluded. In addition to the Musculoskeletal Infection Society criteria, patients in the non-PJI control group were followed for 2 years to assess whether they experienced clinical signs or symptoms of PJI during that period.

Descriptive Data

During the study period, 43 patients were considered potentially eligible. Of these, 11 were excluded: one because of acute PJI, four because of chronic inflammatory disease, four because of insufficient synovial fluid volume, and two because of lack of adequate clinical and laboratory data. Therefore, 32 patients comprised the final study population. Sixteen patients had a confirmed PJI diagnosis according to the Musculoskeletal Infection Society criteria. After 2 years of clinical surveillance, none of the patients in the non-PJI group showed signs of or were diagnosed with PJI. The PJI and non-PJI groups showed no differences in sex, age, or

BMI (Table 1). Four patients in the PJI group presented with a draining sinus. In 13 patients, it was possible to identify the causative microorganisms. *Staphylococcus aureus* was the main causative pathogen, and polymicrobial infections were detected in six patients (Table 2).

Samples

Synovial fluid was obtained during the revision procedure, before arthrotomy, with 1 to 3 mL of fluid drawn into vials for aerobic and anaerobic cultures (Becton, Dickinson and Company). Culture dishes were held for 14 days and discarded after the cultures became positive, according to our laboratory protocol. In addition, five to seven periprosthetic tissue samples were harvested and sent for microbiological analyses. Histopathologic analyses were also performed on periprosthetic membranes, which were classified according to Morawietz et al.'s criteria [14].

We transferred 1 mL of synovial fluid to EDTA-containing tubes and used it to count leukocytes and the percentage of polymorphonuclear cells using flow cytometry with an automated hematology analyzer (Cell Dyn 3700 SL, Abbott Diagnostics). The remaining synovial fluid was centrifuged at 10,000 x g for 10 minutes, and the cell-free supernatant was aliquoted in sterile tubes and stored at -80°C until further analysis, for no more than 6 months.

Table 1. Patient characteristics

	No infection (n = 16)	Infection (n = 16)	p value
Sex			
Male	6	9	> 0.99 ^a
Female	10	7	
Age in years	70 (68 to 80)	67 (60 to 78)	0.2 ^b
BMI in kg/m ²	32 (27 to 37)	29 (24 to 33)	0.4 ^b
Joint			
Hip	2	3	> 0.99 ^a
Knee	14	13	
Previous implant			
Primary	15	12	0.2 ^a
Revision	1	4	
Serum CRP	0.8 ± 1.0	7.4 ± 7.4	< 0.001 ^b
D-dimer	2.0 ± 2.3	3.1 ± 2.3	0.1 ^b
ESR	41 ± 24	80 ± 29	< 0.001 ^b
SF leukocyte	1245 ± 1295	27,990 ± 32,614	0.06 ^b
SF % PMN	20 ± 13	66 ± 25	< 0.001 ^b

Data presented as % (n) or median (IQR).

^aChi-square test.

^bMann-Whitney test.

CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; SF = synovial fluid; PMN = polymorphonuclear leukocytes.

Table 2. Microbiological profile of PJI group (n = 16)

Microorganism	Number of patients
<i>Staphylococcus aureus</i>	7
Coagulase-negative <i>staphylococcus</i>	4
<i>Enterococcus</i> species	2
Gram negative	8
Polymicrobial ^a	6
Culture negative	2

^aPatients with PJI in which at least two different microorganisms were isolated from the culture of synovial fluid or intraoperative tissues.

We quantified cf-DNA in synovial fluid using the Quant It Picogreen dsDNA kit (Thermo Fisher Scientific), as described elsewhere [3].

Specific ELISA kits were used to measure MPO (Thermo Fischer Scientific) and H3Cit (Cayman Chemical). When the absorbance of a patient's reading was lower than that of the buffer blank, the measurement was set at 0 ng/mL.

Primary and Secondary Study Outcomes

Our primary study goal was to investigate whether the NET release process was occurring in the infected periprosthetic microenvironment. To assess this, we evaluated the synovial fluid from patients in the PJI and non-PJI groups through an ELISA assay for the presence of NET-related biomarkers (cf-DNA, MPO, and H3Cit).

Our secondary study goal was to evaluate the diagnostic accuracy of NET-related biomarkers for the diagnosis of PJI. To answer this question, receiver operating characteristic (ROC) curve analyses were performed for all three NET biomarkers that were evaluated, using the Musculoskeletal Infection Society criteria as the gold standard for diagnosis.

Ethical Approval

Ethical approval for this study was obtained from the institutional review board of the National Institute of Traumatology and Orthopedics Jamil Haddad (protocol number: 21100519.1.0000.5273).

Statistical Analyses

The sample size was determined based on a power analysis using the mean and standard deviation from a previous study on cf-DNA quantification [4] to obtain a power of 0.8 and alpha of 0.05. For the analysis, we used mean cf-DNA values of 122.5 ± 57.2 in the chronic PJI group and a mean

of 4.6 ± 2.5 in the aseptic group, based on our previous study [3]. We performed the analyses using the ClinCalc software (MedCalc), requiring a sample of at least 10 participants (five participants per group).

We analyzed data distribution with the D'Agostino-Pearson normality test. To compare biomarker levels between groups, we used the Mann-Whitney U test, and we compared categorical variables using a chi-square test. ROC curves were constructed by calculating the area under the curve (AUC), sensitivity, and specificity at different cutoff levels. The optimum thresholds for each of the biomarkers we evaluated were calculated using the Youden statistic. A significance level of 0.05 was set for all tests. These statistical analyses were performed using MedCalc software version 21 (U MedCalc Software).

Results

Are H3Cit, cf-DNA, and MPO Increased in the Synovial Fluid of Patients With PJI?

We observed that cf-DNA levels were increased in the synovial fluid of the group with PJI (median [range] 130 ng/ μ L [18 to 179 ng/ μ L]) compared with the group without PJI (2 ng/ μ L [0 to 6 ng/ μ L]) ($p < 0.001$) (Fig. 1A). We observed that MPO was also increased in the PJI group (1436 ng/ μ L [55 to 3996 ng/ μ L]) compared with the non-PJI group (0 ng/ μ L [0 to 393 ng/ μ L]) ($p < 0.001$) (Fig. 1B). The concentration of H3Cit was also higher in the group with PJI (2115 ng/ μ L [5 to 2885 ng/ μ L]) than in the group without PJI (3 ng/ μ L [0 to 87 ng/ μ L]) ($p < 0.001$) (Fig. 1C).

What Is the Diagnostic Accuracy of NET Formation Biomarkers for PJI?

The ROC curve analyses for the three biomarkers we evaluated showed the following: the AUC for cf-DNA was 1 (95% CI 0.9 to 1), indicating an excellent performance. The optimal diagnostic cutoff was calculated as 5.9 ng/mL, yielding 100% specificity and sensitivity. MPO had a near-perfect AUC of 0.984 (95% CI 0.9 to 1). A threshold level of 393.6 provided 94% sensitivity and 100% specificity. H3Cit also showed a remarkable performance, with an AUC of 0.94 (95% CI 0.8 to 0.99), with the ideal cutoff set at 27.9. Sensitivity and specificity were both 88% (Table 3).

Discussion

PJI is becoming an important health issue because its incidence is increasing as the number of arthroplasties rises. In this scenario, the diagnosis of chronic PJI remains a

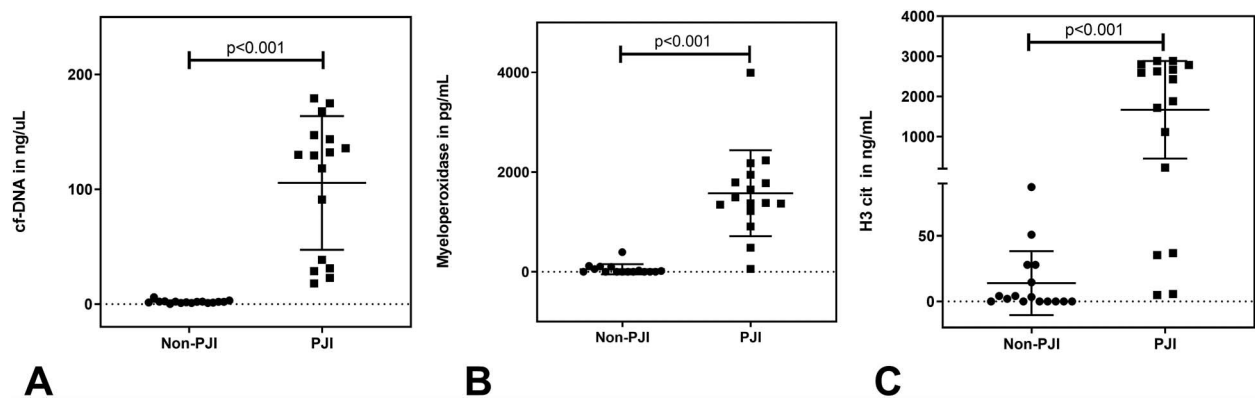


Fig. 1 These images represent quantification of NET-related biomarkers in the synovial fluid of patients with PJI and those without PJI: **(A)** cell-free DNA, **(B)** myeloperoxidase, and **(C)** citrullinated histone H3. *** $p < 0.001$.

challenge because PJI often resembles the clinical presentation of aseptic prosthetic loosening. Until now, there has been no single biomarker for diagnosing PJI, and the available biomarkers have controversial performances; additionally, some of them can be quite expensive [1, 5, 6 19]. Therefore, accurate biomarkers are needed for the diagnosis of PJI. Neutrophils play a major role in the pathophysiology of PJI; however, the mechanism triggered by those cells in the infected periprosthetic environment has not been completely elucidated. Therefore, we intended to investigate whether NET release is one of the mechanisms used by neutrophils to fight bacteria in the infected periprosthetic microenvironment, and if so, whether NET-related biomarkers would show good accuracy for the diagnosis of PJI. Our results indicate that three NET-related biomarkers—cf-DNA, MPO, and H3Cit—are increased in the synovial fluid of patients with PJI, showing the occurrence of this process in the infected periprosthetic microenvironment. In addition, we have shown that the quantification of such biomarkers has remarkable accuracy and can be useful for diagnosing PJI.

Limitations

First, because of the small sample, we could not perform subgroup analyses. It would be particularly interesting to

assess whether the levels of NET biomarkers, and consequently their performance, would vary according to the infecting microorganisms; for example, whether the levels would be lower for coagulase-negative *Staphylococcus*. Second, this analysis excluded patients with acute PJI and those with inflammatory conditions that could lead to NET release independent of PJI. Third, some minor criteria were not evaluated, which could have impacted the non-PJI control group because some patients could have tested positive. To overcome this, patients in this group were followed for 2 years to ensure that PJI had not developed.

Are H3Cit, cf-DNA, and MPO Increased in the Synovial Fluid of Patients With PJI?

We found that three NET-related biomarkers, cf-DNA, H3Cit, and MPO, are increased in the synovial fluid of patients who have PJI. Previous studies have shown that there were higher levels of cf-DNA [4] and MPO [7] in the synovial fluid of patients with PJI. Such studies have identified the NETosis process as a potential source of both biomarkers; however, the occurrence of this process in PJI has not been addressed. NETosis biomarkers to indicate the occurrence of NET release should be evaluated with caution because cf-DNA may derive from events unrelated to NETosis, such as cell death and tissue injury, and enzymes

Table 3. Diagnostic performance of NET formation biomarkers

Biomarker	Sensitivity	Specificity	Accuracy	PPV	NPV
cf-DNA	100 (79-100)	100 (79-100)	100 (89-100)	100	100
MPO	94 (70-100)	100 (79-100)	98 (86-100)	100	98 (88-100)
H3Cit	88 (62-98)	88 (62-98)	88 (71-96)	70 (39-90)	95 (85-99)

Data are provided as the calculated sensitivity, specificity, accuracy, PPV, or NPV (95% CI). PPV = positive predictive value; NPV = negative predictive value; cf-DNA = cell-free DNA; MPO = myeloperoxidase; H3Cit = citrullinated histone H3.

such as MPO may be released because of neutrophil activation that is not related to NET generation [13, 17]. A key step in the NET release process is the generation of H3Cit, mediated by the peptidyl arginine deiminase 4 enzyme, which results in chromatin decondensation, an initial event of NETosis [12]. Although other peptidyl arginine deiminase 4-independent mechanisms have been described, H3Cit is considered a NET-specific biomarker [12, 18]. In this study, we investigated cf-DNA, MPO, and H3Cit, all of which were increased in the synovial fluid of patients with PJI, thus providing strong evidence supporting NET release in the infected periprosthetic microenvironment.

What Is the Diagnostic Accuracy of NET Formation Biomarkers for PJI?

NET-related biomarkers had remarkable accuracy for the diagnosis of PJI, with all of them displaying a near-perfect diagnostic performance, indicating them as promising biomarkers for diagnosing PJI. Neutrophil-related synovial fluid biomarkers, such as the percentage of polymorphonuclear cells, leukocyte esterase, and alpha-defensin, have been widely used for diagnosing PJI but have some limitations. Determining the percentage of polymorphonuclear cells in synovial fluid varies broadly across different institutions, and to date, there is no consensus regarding the best threshold [6]. Leukocyte esterase has shown a sensitivity of 93% and a specificity of 77%, but its utility is impaired by the presence of blood or debris in the synovial fluid [19]. Recent analyses of the performance of alpha-defensin for diagnosing PJI have shown controversial results because the lateral flow test seems to display poorer performance than an immunoassay, with sensitivity and specificity of 80% and 89%, respectively [1, 5]. Our results have shown that NET-related biomarkers had high sensitivity and specificity for the diagnosis of PJI and could be used in combination with traditional biomarkers to increase the accuracy of diagnosing PJI. In this scenario, novel studies are needed to evaluate how such biomarkers could be better used for diagnosing PJI.

Conclusion

We have shown that biomarkers of NET formation are increased in the synovial fluid of patients with PJI, evidencing the occurrence of NETosis in the infected periprosthetic microenvironment. Knowledge about the occurrence of this process may be useful for better planning regarding the use of biomarkers for diagnosing PJI. NET-related biomarkers have high sensitivity and specificity. This finding enables the design of novel studies aiming to find potential biomarkers in the NETosis pathway. In

addition, further studies are needed to confirm our findings and evaluate whether and how such biomarkers can be used for the diagnosis of PJI.

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