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Rapid multiplex micro-ELISA assay for simultaneous measurement of synovial biomarkers

a potential aid in diagnosing periprosthetic joint infection?

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Aims
The diagnosis of periprosthetic joint infection (PJI) remains a challenge, as no single diagnostic test shows high diagnostic accuracy. Recently, the measurement of synovial biomarkers has shown promising results. The aim of this study was to present a novel multiplex micro-enzyme-linked immunosorbent assay (ELISA) method for the rapid and simultaneous measurement of alpha-defensin, interleukin (IL)-6, and calprotectin developed in a model buffer system and human spiked synovial fluid.

Methods
A microfluidics- and chemiluminescence-based multiplex micro-ELISA point-of-care testing system was employed for the rapid and simultaneous measurement of alpha-defensin, calprotectin, and IL-6 developed in a model buffer system and spiked human synovial fluid. Cut-off values of 1.56 μg/ml for alpha-defensin, 50 μg/ml for calprotectin, and 0.031 μg/ml for IL-6 were extracted from the literature as optimal cut-off values for the diagnosis of PJI, and were used for comparison.

Results
Limit of detection (LoD) was determined for each individual biomarker by means of calibration curves with serial dilutions in a model buffer system. LoDs of 0.008, 0.002, and 0.00014 μg/ml were determined for alpha-defensin, calprotectin, and IL-6, respectively. The spiked synovial fluid assay determined LoDs of 0.08, 0.31, and 0.004 μg/ml for alpha-defensin, calprotectin, and IL-6, respectively.

Conclusion
These findings highlight the proposed platform's unique features, including simultaneous measurement of three key synovial biomarkers, minimal sample volume requirements (5 to 50 μl), lower LoDs compared to conventional tests, and a short processing turnaround time of 22 minutes. Further validation studies are necessary to confirm its clinical utility.

Article focus
- To present a novel multiplex micro-enzyme-linked immunosorbent assay (ELISA) for the rapid and simultaneous measurement of biomarkers developed in a model buffer system and human spiked synovial fluid.

Bone & Joint Research

Key messages

- Simultaneous quantification of three key synovial biomarkers (alpha-defensin, calprotectin, and interleukin (IL)-6).
- Lower LoDs compared to conventional tests and speed of processing can give this novel multiplex μ-ELISA platform great diagnostic potential as a point-of-care tool.

Strengths and limitations

- Small sample volume requirements (5 to 50 μl).
- Lower limits of detection compared to conventional tests.
- Need for validation with clinical studies.

Introduction

Periprosthetic joint infection (PJI) is a rare but serious complication after joint arthroplasty.[1,2] The diagnosis of PJI remains a challenge, especially in low-grade and culture-negative cases, as a diagnostic test with high accuracy is lacking. An accurate diagnosis is essential to guide appropriate surgical and antibiotic therapy.[3] Biomarkers represent a set of diagnostic tools for monitoring a biological process or guiding therapeutic decisions. They can be measured and assessed as indicators of biological processes, disease agents, or therapeutic responses to treatment interventions.[4] In the setting of infection, biomarkers are widely employed to enhance the accuracy and timing of diagnosis, assess prognosis, and optimize the choice of tailor-made treatment.[5] The research for synovial biomarkers that can aid in the diagnosis of PJI is an ongoing field, and several molecules are currently under investigation.[6-8] Of these, alpha-defensin, calprotectin, and interleukin (IL)-6 seem to be the most promising.[9] The clinical gold standard for protein biomarker analysis is enzyme-linked immunosorbent assay (ELISA); however, standard ELISA assays are time-consuming (two to four hours) and do not allow random access testing at reasonable costs. Lateral flow immunoassay, mostly based on chromatography, has recently been proposed as a possible alternative to standard ELISA in point-of-care settings due to its ease of use and quick detection.[10] Microfluidic chip-based ELISA methods present another innovative option, combining the ease of use and swift results of lateral flow assays with the precision and reliability of traditional ELISA.[11-13]

Despite extensive investigations, so far, no single biomarker test could provide a definitive and accurate diagnosis of PJI. On the other hand, the approach of merging multiple biomarkers into a single panel seemed to be more successful in ensuring improvement in diagnostic accuracy.[14,15] Moreover, according to recent findings, the diagnostic potential increases if the association involves biomarkers belonging to different categories (cytokines, inflammatory proteins, and antimicrobial peptides).[16]

The aim of this study was to present a novel multiplex micro-ELISA (μ-ELISA) method for the rapid and simultaneous measurement of alpha-defensin, IL-6, and calprotectin developed in a model buffer system and human spiked synovial fluid.

Methods

The multiplex μ-ELISA system

The commercial rapid test system (GENSPEED Biotech, Austria) was used to detect alpha-defensin, calprotectin, and IL-6 on prototype test chips (Figure 1). This technology is a point-of-care chemiluminescence-based multiplex μ-ELISA system that can be employed in various applications.

The test system is based on proprietary technology that combines microfluidic test chips with integrated microarrays, miniaturized optoelectronics, and automated assay procedures. The test chip consists of an injection-moulded top part containing the microfluidic structures and a bottom foil (both polystyrene) onto which the capture antibodies are deposited (Figure 2).

The immobilization of the capture antibodies (Table I) in the corresponding capture areas (1 × 2 mm) is achieved using a sciFLEXARRAYER S3 (SCIENION, Germany) by printing a pattern of spots (diameter 200 μm, pitch 500 μm) using proprietary printing buffers. Finally, the microfluidic top part and the bottom foil with the immobilized capture antibodies are connected by ultrasonic welding.

The design of the microfluidic test chip, which includes an inlet port, a reaction channel, and a waste reservoir, facilitates a sequential, capillary force-driven flow of microlitre volumes of sample and reagents. After addition to the inlet, the fluids are transported through the channel by capillary action, halting when the inlet is empty and resuming with the introduction of new fluids, thereby displacing the preceding solution into the waste reservoir.

Before analysis, a sample filtration is performed, followed by applying a drop of the sample to the test chip's inlet port. After incubation, a drop of biotinylated detection antibody solution is added (Table I). The GENSPEED R2 analyzer then automatically dispenses all necessary reagents for the multiplex μ-ELISA using micro-piezo pumps (mp6; Bartels Mikrotechnik, Germany), with results ready in 15 to 22 minutes, as shown in a previous study.[17]

For data readout, the chemiluminescence reaction's emitted photons are captured by a custom photodiode array located beneath the test chip's reaction channel. The one-dimensional array, with a spatial resolution of 1 mm, comprises 32 pixels and features a low-noise equivalent power (10^{-15} W/√Hz), and is used in combination with an appropriate analogue digital converter to integrate chemiluminescence signals over time. This setup has been successfully applied in standard multiplexing assays to detect various biomarkers or pathogens in a single test across different sample types, such as blood or swab materials. Examples include nucleic acid-based tests for identifying antibiotic resistance,[18] and serological assays for detecting various immunoglobulins in patients infected with SARS-CoV-2.[17]

Data processing

The measurements in this study are expressed as readout in arbitrary units of mean chemiluminescence intensity originating from different locations of the microfluidic chip. They correspond to the positions of the respective immobilized capture antibodies for each of the biomarkers.

Measurement protocol

Either buffer samples or real synovial fluid samples from one patient were analyzed using the chemiluminescence-based multiplex μ-ELISA system in a point-of-care format. First, a dilution series of the corresponding markers (Table I) was

Fig. 1
Analyzer and microfluidic test chip used in this study (for details refer to "Methods").

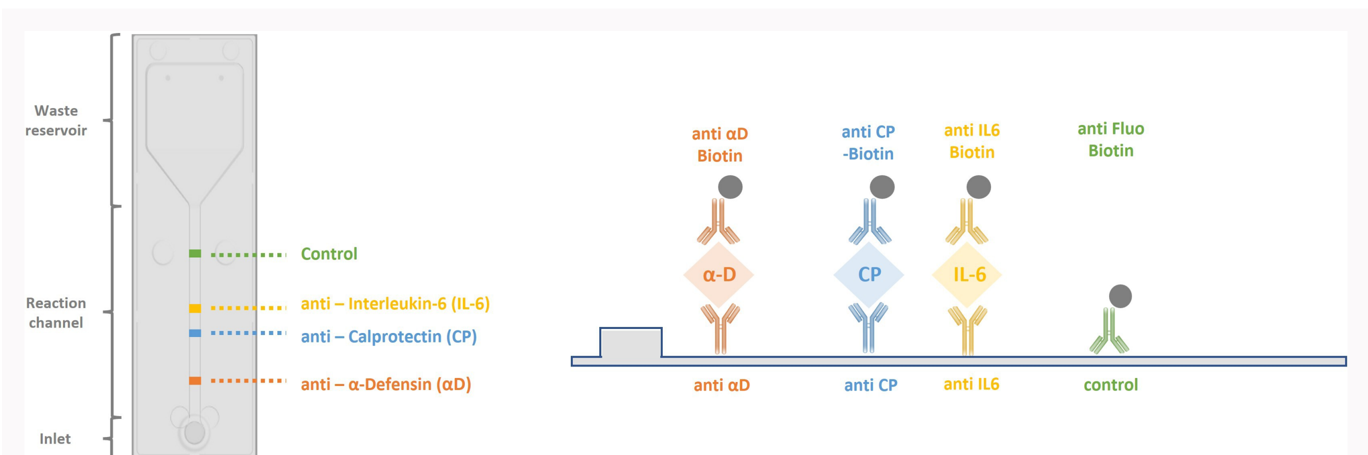

Fig. 2
Left panel: Layout of capture antibodies immobilized on the multiplex microfluidic test chip for the respective biomarke. Sample and reagents enter through the chip inlet, pass through a channel with capture antibodies, and exit into the waste reservoir. Right panel: side view showing the sandwich assay setup for each biomarker: immobilized antibodies captured the biomarker, biotinylated detection antibodies bind, and streptavidin-horseradish peroxidase (HRP) interaction enables chemiluminescence detection. A biotinylated control antibody checks the assay's workflow. α-D, alpha-defensin; CP, calprotecin; Fluo, fluorescein; IL-6, interleukin 6.

Table I. List of materials used for the detection of human alpha-defensin, calprotectin, and interleukin-6 with the GENSPEED microfluidic test chip.

Biomarker	Capture antibody	Detection antibody	Antigen/Standard
Alpha-defensin	Human alpha-defensin 1 DuoSet ELISA (R&D Systems, USA)	Human alpha-defensin 1 DuoSet ELISA (R&D Systems)	Recombinant human alpha-defensin 1 protein (Abcam)
Calprotectin	Anti-S100A9+ Calprotectin (S100A8/A9 complex) antibody (Abcam, UK)	Human S100A8 DuoSet ELISA (R&D Systems)	Recombinant Human S100A8/ S100A9 (R&D Systems)
IL-6	Human IL-6 DuoSet ELISA (R&D Systems)	Human IL-6 DuoSet ELISA (R&D Systems)	Recombinant Human IL-6 (R&D Systems)

ELISA, enzyme-linked immunosorbent assay; IL-6, interleukin-6.

Table II. List of buffer-based and synovial fluid-based samples with different concentrations of alpha-defensin, calprotectin, and interleukin-6.

Sample no.	α-Defensin, μg/ml	Calprotectin, μg/ml	Interleukin-6, μg/ml
Buffer-based samples			
1	1	100	0.1
2	0.1	10	0.01
3	0.01	1	0.001
4	0.001	0.1	0.0001
5		0.01	0.00001
Blank	0	0	0
Synovial fluid-based samples			
1	1,000	10,000	10
2	100	1,000	1
3	10	100	0.1
4	1	10	0.0
5	0.1	1	0.001
Blank	0	0	0

Table III. Automized measurement protocol of GENSPEED R2 Analyzer.

Sample mix					
Sample	Detection antibody solution	Enzyme solution	Washing solution	Chemiluminescence substrate	Total
25 μl	25 μl	30 μl	4 × 10 μl	30 μl	150 μl
10 mins		5 mins	4 × 1 min	2.5 mins	21.5 mins

prepared using either washing solution (WL) or a human synovial fluid sample as dilutor (Table II).

For measurements of buffer-based samples, 78 μl of the sample was mixed with 78 μl of the detection antibody solution consisting of the corresponding biotinylated detection antibodies diluted in blocking solution (2 μg/ml). Subsequently, 50 μl of the sample-mix was transferred to the chip inlet using a laboratory pipette. The prepared sample-mix volume was sufficient to perform three replicates of each marker concentration. The rest of the analysis was obtained by the GENSPEED R2 Analyzer applying the test protocol as described in Table III.

Spiked frozen synovial fluid (4 ml) taken from a patient undergoing arthrocentesis for routine diagnostic process was used and infection was excluded. Joint puncture was performed with sterile technique according to the standard institutional guidelines. The study was conducted according to the guidelines of the Declaration of Helsinki[19] and approved by the Institutional Ethics Committee of the Charité – Universitätsmedizin Berlin, Germany (protocol code EA1/026/20, approved on 26 May 2020). For the measurements of samples based on spiked synovial fluid, a pre-dilution step was done to be able to deal with the high viscosity of the sample material. For samples containing alpha-defensin or calprotectin, a 1:100 dilution with washing solution was performed (5 μl spiked synovial fluid + 495 μl WL), whereas for samples containing IL-6 only a 1:10 dilution was used (50 μl spiked synovial fluid + 450 μl WL). All these dilutions were done in a GENSPEED dropper bottle before a special GENSPEED filter dropper was applied to it. Dropping the sample mix through this filter dropper allowed the removal of inhibiting components of the sample material such as erythrocytes or fibres that could negatively affect the flow rate of the sample through the reaction channel of the test chip. Further processing of the filtered sample material was done as described for buffer-based samples. Cut-off values of 1.56 μg/ml, 50 μg/ml, and 0.031 μg/ml for alpha-defensin, calprotectin, and IL-6, respectively, were extrapolated from literature data as optimal cut-offs for the diagnosis of PJI and, consequently, were used for comparison.[20–22]

Statistical analysis

Mean values and SDs for quantification of limit of blank (LoB) and limit of detection (LoD) were determined. Measure triplicates and respective mean values were considered. Also, the use of five parameter logistic regression for fitting and recalculation of concentrations was employed for statistical comparison in immunoassays. For the evaluation of the biomarker-specific performance of the system, calibration curves were generated using five parameter logistic regression

Fig. 3

Buffer-based dilution series experiment for human alpha-defensin, calprotectin, and interleukin-6 (IL-6) diluted using GENSPEED washing solution measured with multiplex GENSPEED test chips. The figure displays the chemiluminescent signal intensities (AU) across various biomarker concentrations. The data represent mean values from three measurements: a) alpha-defensin; b) calprotectin; c) IL-6; and d) five parameters logistic (PL) regression. PBS, phosphate-buffered saline.

methods.[23] LoB and LoD were calculated using the following formulae:

$$LoB = \o_{blank} + 1.645 \cdot \sigma_{blank}$$

$$LoD = LoB + 1.645 \cdot \sigma_{low\ conc}$$

Statistical analyses and graphics were conducted using Microsoft Excel for Microsoft 365 (Version 2402; Microsoft, USA), alongside the MyCurveFit Excel Add-in (Version 1.0.415.1001 (210304); MyAssays, UK).

Results

A three-plex µ-ELISA based technology for the simultaneous measurement of three biomarkers (alpha-defensin, calprotectin, and IL-6) has been developed in a model buffer system as well as in spiked human synovial fluid sample. A multiplex immunoassay microfluidic test chip has been designed to achieve a rapid and point-of-care result. First, LoD was determined for each individual biomarker by means of calibration curves with serial dilutions in a buffer-based system. Different concentrations of the three biomarkers are shown in Table II. The concentrations of detection antibody and capture antibody were 2 µg/ml and 0.1 mg/ml, respectively.

LoD of 0.008 µg/ml, 0.002 µg/ml, and 0.00014 µg/ml was determined for alpha-defensin, calprotectin, and IL-6, respectively. All measurements were performed in triplicates. The mean values of all three test results are shown in Figure 3.

To assess the performance of this µ-ELISA assay, the experiment was reproduced using spiked human synovial fluid, collected from patients without joint infection. Then, predetermined concentrations of individual biomarker were added (Table II). LoD for alpha-defensin was 0.08 µg/ml and therefore clearly below 1.56 µg/ml, which is an important cut-off threshold for this marker. The same was shown for calprotectin with a LoD of 0.31 µg/ml and a cut-off threshold of 50 µg/ml, as well as for IL-6 with a LoD of 0.004 µg/ml and a cut-off threshold of 0.031 µg/ml. Considering the mean signal intensities of all three replicates, calibration curves for each marker in synovial fluid have been generated as described in the Methods section. The detection ranges are indicated with correspondingly coloured arrows in Figure 4d.

The mean coefficient of variation (CV) values for alpha-defensin, calprotectin, and IL-6 were 10%, 6%, and 5%, respectively (Table IV).

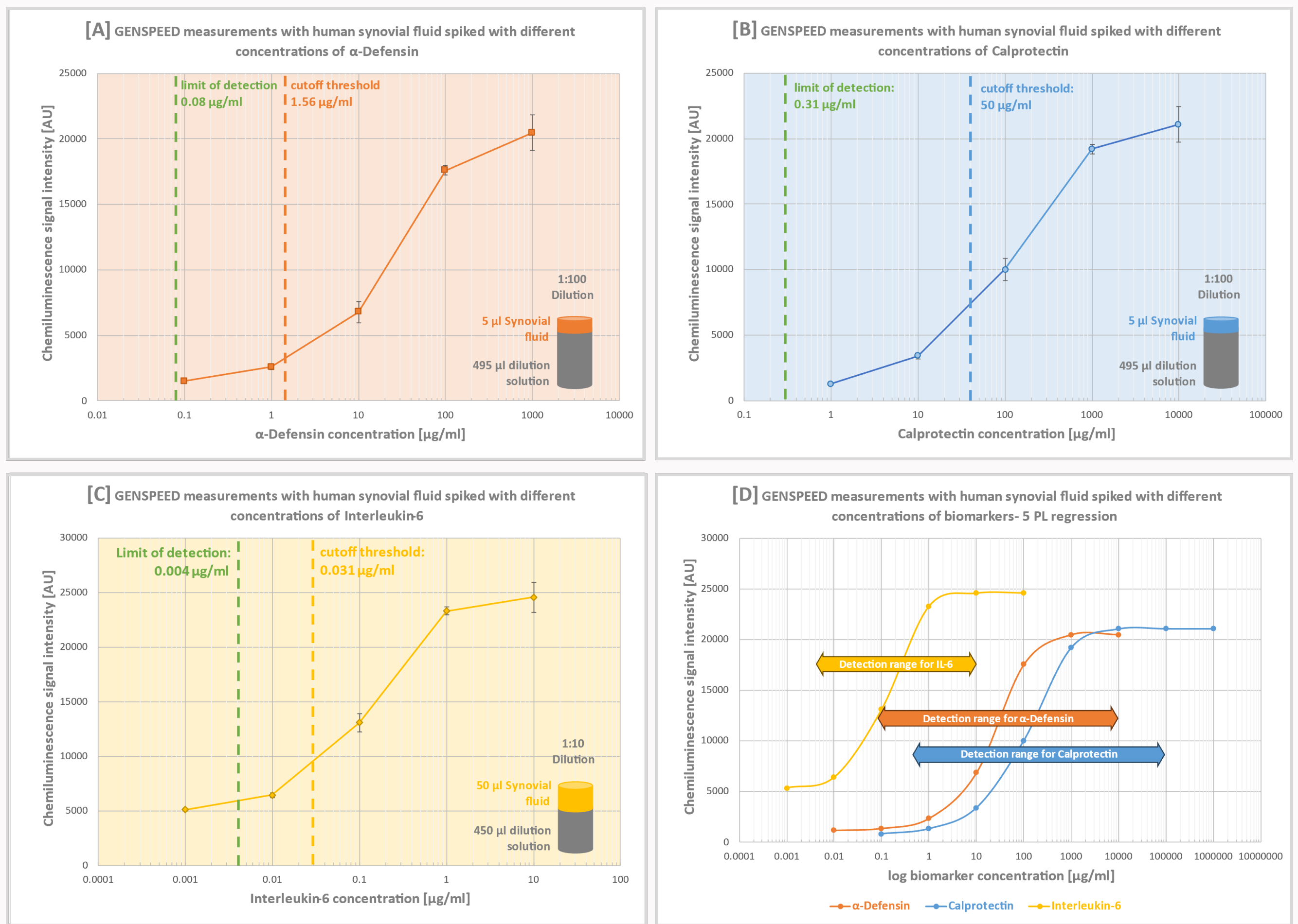

Fig. 4

Dilution series experiments on human synovial fluid spiked with alpha-defension (α-defensin), calprotectin, and interleukin-6 (IL-6) were conducted using multiplex GENSPEED test chips. α-defensin and calprotectin were diluted 1/100, while IL-6 was diluted 1/10 with GENSPEED washing solution. The figure shows chemiluminescent signal intensities (AU) for each biomarker, with all measurements done in triplicates. It includes the limit of detection (LoD) (green line) and a clinically relevant cut-off for periprosthetic joint infection (PJI). Data represent mean values from three tests: a) α-defensin; b) calprotectin; c) interleukin-6; and d) five parameters logistic (PL) regression.

Table IV. Summary of key results evaluating the performance of the multiplex micro-enzyme-linked immunosorbent assay (μ-ELISA) for the simultaneous detection and quantification of alpha-defensin, calprotectin, and interleukin-6 in spiked synovial fluids.

Parameter	PJI-specific cut-off values	Limit of detection	CV*
Alpha-defensin	1.56 μg/ml[22]	0.08 μg/ml†	10%
Calprotectin	50 μg/ml[23]	0.31 μg/ml‡	6%
IL-6	0.031 μg/ml[24]	0.004 μg/ml	5%

*Mean value of all samples.
†Detection limit Synovasure Lateral Flow Test for alpha-defensin detection: 11.7 μg/ml (for comparison).[24]
‡Detection limit Lyfstone Lateral Flow Test for calprotectin detection: 14 μg/ml (for comparison).[25]
CV, coefficient of variation; IL-6, interleukin-6; PJI, periprosthetic joint infection.

Discussion

For accurate diagnosis of PJI, a combination of conventional (total white blood cell count, polymorphonuclear percentage)[26] and emerging synovial fluid biomarkers (such as alpha-defensin or calprotectin) together with microbiological, histopathological, and molecular examinations is required. Despite the combination of multiple parameters, however, the diagnosis remains sometimes inconclusive. Alpha-defensin is the only biomarker included in the 2018 Philadelphia International Consensus Meeting (ICM) and 2021 European Bone and Joint Infection Society (EBJIS) diagnostic algorithms. Therefore, it was included in our panel. In 2019, the USA Food and Drug Administration (FDA) approved the detection of alpha-defensin in synovial fluid using the alpha-defensin lateral flow assay (Synovasure; Zimmer Biomet, USA) as the first biomarker for the diagnosis of PJI.[27] However, a previous meta-analysis has shown that alpha-defensin ELISA immunoassay had superior overall diagnostic value compared with the lateral flow test (area under the curve, 0.98 vs 0.75) with higher sensitivity (96% (90% to 98%) vs 71% (55% to 83%), $p < 0.001$), but no significant difference in specificity (96% (93% to 97%) vs 90% (81% to 95%), $p = 0.060$).[28] The

immunoenzymatic assay was optimized to operate at a cut-off value of 5.2 mg/l, with a lower detection limit of 1.56 μg/ml.[20] Although this cut-off has been used in several studies, it has not been officially recognized.[29] A recent paper has proposed a high-performance liquid chromatography-mass spectrometry (LC-MS) method for the detection of alpha-defensin in synovial fluid, with good results in terms of accuracy. However, the routine use of this promising method is limited by the fact that it is only available in highly specialized laboratories and is time-consuming.[30]

Furthermore, previous published data have shown that the performance of this biomarker may be influenced by the clinical definition of PJI used, with a poorer consistency with EBJIS criteria compared to MusculoSkeletal Infection Society (MSIS).[15,16] Consequently, regardless of the type of technique used (immunoenzymatic assay, lateral flow, or LC-MS), the use of alpha-defensin as a single marker for the diagnosis of PJI should be discouraged.[31–33] Regarding other biomarkers, IL-6 and calprotectin, the choice of these molecules was based on a recent review by Fisher and Patel,[9] which ranks these three markers among the five most promising in the field of PJI.

Synovial fluid pro-inflammatory cytokines have a key role in PJI, since they trigger inflammation in response to an infectious process. Among them, IL-6 has been quoted as promising with sensitivity and specificity ranging from 62.5% to 90% and 85.7% to 94.7% in several papers, with different cut-offs (2,100 pg/ml vs 30,750 pg/ml).[22,34] According to Lenski and Scherer,[22] synovial IL-6 lower than 10,000 pg/ml completely excludes the diagnosis of PJI, while IL-6 levels below 49,000 pg/ml strongly suggests it. However, when between 10,000 and 49,000 pg/ml, the suspicion of PJI remains uncertain, and the simultaneous detection of other markers remains strongly recommended to settle the dilemma of septic versus aseptic inflammation.[22] Furthermore, the fact that standard IL-6 assays are designed to assess cytokine levels in serum rather than synovial fluid should be emphasized. Because of this, it is essential that future studies include IL-6 tests, whose consistency and effectiveness have been previously shown in a synovial biomarker investigation.[34] Regarding inflammatory proteins, calprotectin has recently been successfully investigated in PJI.[35]

Different laboratory techniques have been studied to detect calprotectin in synovial fluid, including ELISA, lateral flow tests, and immunoturbidimetric immunoassays.[21,36,37] A recent meta-analysis performed on eight studies with a sample size of 618 patients showed an outstanding cumulative diagnostic accuracy for PJI, with a sensitivity of 92% and specificity of 93% and a pooled positive and negative likelihood ratio of 9.91 and 0.07, respectively.[38] However, a separate analysis of the single papers revealed a wide discrepancy according to joint site (hip, knee, shoulder prostheses), criteria used for the diagnosis of PJI (ICM 2018, MSIS), laboratory method (ELISA, immunochromatographic assay, immunoturbidimetric), and related cut-offs. Although five of the included studies used 50 μg/ml as the best cut-off using either ELISA,[36,39] lateral flow immunoassay,[40,41] or both,[21] a standardized threshold for calprotectin in the context of PJI has not yet been universally recognized, as well as for the previous biomarkers. As more and more synovial fluid biomarkers have been discovered in recent years, it is becoming increasingly necessary to detect multiple biomarkers from a single sample in a single assay to get appropriate or conclusive findings. For this reason, multipanel technologies are becoming crucial in modern clinical diagnosis, and they are expected to improve the efficacy and accuracy of PJI detection. Therefore, the real challenge would lie in not only developing multiplex biomarker tests that can accurately and consistently distinguish between PJI and aseptic failures, but also in enabling their application in point of care. The most often used point-of-care methods for obtaining quick and 'on-site' results are paper-based diagnostic devices because of their versatility, sample manufacturing, visual interpretation of the result, affordability, and ease of transport. The presented assay represents an extremely promising platform since it offers a simultaneous measurement of three of the most promising biomarkers in the diagnosis of PJI, using only a minimal amount of synovial fluid (5 to 50 μl). The microfluidic architecture of the test chip provides a significant advantage in terms of time requirement for the analytical process. The capillary force-driven fluid flow of sample and reagents through the reaction channel, facilitated by the low height of the reaction channel (only 100 μm) which induces shear forces, reduces the time required for diffusion of the involved components and increases the likelihood of biomolecular interactions between the analytes and the immobilized capture probes as they pass through the channel.[42] In contrast to laboratory-based ELISAs with turnaround times of two to four hours, this microfluidics-based platform allows for assay procedures to be completed within only 15 to 22 minutes and without employing trained laboratory personnel. Furthermore, the results obtained in the experimental model with spiked synovial fluid were extremely promising, since LoDs far lower than PJI-specific thresholds of other assays normally used in clinical practice were detected (Table IV). In summary, LoD for alpha-defensin was 0.08 μg/ml, thus considerably lower than 1.56 μg/ml. The same was shown for calprotectin with a LoD of 0.31 μg/ml and a cut-off threshold of 50 μg/ml, as well as for IL-6 with a LoD of 0.004 μg/ml and a cut-off threshold of 0.031 μg/ml.

Similar to other studies carried out under experimental conditions, the main limitation of this in vitro study is that it is based on spiked synovial fluid, in which known and progressive concentrations of individual biomarkers were added to mimic as closely as possible the joint environment during infection/inflammation. However, of course, the biomarker levels can be different in real patient samples, as a reason why clinical studies are obligatory to finally validate this assay.

In conclusion, the described multiplex μ-ELISA system considerably outperforms standard lateral flow point-of-care tests regarding multiplexing capabilities in terms of assay quality aspects. We therefore believe that the unique features of simultaneous measurement of the three most promising synovial biomarkers in PJI diagnosis using minimal sample volume, lower LoDs compared to conventional tests, and speed of processing can give the novel multiplex μ-ELISA platform, used in this study, great diagnostic potential as a point-of-care tool to help the clinician gain a broader picture for characterizing synovial fluids from PJI/aseptic failure doubtful cases.

However, while the system demonstrates promising benefits, it is important to acknowledge that clinical studies

are necessary to confirm its effectiveness and validate its practical use in real-world clinical settings.

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Data sharing

The data that support the findings for this study are available to other researchers from the corresponding author upon reasonable request.

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Ethical review statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Ethics Committee of Charité – Universitätsmedizin Berlin, Germany (protocol code EA1/026/20, approved on 26 May 2020).

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