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Abstract

Objectives: Several studies have demonstrated that synovial calprotectin is a highly accurate biomarker in diagnosing periprosthetic joint infections (PJI). Assuring reliability is of great importance and coincides with adequate preanalytical handling. This study focuses on potentially interfering factors.
Methods: To assess the stability of synovial calprotectin, the effect of time, storage temperature, EDTA, freeze-thaw cycles, viscosity, and blood and lipid contamination was investigated. In the blood and lipid contamination experiments, hemolyzed and non-hemolyzed blood, homogenized adipose tissue, intralipid and chylomicrons were added. The effect of viscosity was investigated using freeze-thaw cycles, enzymatic pretreatment and sonification.
Results: No effect on synovial calprotectin levels was observed in synovial samples kept at room temperature compared to samples kept at 4 °C for up to seven days of storage. Freeze-thaw cycles did not result in significantly different calprotectin levels, although samples without EDTA resulted in higher recoveries after 1 and 2 freeze-thaw cycles. Blood and lipid contamination did not interfere with accurate synovial calprotectin analysis. Sample pretreatment to reduce sample viscosity by pretreating samples with DNAse and/or hyaluronidase did not influence calprotectin analysis. Sonification, however, resulted in increased calprotectin values.
Conclusions: Synovial calprotectin is a stable biomarker and its analysis is not easily influenced by potential interfering factors.

Keywords: blood; contamination; sonification; stability; synovial calprotectin; viscosity

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Introduction

Periprosthetic joint infection (PJI) is a rare debilitating complication of joint arthroplasty [1]. Proportionately, PJI increases in relevance by the increasing demand for arthroplasty due to the constant increase in life expectancy globally and the fast pace aging trend in western societies [2]. It is a potential threat to 1–2 % of patients undergoing arthroplasty [1]. An early and accurate diagnosis of PJI is essential to start effective treatment as early as possible.

Currently, the diagnosis of PJI is considered a challenge [3]. Negative cultures cannot fully exclude a PJI [4] and take up to 14 days before a final diagnosis can be made [5]. Therefore, other markers are incorporated in diagnosing PJI, such as serum C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), histology and synovial leukocyte count. These biomarkers combined with cultures led to the development of several diagnostic criteria [6, 7]. In addition, new promising biomarkers for the diagnosis of PJI in synovial fluid have been introduced. Wouthuyzen-Bakker et al. were the first to demonstrate the clinical utility of synovial calprotectin in diagnosing PJI by evaluating and validating a quantitative synovial calprotectin lateral flow (LF) assay that is normally used for the measurement of fecal calprotectin in many clinics [8]. A recent meta-analysis demonstrated the accuracy of synovial calprotectin in diagnosing and excluding PJI with a sensitivity of 92 % (95 % CI: 84–98 %), and a specificity of 93 % (95 % CI: 84–99 %) [9].

As for any diagnostic marker, assuring accuracy and reliability is of great importance and coincides with adequate and careful preanalytical handling. It has been reported that 70 % of laboratory errors are due to preanalytical causes [10]. Synovial fluid has specific pre analytical challenges by itself, since it is a heterogenous fluid with variations in viscosity, lipid content, and the frequent occurrence of blood

contamination, all forming a challenge to biomarker detection or cytological analysis of synovial fluid [11, 12]. Moreover, neutrophil responses to infection comprise release of deoxyribonucleic acid (DNA) and proteins to the synovial fluid by the process of NETosis, attributing to the viscous nature of synovial fluid. Diagnostic important biomarkers such as leukocyte esterase, alpha defensins and calprotectin are neutrophil extracellular trap (NET) binding proteins [13], yet little is known concerning the potential analytical interference from NETosis in synovial fluids. This study focuses on these potentially interfering factors, with special attention to the effect of blood and/or lipid contamination, sample stability and sample viscosity.

Materials and methods

Samples

In the University Medical Center Groningen (UMCG), synovial fluid samples were aspirated by using a 21 gauge, 1 in. needle that is attached to a 20 mL syringe, then transported to the laboratory in less than 30 min. The samples were used for routine calprotectin measurements on the same day. The residual synovial samples were stored at −80 °C in the biobank and used for this study. Samples were selected based on calprotectin concentration, synovial fluid volume, and, for the viscosity experiments, viscous aspect.

For the blood contamination and lipid contamination experiments, residual whole blood samples and lipaemic blood samples were used. For the blood contamination experiments EDTA anticoagulated tubes manufactured by Becton Dickonson (BD, Vianen, The Netherlands) are used. Highly lipaemic blood samples were left to stand for 3 h at room temperature and the lipaemic layer, merely containing chylomicrons, was subsequently harvested. In addition, anonymized residual subcutaneous fat tissue obtained after bariatric surgery was homogenized and stored at −20 °C prior to the lipid contamination experiment to release adipocyte intracellular content. The Medical Ethics Review Board (University Medical Centre Groningen (UMCG)) waived the need for ethical approval (METc 2021/168 and M22.302539).

Synovial calprotectin measurements

The levels of calprotectin were determined using the Lyfstone rapid LF test (Lyfstone, Tromsø, Norway), according to the manufacturer's instructions. Within run and between run coefficients of variation (CV) of the assay are 20 and 25 %, respectively. To determine the CV's freshly frozen and aliquoted samples were analyzed according to the CSLI-EP5-A protocol (Simple Precision).

Stability experiment (effect of time, temperature, EDTA and freeze-thaw cycles)

Synovial fluid samples of different synovial calprotectin levels; low (20–40 mg/L), medium (40–100 mg/L) and high (100–200 mg/L), were exposed to different storage conditions.

To investigate the effect of ethylene diamine tetra acetic acid (EDTA), time and temperature on sample stability, native fresh samples were split and either aspirated into an EDTA-containing vacutainer tube or a neutral tube and were either stored at room temperature (RT) or at 4 °C. For samples kept at RT, they were measured at six different time points; directly (t=0), after 3 h, 6 h, 24 h, three days and seven days. For samples kept at 4 °C, they were measured at five different time points; directly (t=0), 6 h, 24 h, three days and seven days. Each level and condition (with or without EDTA, temperature) contained three samples, hence a total of 396 samples were analyzed for the EDTA and temperature experiment.

To investigate the effect of freeze-thaw cycles on sample stability samples were exposed to 1, 2, 3 or 5 freeze-thaw cycles. For this experiment the same levels were used, each level and condition (with or without EDTA) contained three samples, hence a total of 72 samples were analyzed.

To compare the results between conditions and time points independent of the three different calprotectin levels, synovial calprotectin levels were expressed as percentage recovery based on the synovial calprotectin level at t=0.

Effect of blood and lipid contamination

To investigate the extent of blood and lipid contamination of routinely derived clinical synovial samples, our routine clinical database/biobank, containing 83 synovial samples and patient data from 74 individual patients collected since July 2015, was surveyed for the haemolytic index (H-index) and the lipemic index (L-index).

The effect of blood contamination on synovial calprotectin measurement was investigated at three different synovial calprotectin levels as described above, by the addition of A) hemolyzed whole blood and B) non-hemolyzed whole blood. Hemolyzed blood was obtained by three freeze-thaw cycles of an EDTA whole blood sample. The effect of lipid contamination on synovial calprotectin measurement was investigated also at the three different synovial calprotectin levels, by the addition of A) homogenized adipose tissue, B) intralipid (Fresenius Kabi Netherlands B.V., Zeist, the Netherlands) C) chylomicrons.

Synovial samples were separately mixed with either hemolyzed whole blood, non-hemolyzed whole blood, adipocyte homogenate, intralipid or chylomicrons in different ratios, which resulted in the following percentages of contamination: 100 , 50, 25, 12.5, 6.25, 3.12, 1.56, 0.76, 0.38 and 0 % blood or lipid. Each blood contamination condition was analyzed in duplo, whereas the adipocyte contamination experiment was performed in triplicate. In total, for the blood contamination experiment 40 samples and for lipid contamination experiment 108 samples were analyzed. In these experiments synovial samples were used with a mean calprotectin concentration of 87 or 103 mg/L (the adipocyte contamination experiment). This lower value was chosen to investigate the effect of contamination on samples with calprotectin values around the clinical cutoff value of 50 mg/L [3, 8]. The H and L indices were subsequently measured on a Cobas 6000 chemistry analyzer (Roche Diagnostics, Rotkreuz, Switzerland). To compare the results between conditions independent of the three different calprotectin levels, synovial calprotectin levels were expressed as percentage recovery based on the synovial calprotectin level in the sample without blood or lipid contamination.

Effect of synovial viscosity

To examine the effect of viscosity reduction on synovial calprotectin analysis, three pretreatment modalities to liquify vicious samples were investigated: A) five subsequent freeze-thaw cycles, B) enzymatic pretreatment and C) sonification. For these approaches, fresh samples were included that were sufficiently viscous by visual inspection and semi-quantitatively scored (1–5) by two different technicians, independently. Viscosity was scored and calprotectin levels were measured before and after each pretreatment. Synovial calprotectin levels were expressed as percentage recovery based on the synovial calprotectin level in the sample without pretreatment.

Approach A consisted of five subsequent freeze-thaw cycles. For this experiment, the same samples were used as described in the stability experiments.

Approach B consisted of a) no treatment, b) hyaluronidase (Sigma Aldrich, Amsterdam, The Netherlands) treatment, c) DNAse (Sigma Aldrich, Amsterdam, The Netherlands) treatment or d) combined hyaluronidase and DNAse treatment.

Three different levels (68, 85 and 136 mg/L) of calprotectin concentrations were used. For each level, analysis was performed in triplicate. Each synovial fluid sample was further divided and transferred into four different tubes (one for each pretreatment modality). A hyaluronidase solution of 146 U/mL was prepared by dissolving 10 mg of bovine testicular hyaluronidase solution (439 U/mg) in 30 mL of saline buffer (0.9 g/L). A DNAse solution of 150 U/mL was prepared by dissolving one DNAse-containing tablet (10,000 U/tablet) in 66.6 mL of distilled water.

The untreated synovial sample was mixed in a 2:1 (sample:enzyme) ratio with the hyaluronidase (150 U/mL) solution and/or the DNAse solution. After incubation at 37 °C for 30 min with hyaluronidase, DNAse or both, synovial calprotectin was measured.

In total 36 samples were analyzed. Approach C consisted of sonification of the samples, for 10 s using a Vibra cell, model VC60 sonificator (Sonics & Materials Inc, Newtown, USA). Twenty-five subsequent fresh routine patient samples were used. Before and after sonification, viscosity was scored as described above, and synovial calprotectin was analyzed.

Statistics and data analysis

The mean and confidence interval of the H-index and L-index were calculated. Repeated measurements of calprotectin levels over time were analyzed using mixed model analysis, where group (i.e., preanalytical condition) and measurement (calprotectin recovery) were both added as fixed variables. The interaction term group × measurement was added to analyze differences between groups over time. A Wilcoxon matched-pairs signed rank test was used to compare results before and after sonification. For all comparisons, a p-value<0.05 was regarded as significant. Data were analyzed using the software program GraphPad Prism (GraphPad Software, LLC, version 8.4.2, Boston, USA) and SPSS (version 29.0; SPSS, Chicago, USA).

Results

Stability experiment (effect of time, temperature, EDTA and freeze-thaw cycles)

No significant decrease or increase of measured calprotectin was observed in synovial samples kept at room temperature compared to samples kept at 4 °C for up to seven days of storage. After seven days of storage, the addition of EDTA, in samples kept at 4 °C resulted in a slight, non-significant reduction of values (p-value=0.07) (Figure 1).

Five successive cycles of freeze-thawing did not influence the levels of measured calprotectin in EDTA-containing samples. In samples without EDTA, calprotectin levels increased significantly after the first and second freeze-thaw cycles (p-value=0.011 and 0.003, respectively), this difference obliviated after three or more freeze-thaw cycles (Figure 2).

Effect of blood and lipid contamination

In the routine clinical data and biobank, the median H-index was 34 with a range of 0–6,834, while the median L-index was 40 with a range of 4–900 (Figure 3).

Synovial calprotectin LF assay appears to report lower calprotectin values in synovial samples contaminated with hemolyzed blood. However, no statistical relation could be found between the level of H index, contamination percentage and the recovered values of calprotectin (Figure 4A).

The use of non-hemolyzed whole blood in these experiments resulted in higher H indices than the use of hemolyzed blood, 8,180 and 980, respectively. Synovial calprotectin LF assay appears to report lower values in the presence of whole blood, yet this difference was not significant. No significant correlation could be found between the level of H-index and the recovered values of calprotectin (Figure 4B).

Adipose tissue or chylomicrons contaminated synovial fluid resulted in higher L indices than in the intralipid experiment, a maximum of 7,260, 6,690 and 4,330, respectively. In the lipid contamination experiment, the synovial calprotectin LF assay shows no significant increase or decrease in calprotectin levels. Furthermore, there is no clear or significant relation between the level of adipocyte, intralipid or chylomicrons contamination and the recovered values of calprotectin (Figure 5A–C, respectively).

Effect of synovial viscosity

After five subsequent freeze-thaw cycles the viscosity of the samples did not decrease, as judged visually using the 5-point scoring system (data not shown).

After hyaluronidase/DNAse treatment, the viscosity of the samples reduced from a score of 4–1 (Figure 6). DNAse alone has a smaller effect in reducing the viscosity of the synovial fluid as measured using the viscosity score. Calprotectin levels did not differ significantly between the different pretreatment modalities.

Twenty-five subsequent fresh synovial samples were analyzed before and after sonification. Sonification had a

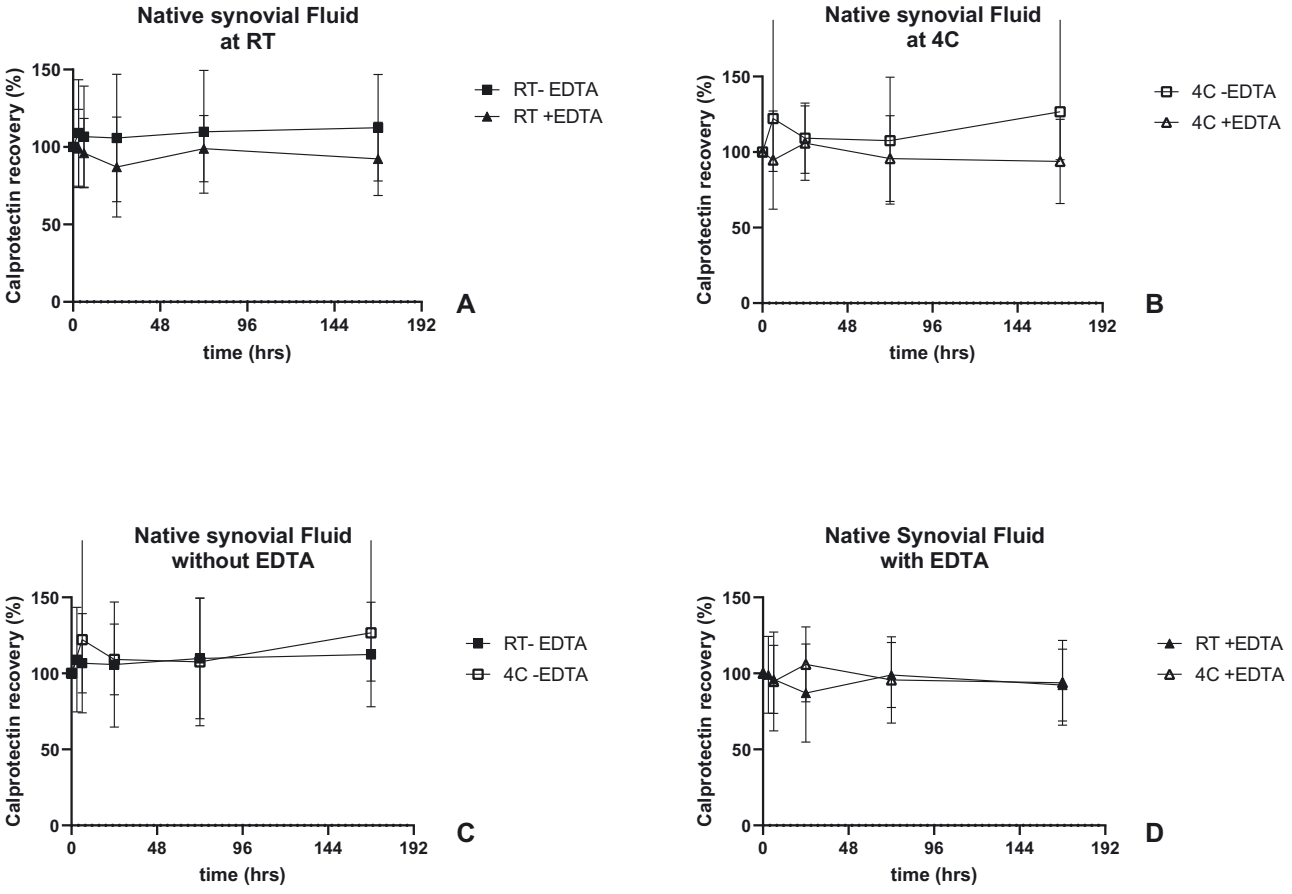

Figure 1: Percentage recovery of synovial calprotectin (y-axis) in relation to storage duration (x-axis), effect of EDTA and temperature. Duration is depicted in hours (h). Results expressed as mean and standard deviations (SD, errors bars). (A) Effect of EDTA on percentage calprotectin recovery at room temperature (RT). (B) Effect of EDTA on percentage calprotectin recovery at 4 Celsius (4 °C). (C) Effect of the two storage temperatures on percentage calprotectin recovery in EDTA free synovial fluid samples. (D) Effect of the two storage temperatures on percentage calprotectin recovery of calprotectin in synovial fluid samples with EDTA.

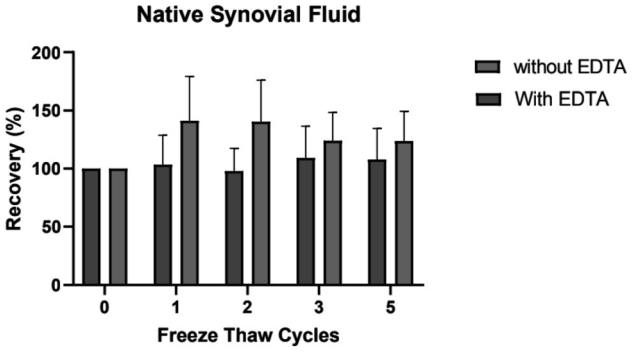

Figure 2: The percentage recovery of synovial calprotectin (y-axis) in relation to the number of freeze-thaw cycles (x-axis), in synovial samples with (dark gray) and without (light gray) EDTA.

significant effect on sample viscosity; mean viscosity scores decreased from 3.2 to 1. In addition, median calprotectin concentration increased significantly, from 59 mg/L before sonification to 100.5 mg/L after sonification ($p<0.01$) (Figure 7).

Discussion

Although synovial calprotectin is a highly sensitive and specific marker in diagnosing PJI [9, 14], there is a knowledge deficit on the influence of preanalytical handling on calprotectin detection. In this study we investigated different preanalytical conditions that occur frequently in clinical practice and that may influence calprotectin reliability. We demonstrated that calprotectin levels are stable for up to seven days regardless of storage duration and temperature in synovial samples with and without EDTA. Subsequent freeze-thaw cycles did not result in significantly different calprotectin levels, although samples without EDTA seemed to result in significantly higher recoveries of over 100 % after 1, 2 freeze-thaw cycles. A possible explanation for the significant results is the between-run assay variation of 25 % that is inherent to LF assay, in addition to the nature of the experiment itself. Freeze-thaw cycles can

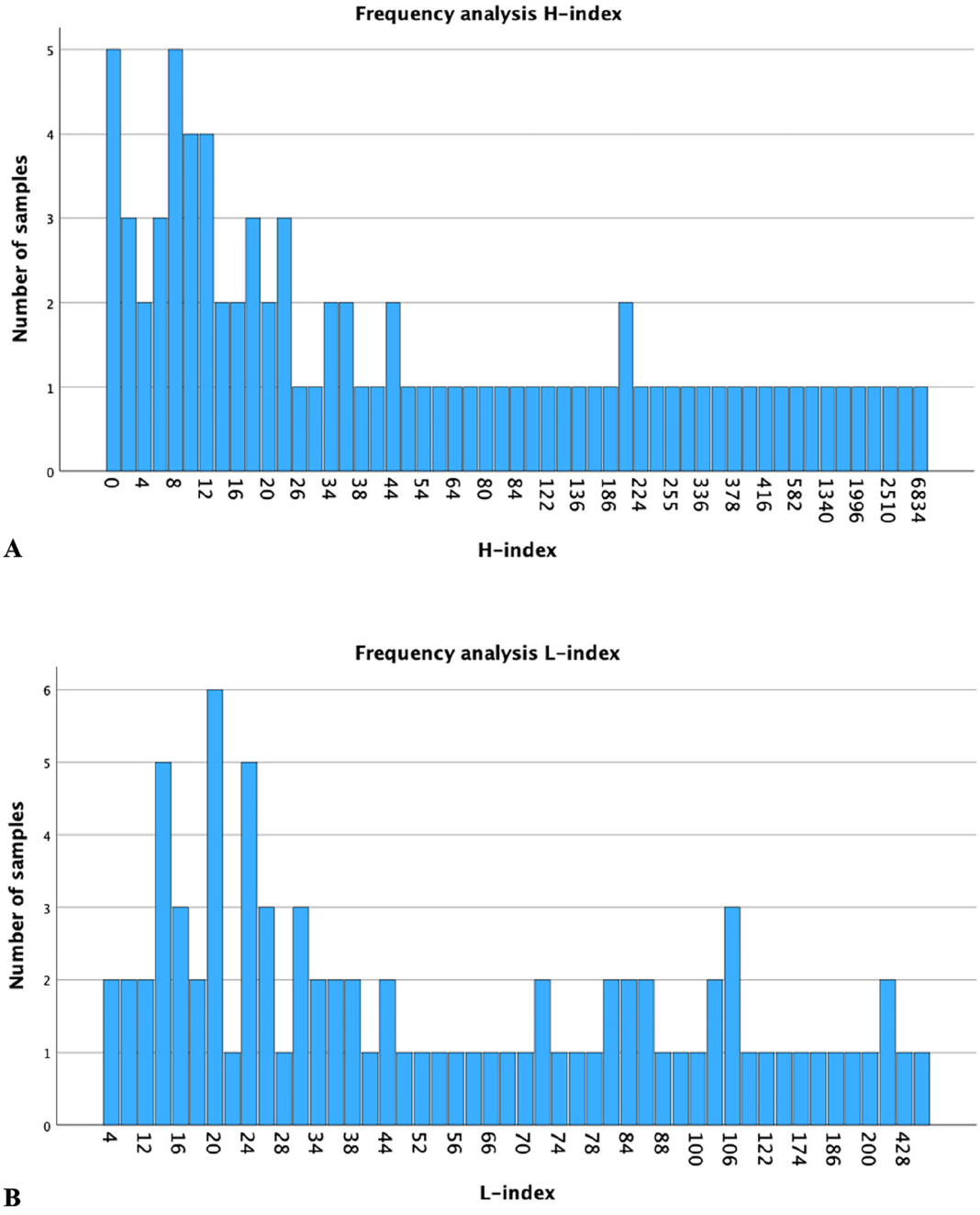

Figure 3: Frequency analysis of (A) H-index and (B) L-index in 83 synovial fluid samples.

lead to neutrophils destruction releasing their content including calprotectin, yet repetitive freeze-thaw cycles also affect stability and integrity of calprotectin, which could have equalized the effect. Blood and/or lipid contamination did not interfere with a reliable calprotectin analysis. Finally, sample pretreatment to reduce sample viscosity can be done by pretreating samples with DNAse and/or hyaluronidase without influencing calprotectin analysis. However, sonification as means to reduce sample viscosity, results in increased calprotectin values. Clinically, our findings suggest that synovial fluid could be aspirated into a standard syringe without specific precautions and kept and transported at room temperature until calprotectin analysis.

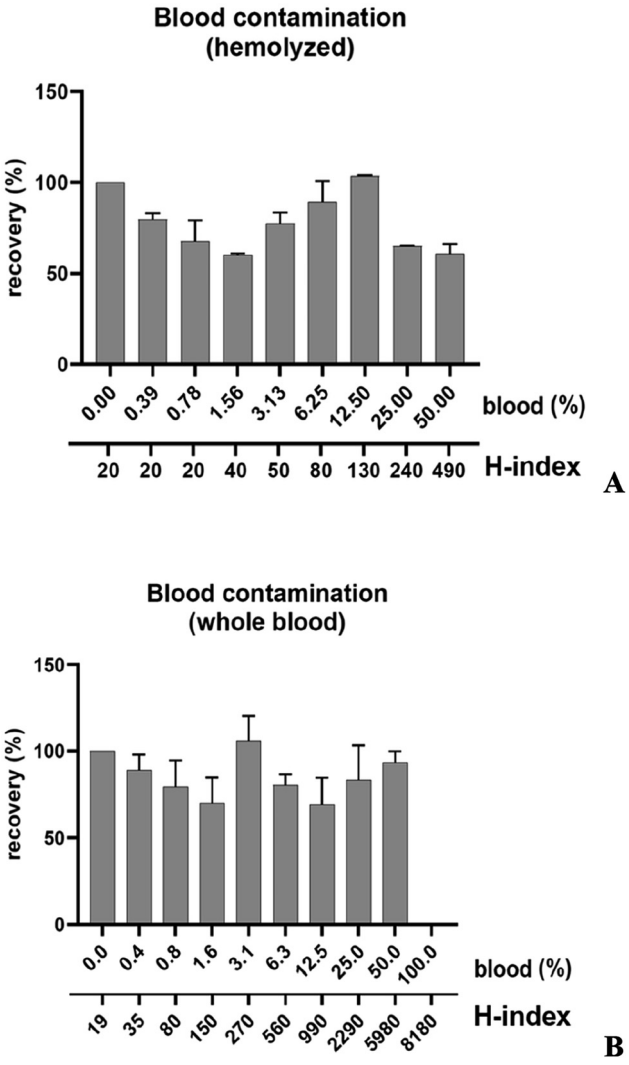

Figure 4: Percentage recovery of synovial calprotectin (y-axis) in relation to the H-index and the percentage of contamination (x-axis) (A) hemolyzed blood and (B) non hemolyzed blood.

Blood and lipid contamination of synovial fluid occurs frequently in clinical practice (see Results section). However, the level of blood and/or lipid contamination is mostly very low with a median H-index of 34 and L-index of 40, respectively. Lipid contamination level in synovial fluids is expected to increase due to the expected upward trend of obesity prevalence in the world and especially in the western countries [15]. Although in the blood and lipid contamination experiments, the degree of calprotectin fluctuation did not significantly correlate with the level of contamination or the levels of H index and L index, respectively, values around the cut-off value should be interpreted with caution. Therefore, according to our findings, calprotectin analysis in daily practice is not influenced by blood and/or lipid contamination of the synovial sample.

Occasionally, synovial samples are highly viscous, which may hamper accurate pipetting and consequently may lead to less accurate measurement. In our attempt to reduce viscosity, DNAse and/or hyaluronidase pretreatment reduced the viscosity of the synovial fluid without a significant effect on the calprotectin levels. Pretreatment with sonification resulted in decreased viscosity, although resulted in a significant increase in calprotectin levels Pretreatment with sonification resulted in decreased viscosity. Although sonification pretreatments resulted in a significant increase in calprotectin levels, there are few individual samples that showed stable and even decreased calprotectin levels. We speculate that this discrepancy could be attributed to sampling error. Assuming that highly viscous samples could impede proper pipetting, NETs could possibly not be distributed in a homogeneous fashion in the sample and therefore could also affect the concentration of calprotectin. We assume that sonification pretreatments result in the release of calprotectin from neutrophil extracellular traps (NETs) present in the synovial fluid, which explains the predominant trend in our sonification experiment [16]. Upon neutrophil activation, neutrophils release granule proteins and chromatin to form an extracellular fibril matrix known as NETs. NETs are networks of extracellular fibers, primarily composed of DNA and proteins from neutrophils, which neutralize pathogens. However, pretreatment of synovial samples with DNAse and/or hyaluronidase did not result in a significant increase of calprotectin levels, which one would expect when NETs are enzymatically cleaved by DNAse/hyaluronidase. Another explanation for the discordant results of calprotectin levels after pretreatment by sonification pretreatment may be because sonification may result in disruption of intact neutrophils present in the NETs, thereby releasing intracellular calprotectin, whereas enzymatic pretreatment leaves neutrophils in the sample intact [17].

Limitations and considerations

Apart from the limitation of some of the experiments being limited to a small sample size which could affect the generalization of the results, this study is the first of its kind to investigate and demonstrate an extensive overview of the potential interfering factors that could affect the analysis synovial calprotectin.

Although most of the interfering factors did not have a statistically significant effect on the calprotectin levels in synovial fluid, quite frequently, recoveries of 80 and 120 % were observed in our results. Together with analytical

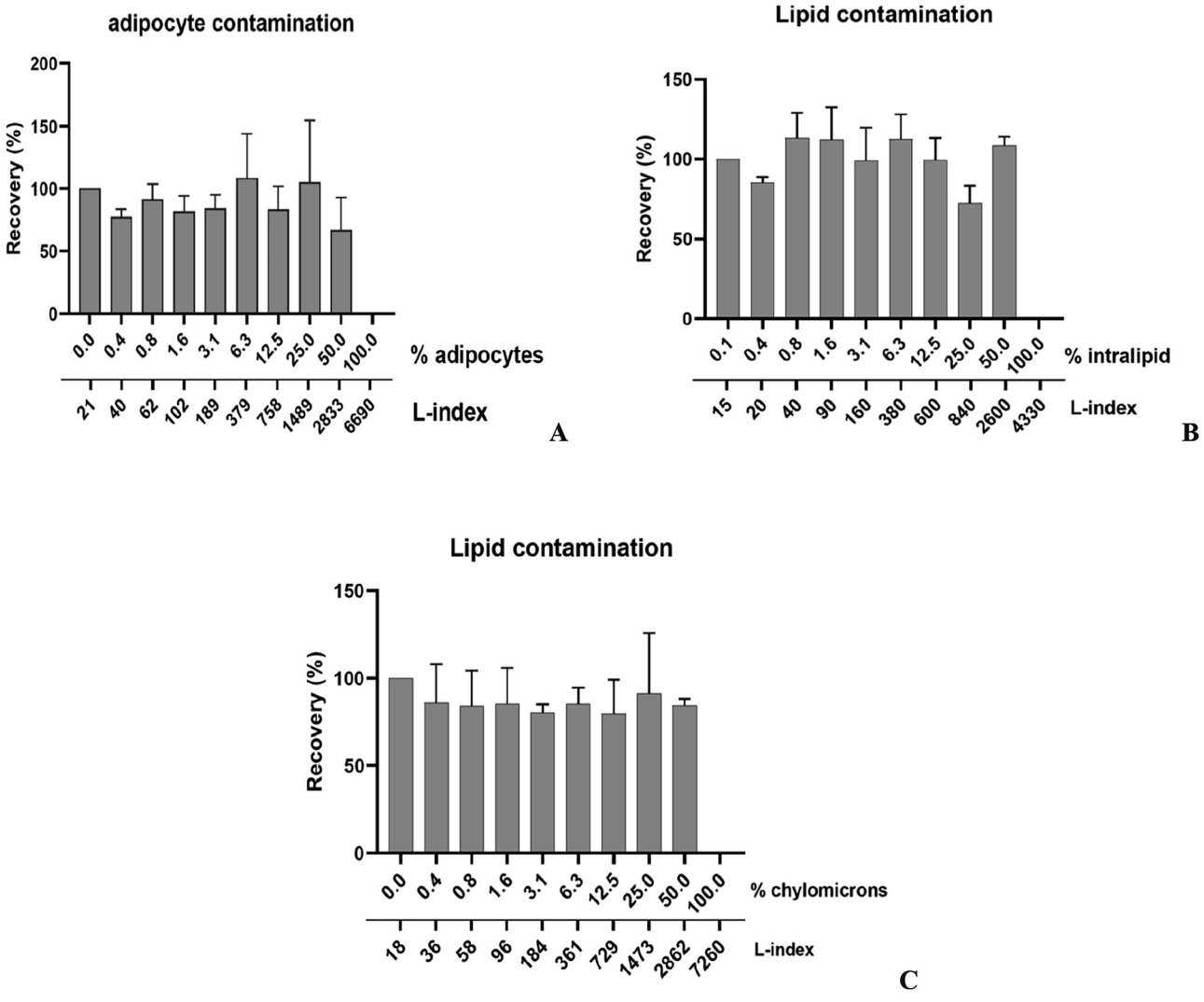

Figure 5: Percentage recovery of synovial calprotectin (y-axis) in relation to the L-index and the percentage of contamination (x-axis) with (A) homogenized adipose tissue (B) intralipid (C) chylomicrons.

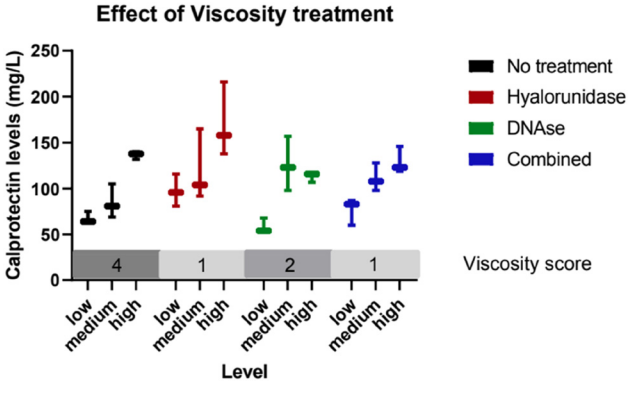

Figure 6: Calprotectin value in mg/L and viscosity score of synovial samples in a control group and three enzymatically pretreated groups. The results are depicted in three different calprotectin levels; low, medium and high (x-axis).

variation, we propose caution when interpreting synovial calprotectin values around the cut-off value without taking into account other clinical parameters.

Since sonification results in higher calprotectin values than untreated samples, probably due to the release of calprotectin from NETs and/or neutrophils present in the sample, one may argue whether this improves the sensitivity of the test. The presence of these intact neutrophils is overlooked when no or other sample pretreatments, such as DNAse/hyaluronidase pretreatment, is used. The presence of these neutrophils, beside the free calprotectin levels in the synovial fluid, indicate that an active inflammatory process is ongoing. When choosing this sample pretreatment as the standard pretreatment, new clinical cut-off values should however, be established.

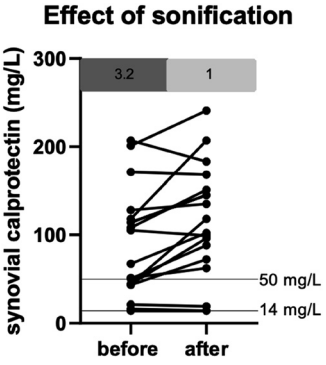

Figure 7: Calprotectin value in mg/L (y-axis) and viscosity degree of synovial samples before and after sonification (x-axis).

Conclusions

Synovial calprotectin is a stable biomarker and not likely to be influenced by blood and/or lipid contamination. Nevertheless, values around the cut-off value should be interpreted with caution. Synovial viscosity may interfere in correctly pipetting the sample albeit may be circumvented by pretreating synovial samples with DNAse and/or hyaluronidase. For clinicians, researchers and laboratory specialists our findings suggest that synovial calprotectin is a stable and reliable tool in the diagnosis of PJI without the need of special impractical specific preanalytical conditions.

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Research ethics: The Medical Ethics Review Board (University Medical Centre Groningen (UMCG)) waived the need for ethical approval (METc 2021/168 and M22.302539).

Informed consent: Not applicable.

Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Competing interests: Lyfstone (Lyfstone, Tromsø, Norway) supplied the reagent kits in kind, used in the present study. This organization played no role in analysis and interpretation of data, writing of the report, or in the decision to submit the findings for publication. None of authors have financial disclosures or conflicts of interest.

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Data availability: Not applicable.

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