Diagnostic Utility of a Novel Point-of-Care Test of Calprotectin for Revision Total Knee Arthroplasty

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STUDY PROTOCOL

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I. Synopsis

Title	Diagnostic Utility of a Novel Point-of-Care Test of Calprotectin for Revision Total Knee Arthroplasty
Sponsor	Orthogenics
Sites	Cleveland Clinic Main Campus (Cleveland, OH) and Cleveland Clinic Florida (Weston, FL)
Objectives	To investigate whether there are quantifiable differences in the level of calprotectin in the synovial fluid that allow separation of different modes of joint implant failure (e.g. infected, aseptic loosening). A subset of primary TKA patients (with history of OA) will be included as a baseline.
Design	Prospective clinical trial involving patients undergoing primary or revision TKA.
Test Product	Calprotectin POC test is a lateral flow device that detects calprotectin, a marker of inflammation, in the synovial fluid.
Number of Patients	N=30 primary TKA N=120 revision TKA (of which, n=70 aseptic and n=50 septic)
Procedure	Synovial fluid will be collected pre-operatively or intra-operatively from patients meeting the inclusion/exclusion criteria, and will be tested for infection using the Calprotectin POC test, which will be validated against then CALPRO Calprotectin ELISA test.
Statistical Methods	Diagnostic parameters of Calprotectin POC test will be assessed using the MSIS criteria as the gold standard.

II. Background

Total knee arthroplasty (TKA) is considered to be a very successful procedure which has changed treatment of severe degenerative joint disease, improving the quality of life of millions[1,2]. However, infection of these implants, called periprosthetic joint infection (PJI), is a serious complication of arthroplasty[3]. PJI can lead to additional surgeries, revision, fusion, and other adverse events. It is important to accurately diagnose PJI, because its management differs from that of other causes of arthroplasty failure.

Unfortunately, no single test or clinical sign is currently available to diagnose infection. The classic signs of infection such as redness, warmth, swelling and pain can result from a variety of septic and aseptic etiologies. Additionally, some cases of PJI, especially those caused by indolent organisms, are often associated with relatively little redness, warmth, swelling and pain. Though rare, the only clinical sign that has been strongly associated with PJI is a sinus tract leading from the skin to the joint. Although clinical signs and symptoms should alert a clinician to the possibility of infection, there is general agreement in the field that they are not useful in making a definitive diagnosis. Therefore, a combination of laboratory testing techniques has guided the diagnostic decision-making related to PJI[4–6].

Recognizing the limitations of any one test for diagnosing PJI, the Musculoskeletal Infection Society (MSIS) has generated a criteria-based definition of PJI for diagnosing PJI[6]. This criteria-based definition of PJI places emphasis on culture techniques that identify pathogens. However, because the diagnostic sensitivity of cultures is about 70%[7,8], and because tissue culture results from intraoperative samples are only available after surgery, the MSIS definition also includes serological and synovial inflammatory markers that can be used to diagnose PJI. A major disadvantage of the MSIS criteria-based definition of PJI is that two of the essential criteria, tissue culture and histological analysis, are only available after surgery. In cases where the surgical samples are critical for the diagnosis of infection, the physician may need to adjust his or her treatment post operatively for the patient based on the treatment provided. The availability of a preoperative test that correlates well with the MSIS criteria could improve patient care. Although the currently available pre-operative tests have been shown to be useful, these tests often require laboratory expertise, and the results may not be available in a timely fashion. Therefore, there has been growing interest in point of care (POC) test for the diagnosis of infection.

Currently, some of the POC tests available include the alpha defensin and leucocyte esterase (LE) tests. Both these test measure markers of infection present in the synovial fluid. The LE test, though cheap and easy to perform, is easily affected by the presence of blood in the synovial fluid[9,10]. Although centrifugation of the bloody samples might improve the accuracy of the LE test, it is cumbersome, and not easily available. As a result, a large number of bloody samples are

unable to be analyzed by the LE test. The alpha-defensin lateral flow device is another promising POC test which has sensitivity of about 70%[11]. Although the clinical results of this are promising, they are not as good as previous studies using alpha-defensin levels measured in a laboratory[11]. Moreover, there are concerns that the alpha defensing test will yield false positive results in cases of metal on metal revisions[12]. Therefore, better tests are required.

III. Rationale

Calprotectin is a biomarker closely associated with leucocytes in general, and is present in high volumes in neutrophil cells[13–15] . Calprotectin is also produced by infiltrating monocytes and macrophages, where calprotectin is released upon phagocytosis[16].In neutrophils, calprotectin is stored intracellularly and are released upon activation of the cell. The determination of number of neutrophils and proportion of neutrophils out of total number of inflammatory cells is a diagnostic strategy commonly used in diagnosis of infection[6,17]. Upon encounter with a pathogen, neutrophils have several strategies to fight infections , and produce high-levels of calprotectin [13,18]. Activation of neutrophils, and release of calprotectin, can be for any reason causing activation of the complement system and aseptic Inflammatory responses[13]. Moreover, Calprotectin is a danger associated molecular patterns (DAMP) signal influencing the inflammatory responses[14]. The level of activated neutrophils in PJI provide basis of the presence of calprotectin in the synovial fluid of PJI patients, and thus, for calprotectin as a potential biomarker for PJI. Calprotectin-levels in the synovial fluid do not merely reflect the level of leucocytes and neutrophils present in the synovial fluid, but levels are correlated to the WBC content. Calprotectin is likely to reflect the number of activated cells and surpass the diagnostic accuracy of total WBC counts and neutrophil percentage for PJI diagnosis.

Wouthuyzen-Bakker et al. [19] demonstrated that a level of calprotectin of 50 mg/L in the synovial fluid has very good diagnostic accuracy for PJI, supported by area under the curve values of more than 0.9. This break point produced a negative predictive value (NPV) of 95% for all 42 patients in the study. In a subgroup analysis for patients with chronic PJI, a NPV of 97% was observed[19]. The excellent NPV may assist in the orthopaedic clinic to rule out the presence of infection and consider diagnostic alternatives for aseptic loosening and pain revision of the joint patient. A rapid and accurate distinction between these two causes is important as PJI and aseptic loosening are managed differently with regards to surgical Intervention and follow-up.

Point of Care Test diagnostics by lateral flow devices provides reliable test results within minutes of sample collection. Currently, Calprotectin can be detected by such lateral flow devices (developed by Orthogenics, Tromsø, Norway). The speed and ease of use of this test allows for diagnosis at patient’s bed side. These tests can be applied in the physician's office,

operating room, an ambulance, the home, the field, or in the hospital. As the results are timely they allow rapid diagnostic and identifies treatment alternatives for the patient. This technology empowers clinicians to make decisions at the "point-of-care", and can have significant impact on health care delivery and ability to address challenges of health disparities. However, it is important to validate the diagnostic utility of calprotectin POC in a diverse set of patients undergoing revision arthroplasty.

IV. Hypothesis

The study hypothesis is that the use of Calprotectin POC test will help differentiate between septic and aseptic failure following TKA.

V. Aims

Compare levels of calprotectin in these different patient groups:

- septic revisions v aseptic loosening
- aseptic loosening v other non-infected failure modes
- primary knees stratified by OA grade (or simply for baseline comparison?)

VI. Study Setting

This will be a prospective clinical trial. Patients undergoing total knee arthroplasty will be eligible to participate in this study. The clinical trial will be conducted with samples collected at a Cleveland Clinic Main Campus (Cleveland, OH) and Cleveland Clinic Florida (Weston, FL) with high volumes of revision surgery. The goal is to enroll n=30 primary TKA patients, n=70 aseptic revision TKA patients, and n=50 septic revision TKA patients.

VII. Eligibility Criteria

Subject Inclusion Criteria

- Subject is ≥18 years of age.
- Patient with a diagnosis of OA (for primary TKA only)
- Subject has had no recent injections or surgeries of the joint (within past 6 weeks).
- Subject has or will have all of the medical tests required to allow MSIS classification (revision cases only)

- Subject signs informed consent form. English Speaking

Subject Exclusion Criteria

- MSIS criteria labs no older than 60 days (revision cases only)
- Results are not available for medical tests required to perform MSIS classification (revision cases only)
- Sample was obtained via lavage
- Quantity not sufficient (at least 1 ml required).

VIII. Study Procedure

Synovial fluid samples will be collected from the affected joint either during pre-operative work-up or intraoperatively. Sample will be transported to testing site. Once the sample is received by the laboratory or research personnel, fluid will be first aliquoted for use in the physician-ordered diagnostic laboratory testing. The remainder of the specimen will be used for Calprotectin testing using the testing protocol described by Orthogenics. The specimen for the MSIS criteria labs need not be collected at the same time as specimen used for the Calprotectin POC test. In brief, the Calprotectin POC testing technology utilizes the natural occurring liquid movement through membranes, and by combining this with lines of specific antibodies and nanoparticles (gold or latex), biomarker detection in the fluid can be achieved. When Calprotectin is present in the fluid, a test line will form together with a control line that demonstrate the validity of the test. The test will be read as: green (no inflammation), yellow (moderate inflammation) or red (massive inflammation). This will then be validated by comparison against the CALPRO Calprotectin ELISA test. ELISA samples will be diluted 20x for a primary TKA sample and 1000x for a revision TKA sample.

The septic revision cases will be defined using MSIS criteria. All non-infected revision patients are considered as aseptic cases. MSIS criteria-defined PJI diagnosis will be determined by an independent three-physician adjudication panel with expertise in infection who have access to all the necessary patient data for clinical diagnosis (eg, all MSIS criteria and patient history). The adjudication panel will be blinded to the results of the Calprotectin POC test. There are two major criteria, and five minor criteria in the MSIS definition of PJI. The existence of one positive major criterion is sufficient for the diagnosis of PJI. Similarly, the existence of any three of the five positive minor criteria is considered sufficient for the diagnosis of PJI. Table 1 presents the MSIS criteria based definition of PJI.

Table 1: MSIS Workgroup Standard Definition for PJI [6]

One of the following must be met for diagnosis of PJI.

1. A sinus tract communicating with the prosthesis (major criteria);
A pathogen is isolated by culture from two separate tissue or fluid samples obtained from the affected prosthetic joint (major criteria);
3. Three of the following five minor criteria exist: a. Elevated ESR and CRP (ESR≥30mm/hr; CRP≥10mg/L) b. Elevated synovial fluid WBC count (≥ 3000) c. Elevated synovial fluid neutrophil percentage (≥80%) d. Isolation of a microorganism in one periprosthetic tissue or fluid culture Greater than 5 neutrophils per high-powered field in 5 high-power fields observed from histological analysis of periprosthetic tissue at 400 times magnification

All samples will be classified based on the MSIS criteria into positive (infected) or negative (not infected). The MSIS criteria will be considered as the gold-standard for diagnosis.

The interpretation of Calprotectin POC test will be made in a quantitative way by classifying all patients into a positive or a negative test. A positive test will be the presence of yellow or red line. This is expected to improve the sensitivity as Calprotectin POC test is intended to use as a screening tool. However, additional analysis will be performed for different thresholds.

The aseptic revision cases will be identified first by gross loosening on xray. If no loosening evident, a bone scan may be performed.

IX. Stability Experiment

Stability Experiment Protocol

Stability Definition	The length of time an analyte can be stored at a specific condition without a significant change in value and the temperature a specimen with the analyte can be stored at a specific condition without a significant change in value. This is an important quality indicator for sample acceptability and result interpretation after specimen storage.
Experiment Overview	Four temperature conditions will be tested (room temperature, refrigerated, frozen and freeze/thaw cycles) over a designated period of time on at least three (3) calprotectin levels, spanning the device range of 14-300 mg/L. Each specimen will be tested in triplicate for each condition and at baseline and calprotectin will be measured with the Lyfstone Rapid Test.
Experiment Details	1. Create at least three samples with concentrations in the Low, Mid and High ranges of the calprotectin test. These samples will be prepared using one or more samples with high calprotectin concentrations. The high sample(s) will be leftover from de-identified patient(s) enrolled in his study after the test is

performed per the study protocol. The high sample (s) will be diluted as needed to reach the target concentrations using never frozen leftover synovial fluid from the clinical laboratory as the diluent. The use of leftover de-identified synovial fluids was approved by the section head of the laboratory section. Up to 40 mLs synovial fluid will be used.

2. The following conditions will be tested for each sample in triplicate:
    a. Time Zero (T0), baseline result
    b. Room Temperature (RT), room temperature near 25°C, tested at two (2), four (4) and eight (8) hour intervals
    c. Refrigerated (REF) (2-8°C), tested at ten (10) and twenty-four (24) hour intervals
    d. Frozen (F) (-90 to -70°C) with two (2) freeze thaw cycles. Freeze thaw cycle defined as: Samples will be completely frozen then removed to RT and completely thawed. Upon sample being thawed, one set will be tested (1X) and the other sample will be placed back in the freezer to completely freeze. Sample will then be removed from freezer to RT to completely thaw and be tested for analyte (2X)
3. Assay specimens for Calprotectin concentrations, obtain both qualitative and quantitative results, in triplicate following the IFU instructions.

Notes: All raw data must be saved. Record performance time of assay, stability condition of specimen, and qualitative and quantitative values documented. Residual synovial fluid specimens will be used as matrix for this experiment to dilute a known high value of calprotectin from already tested knee synovial fluid samples as described above. The standard calprotectin (64.25 mg/L CALPRO AS, Norway) may also be spiked as needed to obtain the target concentrations.

Data

- Qualitative agreement: After storage, the qualitative result is in agreement with the baseline (T0) result for all samples.
- Quantitative agreement: Samples will be analyzed for % bias from baseline (T0) using the following equation

$$\%Bias = \frac{(Initial\ Average\ condition\ (XX)\ result - T0\ condition)}{T0\ condition} x100$$

- Samples are considered stable if the value is within ±30% bias; larger bias will be investigated to rule out experimental issues. Due to the higher imprecision observed in previous experiments, this target may be revisited after obtaining the data.
- Analyze data using a statistical software.

Notes This experiment represents the minimum verification necessary, and the medical director may require additional steps. An open conversation should be maintained during the entire verification.

X. Data Collection

Data will be collected preoperatively, and during surgery or the hospital stay. All data will be entered and maintained in REDCap, an electronic data capture tools hosted at Cleveland Clinic[20]. REDCap (Research Electronic Data Capture) is a secure, web-based application designed to support data capture for research studies, providing 1) an intuitive interface for validated data entry; 2) audit trails for tracking data manipulation and export procedures; 3) automated export procedures for seamless data downloads to common statistical packages; and 4) procedures for importing data from external sources.

The following will be collected on all patients in the study:

1. Demographics –MRN, Age, Gender, BMI, Race, Smoking status, Joint type, current anti-inflammatory medications, history of inflammatory disease, ongoing antibiotic therapy, history of infectious disease
2. Comorbidities – Charlson or Elixhauser cormorbidity index
3. Surgical history- Previous joint replacement related surgeries, severe synovitis status
4. Laboratory values- ESR/CRP, WBC, RBC, PMN%, LE(optional), Culture, Histology, hemolysis index, synovasure (alpha defensin) test when available,
5. Imaging – Xrays and bone scans (if available)
6. Calprotectin results (POC and ELISA result)

Schedule of Events

Evaluation	Screening*	Aspiration	TKA
Demographics	X		

Medical/Surgical history	X		
Sample screening		X	X
Introp data collection			X
Laboratory details		X	
Calprotectin ELISA and POC	X		
CRP and ESR	X		
Cell counts	X		
Cultures	X		
Histology	X		
Leukocyte esterase	X		
Hemolysis index	X		
Radiographic details			
Xrays	X		
Bone scan (when appropriate)	X		

* from timepoint closest to surgery

XI. Analysis

A. Statistical analysis

Descriptive statistics will be generated for demographics and medical history. Categorical variables will be summarized by frequency counts and percentages. Continuous variables will be summarized by number of observations (N), mean, standard deviation (SD), median, minimum and maximum, and 95% confidence intervals (CIs).

For testing of diagnostic utility, MSIS criteria will be used at the gold-standard for PJI. The following diagnostic parameters will be evaluated: sensitivity, specificity, positive predictive value, negative predictive value and accuracy. Additionally, receiver operating characteristics curves will be plotted. Calprotectin test will be considered to be positive when there is at least moderate or massive inflammation (i.e. yellow or red). The diagnostic parameters will also be evaluated considering only massive inflammation (red) as positive.

The Calprotectin test will be validated by comparison to the CALPRO Calprotectin ELISA test using established diagnostic values from Lyfstone.

Subgroup analysis will be performed based on the infecting organism type, joint, and presence of inflammatory conditions.

XII. Data Safety

The sample obtained from the patient will be stored at the testing site at -80°. At the end of the study, the samples will be transported to the site of principal investigator. Only members of the study team (i.e. the personnel listed on the IRB application) will have access to protected health information of patients included in this study. All data procurement in this research project will utilize the standard of care follow-up visits to collect study data. Only secure computers will be used on main campus, all will be password protected. Only investigators approved by the IRB will be allowed access to this data. The data will be maintained in the patient's medical records, unaltered from its form prior to the study.

XIII. Co-enrollment

Patients from IRB 16-549 may be co-enrolled in this trial. As this trial is observational no special consideration need to be made for recruitment and enrollment. Patients will be identified independently using a search of scheduled cases on EPIC by the clinical research fellow who is running each individual study. Patients who are eligible for both studies will then be contacted by phone several days before surgery to go over the consent form and explain the trial for each study independently. Patients will also be sent a copy of the consent form for the given trial for their review via mail or email, based on their preference. Patients will then formally consented with all signatures necessary obtained on the day of surgery for each study independently using a consent form for each study.

XIV. References

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