

UNIVERSITÀ DEGLI STUDI DI MILANO  
SCUOLA DI DOTTORATO  
*MEDICINA SPERIMENTALE E BIOTECNOLOGIE MEDICHE*  
DIPARTIMENTO  
*Biotecnologie Mediche e Medicina Traslazionale*  
CURRICULUM / CORSO DI DOTTORATO  
R22 - XXIX° CICLO

TESI DI DOTTORATO DI RICERCA

New Biomarkers in the Clinical Process of Prosthetic Joint Infection

sigla del settore/i scientifico disciplinare/i  
MED/05 - Patologia Clinica

Monica Gioia MARAZZI  
R10436

TUTOR:  
Chiarissimo Prof. Massimiliano M. CORSI ROMANELLI

Co-TUTOR:  
Dottoressa Emanuela Rita GALLIERA

COORDINATORE DEL DOTTORATO:  
Chiarissimo Prof. Massimo LOCATI

A.A.  
2015/2016

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*File attached #2: Soluble urokinase-type plasminogen activator receptor (suPAR) as new biomarker of the prosthetic joint infection: correlation with inflammatory cytokines.*

## **Abstract**

Background: Post-operative prosthetic joint infection (PJI) is the most common cause of failure of total joint arthroplasty, requiring revision surgery, but a gold standard for the diagnosis and the consequent treatment of PJI is still lacking. Among the scenario of infections diagnosis, an emerging molecule is Presepsin, the soluble fraction of CD14, recently described as a powerful diagnostic tool, not only to detect sepsis but also to discriminate different grade of sepsis severity.

Question / purpose: We asked:1) is Presepsin a good diagnostic marker for PJI? 2) Does Presepsin correlate with other emerging infection markers such as TREM-1, CD-163, SuPAR, OPN and MMP-9, sTLR2 and CCL2 and IL-6?3) Is Presepsin a good prognostic marker of PJI?

Methods: The population of 100 selected patients undergoing prosthesis revision was enrolled and subdivided into two groups: 48 patients having bacterial infection and 52 patients with no infection, undergoing aseptic loosening of the implant. CRP was measured using immunoturbidimetry on an automated biochemical analyzer (Olympus CRP-Latex assay, Central Valley, PA, CA, USA) Serum IL-6, TREM-1, CCL2, MMP-9, CD-163 and OPN were measured using an ELISA sandwich Quantikine Assay, while TLR2 was measured using an ELISA Duo Set Assay, according to manufacturer protocol (R&D System, Minneapolis, MN, USA). SuPAR was measured by SuPARnostic ELISA Assay, according to manufacturer protocol (Virogates, Denmark). Presepsin plasmatic levels were measured by Presepsin/ PATHFAST according to manufacturers' protocol (Mitsubishi Chemical). PATHFAST Presepsin is a chemiluminescent enzyme immune assay (CLEIA) for the quantitative measurement of Presepsin concentration in whole blood or plasma.

Results: Presepsin has a greater diagnostic value than CRP and IL-6 in the diagnosis of PJI, as assessed by ROC CURVE analysis. OPN, CCL2, TLR2 and SuPAR displayed good diagnostic values in PJI, while CD163, TREM-1 and MMP-9 displayed very low diagnostic potential. Presepsin, as well as OPN, CCL2, SuPAR and TLR2, displayed a significant decrease at the increasing time of recovery

after surgery in PJI patients, while it remains unaltered and significantly lower ( $p < 0.0001$ ) in the patients undergone to revision surgery for aseptic loosening of the implant. These results clearly indicate that Presepsin have a good prognostic value, because it decreases gradually as the inflammatory process in response to the prosthetic infection is resolved.

Conclusions: Taken together, these results indicate that Presepsin can be considered a useful tool for the diagnosis and clinical monitoring of PJI, and it can also be supported by a panel of new inflammatory makers involved in monocyte/macrophage mediated inflammatory response such as OPN, CCL2, TLR2 and SuPAR.

## **1. Introduction: Definition of Prosthetic joint infection (PJI)**

Pathogen infection is one of the main adverse events of surgical procedures, which can lead to an excess of systemic inflammatory response and sepsis, causing multi organ failure and septic shock. In particular, in orthopedic field, one of the most challenging complication associated with surgical procedures is periprosthetic joint infection (PJI).

PJI is the most common cause of failure of total joint arthroplasty, requiring revision surgery.

PJI is mainly due to Gram-positive bacteria, in particular, *Staphylococcus Aureus*, and more rarely by Gram-negative bacteria such as *Pseudomonas Aeruginosa*. [1,2]

A small percentage of hip (1.7% - 3.2%) and knee (2.5%-5.6%) prosthetic joint replacement surgery undergoes prosthetic infection, therefore an appropriate recognition and clinical approach are essential to preserve or re-establish adequate function and limit excess morbidity.

The reasons of the failure include aseptic loosening at the bone-cement surface, periprosthetic fracture, fracture of the prosthetic device itself, wear, implant malposition, dislocation-instabilities, hard material and Prosthetic Joint Infection.

Prosthetic joint infection (PJI), also known as periprosthetic infection, is defined as infection involving the articulation of the prosthesis and adjoining tissue. Advances in the understanding of the epidemiology, diagnosis, management and prevention of PJI over the past decades have led to an improvement in the resolution of this challenging infection.

It's extremely important to identify periprosthetic joint infection as early as possible, in order to start promptly antibiotic treatment and, in worse case, program a surgical revision arthroplasty.

Different definition of PJI have been proposed so far, but at the moment there is not a uniform definition. The diagnosis of PJI is currently based on several criteria provided by Musculoskeletal Infection Society (MSIS), in 2011.[1,3]

## **1.1 PJI Diagnosis**

The diagnosis of PJI is difficult because the clinical presentation is various in the patients and at the moment a single gold standard able to identify Periprosthetic Joint Infection is still lacking.

Currently the diagnosis of PJI is based on proposed criteria consensus by Musculoskeletal Infection Society (2011) and Philadelphia accord on the PJI determination (2013).

PJI exist when the following major criteria is present:

*“isolation of pathogens by culture from two or more separate tissue or fluid samples obtained from the affected prosthetic joint”*

Or when four of the sequent minor criteria are present:

1. Elevated serum erythrocyte sedimentation rate (ESR),
2. Elevate serum C-reactive protein (CRP) concentration,
3. Elevated synovial white Blood Cells (WBC) count,
4. Elevated synovial neutrophil percentage (PMN%),
5. Purulence in the affected joint,
6. Higher than five neutrophils for high-power field in five high-power fields analyse from histologic section of periprosthetic tissue at ×400 magnification. [3,4]

Currently the PJI identification is based on: clinical manifestation, microbiological test, histopathological analysis and imagining tools, but this types of investigation are invasive tests for the patients.

Moreover, microbiological culture tests often provide false negative results.

In addition to these inclusion criteria, serum parameters can also be evaluated, in particular inflammatory markers (Procalcitonin, C reactive protein and IL-6).

Recent evidences indicated that IL-6 and CRP are useful in the diagnosis of PJI, while Procalcitonin had not a great diagnostic value in PJI. [5]

For this reason, in order to optimize the diagnostic process, infection biomarkers with fast response and high sensitivity and specificity for PJI Infection are needed.

## **2. Ideal Biomarker in PJI Diagnosis**

Currently, a large number of test are available for PJI diagnosis, ranging from hematological markers of infection and inflammation, intraoperative culture and histology analysis, and many can be used in combination. Nevertheless, there is still a lack of gold standard for the early diagnosis and the consequent treatment of PJI. [6]

The assay used to measure an ideal infection marker should:

- Be reliable and reproducible in different settings,
- Provide an information from a different type of sample that can be obtained easily,
- Provide rapid information,
- Identify presence or absence of sepsis,
- Aid in risk stratification and/or identify those patients that will benefit from a specific therapeutic intervention,
- Monitor the response to an intervention and/or aid in titrating the intervention.

But:

- No single biomarker can assist the physician at the bedside in sepsis detection or risk stratification,
- Biomarker panels (including RNA's) may be more discriminant,
- Rapid point-of-care tests are needed to implement the use of biomarker in clinical practice,

To date in the clinical practice there isn't a biomarker panel able to identify PJI infection as early as possible.

For these reason my PhD thesis was focused on to investigate the diagnostic and prognostic potential of emerging biomarkers involved in the inflammatory response, such as the new



emerging biomarker Presepsin, in particular investigating the inflammatory mediators involved such as the chemokine CCL2 and the inflammatory cytokine, IL-6.

The diagnostic and prognostic value of Presepsin in PIJ was also investigated correlating Presepsin values with other emerging infection marker such as TREM-1, CD-163, SuPAR, OPN, TLR2 and MMP-9 which could be involved in the progression of PJI.

## **2.1 *Biomarkers currently used in PJI Diagnosis***

In this context, a previous study of our group investigated the possibility to improve the diagnosis of PJI, investigating role of Procalcitonin, C reactive protein and IL-6 as markers of post-operative orthopedics joint prosthesis infection, indicating that IL-6 and CRP were useful in the differentiation between patient with or without post-operative PJI, while Procalcitonin had not a great diagnostic value in this case. Therefore, there is still need to improve the diagnostic tools for the early detection of PJI. [5]

### **2.1.1 C Reactive Protein (CRP)**

C reactive protein is a pentameric protein produced by the liver and it is found in plasma.

it is an acute-phase protein and its plasmatic level increase when there is an inflammatory process.

CRP is expressed on the surface of dead or dying cells and its increases follows interleukin-6 secretion by macrophages and T cells. CRP plays a role in innate immunity as an early defense system against infections. When CRP is expressed, there is an activation of the complement system, promoting the phagocytosis by macrophages, which remove necrotic and apoptotic cells and bacteria. In particular CRP binds to phosphocholine on micro-organisms, in order to stimulate complement binding to bacteria and enhancing phagocytosis by macrophages (opsonin-mediated phagocytosis), which express CRP receptor.

This acute phase response occurs as a result of an increase of IL-6, which is produced by macrophages and adipocytes in response to a wide range of acute and chronic inflammatory conditions such as bacterial, viral, or fungal infections, rheumatic and other inflammatory diseases, malignancy, tissue injury and necrosis. [7]

These conditions lead to the production of interleukin-6 and other cytokines that trigger the synthesis of CRP and fibrinogen by the liver.

The cut off value for the clinical diagnosis of inflammatory is 1 mg/dL. [7,8]

CRP increases within two hours of the onset of inflammation, up to a 50,000-fold, and peaks at 48 hours. Its half-life of 18 hours is constant, therefore its level is determined by the rate of production and hence by the severity of the precipitating cause. CRP is thus a marker for inflammation.

CRP plasma level has not a strong specificity because in patients with chronic inflammation, metabolic syndrome, smoking and obese subjects the value of CRP may be a little bit higher than 1 mg/dL.

In plasma CRP as diagnostic marker displays the following features:

- Sensibility of 0,71
- Specificity of 0.97
- Accuracy of 0.88
- Positive Predictive Value of 0.93
- Negative predictive Value of 0.87
- Likelihood ratio for positive results 23.6

In prosthetic joint infection patients, the levels of CRP are lower compared to the higher value measured in inflammatory condition, where the values are greater than 10 mg/dl. For this reason, even if there is a statistical significant increase in PJI patients compared to not infected ones, CRP is not sufficient to identify PJI and it cannot be considered a useful tool for PJI diagnosis. [8,9]

### **2.1.2 PROCALCITONIN (PCT)**

Procalcitonin (PCT) is a peptide of the hormone calcitonin, involved with calcium homeostasis. PCT is produced from thyroid cells and neuroendocrine cells.

Blood level of procalcitonin in healthy individuals is lower than the cut off value of <50 pg/mL.

The level of PCT increases over the cut off value in response to a pro-inflammatory stimulus, especially of bacterial origin. There isn't a statistically significant increase of PCT serum levels in viral or non-infectious inflammations. Therefore, procalcitonin can be considered specific for bacterial infection. In serum, PCT has a half-life of 25 to 30 hours. [7,10]

PCT is a new generation marker showing properties different from other inflammatory markers currently used:

- PCT is selectively produced in case of bacterial infection, sepsis and multi organ dysfunction syndrome,
- PCT cut off is very low (50 pg/ml), therefore increasing PCT assay sensitivity
- PCT plasma levels are very high in severe bacterial infection (100 ng/ml).

Procalcitonin blood concentration is used as a marker of bacterial infection and in case of severe sepsis. Compared to other markers such as IL-6, C-Reactive Protein and TNF-alpha, procalcitonin shows the highest levels of sensitivity (85%) and specificity (91%) in the differential diagnosis between patients and SIRS from patients with sepsis.

PCT is a specificity marker for systemic bacterial infections but recent studies showed that plasma levels of PCT displayed no significant difference between infected and not infected patients.

Therefore, in this case PCT plasma levels resulted not discriminant in PJI diagnosis. [5]

### **2.1.3 Interleukin 6 (IL-6)**

Interleukin-6 is an endogenous chemical product that acts as both pro-inflammatory cytokine and anti-inflammatory myokine.

Interleukin-6 (IL-6) is released by monocytes and macrophages in response to other inflammatory cytokines which include interleukin-11 (IL-11), and tumor necrosis factor-beta (TNF- $\beta$ ).

IL-6 is involved in inflammatory condition during B cell maturation and it is secreted by T cells and macrophages to stimulate immune response.

IL-6 is produced in the body in case of inflammation, either acute infection or chronic infection and interacts with interleukin-6 receptor alpha, to induce transcription of inflammatory gene products.

The IL-6 receptor is present on normal T-lymphocytes cells in the resting phase, myeloid cells and hepatic cell line. [11]

IL-6 has an important role in inflammatory processes, in particular in the acute phase response, in bone metabolism and in cancerogenesis processes.

In PJI patients IL-6 can be used as biomarkers to discriminate between infected and non-infected patients, and recent evidences show that the levels of IL-6 can be higher than the normal range in patients who had a previous inflammation. [12]

### **3. *New generation marker of infection***

In order to prevent prosthetic infection, aseptic surgical technique and antibiotics prophylaxis are used, but infection still occur in a quite relevant number of primary and revision arthroplasty.

Treating prosthetic infections is very difficult, because pathogens that infect the site create biofilm on implanted material that hinders the leukocyte recruitment to the site of infection and the action of antibiotics. [13,14]

Therefore, the early diagnosis of PJI still remains the best tool to promptly start the antibiotic treatment but a diagnostic gold standard is still lacking. [6,15]

Our group investigated the diagnostic potential of new generation serum biomarkers for the diagnosis of PJI. In order to optimize the diagnostic process, infection biomarkers with fast response and high sensitivity and specificity for bacterial infection are needed. Biomarkers are defined as biological molecules that are characteristic of normal or pathogenic process and, therefore, they can be as useful in the clinical practice for the identification of the pathological process and the monitoring of the disease progression [6,13]

The ideal serum markers for PJI should be able to:

- Have direct correlation with bone metabolism/infection,
- Have more sensible to bone turnover alterations /infections,
- Be less invasive on patients (blood),

- Consent regular, short interval monitoring

Among this scenario, different molecules are emerging as potential biomarkers of PJI, as listed in the following sections.



### **3.1 Soluble Urokinase Plasminogen Activation Receptor (suPAR)**

The first biomarker analyzed for the diagnosis of PJI is suPAR, the soluble urokinase plasminogen activation receptor, recently described as a powerful diagnostic and prognostic tool, able not only to detect sepsis but also to discriminate different grade of sepsis severity.

The urokinase-type plasminogen activator receptor (uPAR) is a glycoprotein released during inflammation and infection. The soluble form of the receptor (suPAR) is obtained by proteolytic cleavage of uPAR from the cell surface and it is released in blood and other organic fluids such as plasma, urine, and cerebrospinal fluid. [16–18]

suPAR is a biomarker of activation of the inflammatory and immune systems: suPAR levels are positively correlated with pro-inflammatory biomarkers, such as tumor necrosis factor- $\alpha$ , leukocyte counts, and C-reactive protein. Elevated levels of suPAR are associated with increased risk of systemic inflammatory response syndrome (SIRS). [19]

SuPAR is a marker of disease severity and aggressiveness: high plasma suPAR levels have been described to be able to predict severity of disease outcome in various infection. [20,21]

### **3.2 TREM-1**

TREM-1 is a triggering receptor expressed on myeloid cells-1, and it is a member of the immunoglobulin superfamily. Its expression on phagocytes is upregulated by exposure to bacteria and fungi.

The soluble form of TREM-1 (sTREM-1) can be found in body fluids, such as plasma, pleural fluid, Broncho alveolar lavage fluid, urine, and cerebrospinal fluid, where it can be assayed by using commercial immunoassay ELISA kits. [22,23]

### **3.3 MMP-9**

Matrix metalloproteinase 9 (MMP-9) is a matrix of the class of enzymes that belong to the zinc-metalloproteinase family involved in degradation of the extracellular matrix.

MMP9 plays several important functions within neutrophil action, such as degrading extracellular matrix, activation of IL-1 $\beta$ , and cleavage of several chemokines. [24]

### **3.4 CCL2**

Chemokine ligand 2 (CCL2) is also referred to as monocyte chemoattractant protein 1 (MCP1) and small inducible cytokine A2.

CCL2 is primarily secreted by monocytes, macrophages and dendritic cells. To become activated CCL2 protein has to be cleaved by metalloproteinase MMP-12. [19] Once activated, CCL2 recruits monocytes, memory T cells, and dendritic cells to the sites of inflammation.

### **3.5 Osteopontin OPN**

Osteopontin (OPN) is a multifunctional glycoprotein with pro-inflammatory properties and it is correlated with SuPAR during inflammatory response. [25]

In severe sepsis, OPN plasma level are significantly higher in non-survivors than in survivors. Recent studies indicated that OPN is associated with greater inflammatory response and increase mortality. [26,27]

Therefore, we evaluated the level of OPN at following time point after surgery for infection eradication in order to analyze the diagnostic and prognostic potential of this marker in PJI, in comparison with Presepsin.

### **3.6 Cluster of Differentiation 163 (CD163)**

Another emerging serum marker of infection is the hemoglobin (Hb) scavenger receptor, CD163. This is a macrophage-specific protein and the upregulated expression of this receptor is one of the major changes in the macrophage switch to alternative activated phenotypes in inflammation. Accordingly, a high expression in macrophages is a characteristic of tissues responding to inflammation.

In addition to this biological role in inflammation, CD163 is a potential inflammation biomarker and a therapeutic target. The biomarker CD163 is the soluble plasmatic form sCD163 result from proteolytic cleavage of the membrane bound CD163, resulting into the shedding of CD163. [28,29]

### 3.7 TLR2

Toll-like receptors (TLRs) are a class of proteins that play an important role in the innate immune response. They are single, membrane-spanning, non-catalytic receptors, usually expressed in sentinel cells such as macrophages and dendritic cells. TLRs receptor have the ability to recognize molecules that are broadly released by pathogens. [22] . In this way TLRs represent the first line of defense again invading pathogen, recognizing many bacterial, fungal and viral moecules. [30] When the TLR recognized the pathogen, it active the inflammatory response aimed to eliminate the pathogen and repair the damaged tissue.

TLRs is expressed on antigen presenting cells (plasmacytoid dendritic cells, B cells, monocytes, and macrophages), T cells (activated CD4+, CD8+, memory, and T-reg), renal tubule epithelial cells, trophoblasts, and decidual and amnion epithelial cells. Soluble forms of TLRs retain ligand binding capability and function as decoy receptors that suppress TLRs mediated inflammation.

TLR2 is one of the toll-like receptor and it plays an important role in the immune system.

TLR2 is the most pleiotropic among the human TLR, as recognizes several components of Gram positive bacteria, such as peptidoglycan, the major component of Staphylococcus Aureus and other Gram positive bacteria, lipoteichoic acid, lipopeptides from Gram positive bacteria, as well as components of mycobacteria, fungi and yeast. [31]

Since TLRs are responsible for the initial recognition of the pathogen and the engagement of the inflammatory response, they could provide useful information to the diagnosis of PJI.

Indeed, the measurement of TLR expression has already been proposed in different inflammatory conditions, such as arthritis [30] and infection, ranging from skin [32] to bone infection. [5,33]

### **3.8 Presepsin**

One of the new emerging infection biomarkers is Presepsin, a fraction of the soluble form of CD14, recently described as a powerful diagnostic tool, able not only to detect sepsis but also to discriminate different grade of sepsis severity. [34]

Cluster of differentiation 14 (known as CD14) is a glycoprotein expressed on the membrane surface of monocytes and macrophages and it binds lipopolysaccharides (LPSs) and LPS-binding proteins (LPBs).

CD14 binds to lipopolysaccharide (LPS) complexes and LPS binding protein (LPB), which triggers activation of toll-like receptor 4 (TLR4), thereby inducing the production of several pro-inflammatory cytokines. CD14 complex can also be released into the bloodstream as its soluble form (sCD14).

sCD14 is in turn cleaved by a plasma protease to generate the sCD14 fragment sCD14-subtype (sCD14-ST) also called Presepsin. [35]

Presepsin is a fraction of the soluble form of CD14, which is shedded from monocytes surface during inflammatory response and then released into blood.

Plasma levels of Presepsin can be measured using an automated chemo luminescent assay. Therefore, Presepsin can be used a circulation marker of infection. sCD14 subtype (sCD14-ST), or Presepsin, is present in low concentrations in the plasma of healthy subject and it has been shown to be increased in response to bacterial infections. [34–36]

#### **4 Aim of the PhD thesis**

The aim of this thesis was to investigate the potential diagnostic and prognostic value of Presepsin in the inflammatory response compared to other inflammatory mediators such as the chemokine CCL2 and the inflammatory cytokine, IL-6, in order to develop new diagnostic approaches for an integrated platform of point of care diagnosis of infection.

The diagnostic and prognostic value of Presepsin on PIJ was also investigated correlating Presepsin values with other emerging infection markers such as TREM-1, CD-163 and SuPAR, OPN, TLR2 and MMP-9 which could be involved in the progression of PJI.

## **5 Methods**

### **Study population**

The population of 100 selected patients undergoing prosthesis revision was enrolled from IRCCS Istituto Ortopedico Galeazzi (Milan), IRCCS Istituto Ortopedico G Pini (Milan), and IRCCS Policlinico San Donato (Milan) and subdivided into two groups according to the cause of prosthesis implant failure:

- 48 patients having bacterial infection (confirmed by positive culture test, with isolation of the causal agent in the infectious focus)
- 52 patients with no infection, undergoing aseptic loosening of the implant.

In the first group prosthetic infection was confirmed by clinical and laboratory data typical of bone joint infection: swelling, erythema, joint pain and secretion of purulent material, positive cultures, with isolation of the causal agent in the infectious focus.

All procedures followed were in accordance with the 1975 Declaration of Helsinki, as revised in 2000 and 2008. Informed consent was obtained from all participants. The study approved by the local ethics committee (CE of IRCCS San Raffaele Hospital, Milan, CE/99/int/2015). Details that might disclose the identity of the subjects under the study were omitted, in accordance with HIPAA.

Blood drawing was performed from all patients at different times points:

- T0 (before surgery),
- T1 (48 hours after surgery)
- T2 (one month after surgery)
- T3 (three months after surgery)

Blood was drawn from all patients for PLASMA + EDTA separation, liquored and stored at -80°C until further analysis.



## **Biomarkers evaluation**

CRP was measured using immunoturbidimetry on an automated biochemical analyzer (Olympus CRP-Latex assay, Central Valley, PA, CA, USA)

IL-6, TREM-1, CCL2, MMP-9, OPN, TLR2 and CD163 were measured using an ELISA sandwich Quantikine Assay, according to manufacturer protocol (R&D System, Minneapolis, MN, USA)

SuPAR was measured by Suparnostic ELISA Assay, according to manufacturer protocol (Virogates, Denmark)

Presepsin levels was measured by Presepsin/ PATHFAST Immunoassay (Mitsubishi Chemical).

PATHFAST is a compact immunoanalyzer analysis system for laboratories, hospitals and medical offices available wherever fast quantitative results (with full-scale lab quality) are required.

PATHFAST Presepsin is a chemiluminescent enzyme immunoassay (CLEIA) for the quantitative measurement of Presepsin concentration. Monoclonal antibodies and polyclonal antibodies recognizing Presepsin are used in the assay. Using the PATHFAST analyzer Presepsin concentration can be determined within 17 minutes in six samples simultaneously.

The test principle is based on non-competitive CLEIA combined with MAGTRATION technology (MAGTRATION is technology of bound/free (B/F) separation where magnetic particles are washed in pipette tip).

Magnetic particles were coated by anti Presepsin polyclonal antibody and monoclonal antibody.

During incubation with plasma, they form immunocomplexes with Presepsin present in the sample.

After incubation the PATHFAST transfer sample immunocomplex, with anti Presepsin polyclonal and monoclonal antibody coated on magnetic particles bounded whit presepsin present in the sample, in the new well.

A chemiluminescent substrate (CDP-Star Chemiluminescent Substrate<sup>1</sup>) is added. After a short incubation, the luminescence intensity generated by the enzyme reaction is measured. The luminescence intensity is directly correlated to Presepsin concentration in the sample which is calculated by means of the standard curve.

### **Statistical analysis**

For all the parameters analyzed, normality of distribution of the groups was verified by KS normality.

Statistical analysis was performed using One-way ANOVA test,  $p < 0.05$  was considered significant and  $p < 0.005$  very significant. Data are expressed as the mean  $\pm$  standard deviation (SD).

Correlation analysis was measured using PRISM 3.0 software, by performing linear regression analysis between the different groups of data and calculating the 95% confidence interval of the regression line.

The Pearson correlation coefficient ( $r^2$ ) was calculated to determine correlation between values measured by the different assays. The Mann–Whitney test was used for comparing values from patients and donors

Statistical analysis of Receiver Operating Characteristic (ROC) curves and Area Under the Curve (AUC) was performed by MedCalc 13.2.2 Software, (Ostend, Belgium)

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<sup>1</sup> Disodium 2-chloro-5-(4-methoxyspiro[1,2-dioxetane-3,2'-(5-chlorotricyclo[3.3.1.1<sup>3,7</sup>]decan])-4-yl]-1-phenyl phosphate

## **6 Results**

In the first part of my PhD project, the two biomarkers sTLR2 and suPAR were evaluated in the diagnosis of prosthetic joint infection.

They were subsequently compared to Presepsin, as well as other new generation inflammatory markers, in the prognostic evaluation of prosthetic joint infection.

### **6.1 Toll-like receptor 2 in serum: a potential diagnostic marker of prosthetic joint infection?**

Galliera E, Drago L, Vassena C, Romanò C, Gioia Marazzi M, Salcito L, Corsi Romanelli MM. J Clin Microbiol. 2014 Feb;52(2):620-3. doi: 10.1128/JCM.02727-13.

The interplay between the immune system and bone metabolism has been recognized as important for both of these systems. Toll-like receptors (TLRs) are a class of proteins that play an important role in the innate immune response activating the inflammatory reactions.

TLR2 and TLR4 are also expressed in bone cells, and their activation affects osteoclasts differentiation and activity. Moreover, in osteoblasts TLRs induce the production of osteoclastogenic cytokines (RANKL and TNF- $\alpha$ ), thereby contributing to TLR ligand-induced osteoclastogenesis.

These processes are involved in bone loss observed in variety of infectious diseases. Various factors produced and released during immune responses markedly affect bone cells and bone metabolism.

Implant infection is a severe complication requiring implant replacement. For example, in bone osteolysis the local bone loss is due to the inflammation in response to pathogen infection. Prosthetic joint infection (PJI) is a major problem in patients undergoing arthroplasty, but since the clinical presentations are variable and the efficacy of diagnostic approaches are low, there is still need to improve diagnostic methods. PJI is mainly due to *Staphylococcus Aureus*, a Gram positive bacterium recognized by TLR2, and more rarely by Gram negative bacteria such as *Pseudomonas*, recognized by TLR4.

In order to evaluate the potential diagnostic role of TLR2 and TLR4 in PJI, in this study TLR2 and TLR4 serum levels, as well as inflammatory marker (IL-6, TNF- $\alpha$  and IL-1, PCR) were evaluated in 32 selected patients undergoing revision of total hip or total knee joint arthroplasty and displaying prosthetic chronic infection for at least 6 months, as demonstrated by clinical and laboratory signs typical of bone joint infection: swelling, erythema, joint pain, and secretion of purulent material . As a control group, we selected 28 non infected patients undergoing routine orthopedic surgery without any other underlying disease or infection of inflammation and showing no comorbid conditions that could affect the expression of TLR2 and other markers, as well as no antibiotic therapy in progress, and no diabetes mellitus type 2 or obesity.

While TLR4 displayed no significant differences, TLR2 level resulted higher in septic patients and correlated with IL-6 and PCR levels. Accordingly, IL-1 $\beta$ , the main TLR2 co-player of the inflammatory response to *Staphylococcus Aureus*, resulted higher in septic patients.

These results indicated that TLR2 play an important role in the inflammatory response to prosthetic joint infection. Therefore, the measure of TLR2 serum level could be considered a potential diagnostic tool that could be used in association with canonical inflammatory parameters for the early detection and diagnosis of prosthetic joint infection.

File attached #1

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**Title:** Toll-Like Receptor 2 in Serum: a Potential Diagnostic Marker of Prosthetic Joint Infection?

**Author:** Emanuela Galliera, Lorenzo Drago, Christian Vassena et al.

**Publication:** Journal of Clinical Microbiology

**Publisher:** American Society for Microbiology

**Date:** Feb 1, 2014

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## **6.2 Soluble urokinase-type plasminogen activator receptor (suPAR) as new biomarker of the prosthetic joint infection: correlation with inflammatory cytokines.**

Galliera E, Drago L, Marazzi MG, Romanò C, Vassena C, Corsi Romanelli MM.

Clin Chim Acta. 2015 Feb 20; 441:23-8. doi: 10.1016/j.cca.2014.11.029.

There is a universally recognized need to identify new, reliable markers of inflammation that can aid in the rapid diagnosis of orthopaedic joint prosthesis infections (PJI). PJI is the most common cause of failure of total joint arthroplasty, requiring revision surgery, but a gold standard for the diagnosis and the treatment of PJI is still lacking.

PJI is mainly due to *Staphylococcus Aureus*, a Gram positive bacterium and more rarely by Gram negative bacteria such as *Pseudomonas*.

This study aimed examine the diagnostic value of SuPAR in post-operative prosthetic joint infection, in order to explore the possible application of this new biomarker in the early diagnosis of PJI.

The urokinase-type plasminogen activator receptor (uPAR) is a glycoprotein released during inflammation and infection. The soluble form of the receptor (suPAR) is obtained by proteolytic cleavage of uPAR from the cell surface and it is released in blood and other organic fluids such as plasma, urine, and cerebrospinal fluid.

In this study, a population of 60 selected patients was enrolled and subdivided into two groups: 32 patients having bacterial infection (confirmed by positive culture test, with isolation of the causal agent in the infectious focus) and control group of 28 not septic patients, undergoing orthopedic surgery, in order to explore the possible application of this new biomarker in the early diagnosis of PJI. The level of SuPAR has been determined using commercial double monoclonal antibody

sandwich immunoassay and correlated with canonical inflammatory markers, such as C-reactive protein, IL-6, IL-1 and TNF $\alpha$  and the chemokine CCL2 measured in the two groups of patients.

Serum suPAR level displayed a strongly significant increase in PJI patients compared to not infected controls, and a significant positive correlation with C-reactive protein, IL-6, IL-1, TNF $\alpha$ .

Also serum level of CCL2 showed a statistically significant increase in PJI patients and it displayed a strong positive correlation with serum suPAR. Moreover, the ROC analysis and AUC indicated a good diagnostic potential of suPAR in PJI determination.

This study provides a clear indication of the diagnostic potential of suPAR, in association to routine inflammatory parameters, such as CRP and IL-6, in the diagnosis of Prosthetic joint infection.

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### **6.3 Evaluation of diagnostic values of Presepsin in PJI**

Presepsin resulted significantly higher in PJI patients compared to controls. In particular, Presepsin in PJI was  $583,7 \pm 250,64$  pg/ml, while in not infected patients resulted  $196,54 \pm 141,68$  pg/ml, with a very highly statistically significance ( $p < 0.001$ , **FIGURE 1 PANEL A**). In order to compare the diagnostic value of Presepsin with inflammatory markers currently used in the clinical detection of PJI, we measured C-Reactive Protein and IL-6.

CRP displayed a statistically significant increase ( $p < 0.001$ , **FIGURE 1 PANEL B**) in PJI patients ( $2.63 \pm 1.79$  mg/dL), compared to controls ( $0.5 \pm 0.2$  mg/dL). Even considering this increase, CRP value display a little increase compared to the cut off value of 1 mg/dL, therefore the single evaluation of CRP cannot be considered clinically determinant for PJI diagnosis.

IL-6 displays a significant increase  $p < 0.001$  in PJI patients ( $11.12 \pm 2.36$  pg/mL) compared to not infected patients ( $1.81 \pm 0.85$  pg/mL), **FIGURE 1 PANEL C**), confirming the ongoing inflammatory response in PJI patients



Figure 1

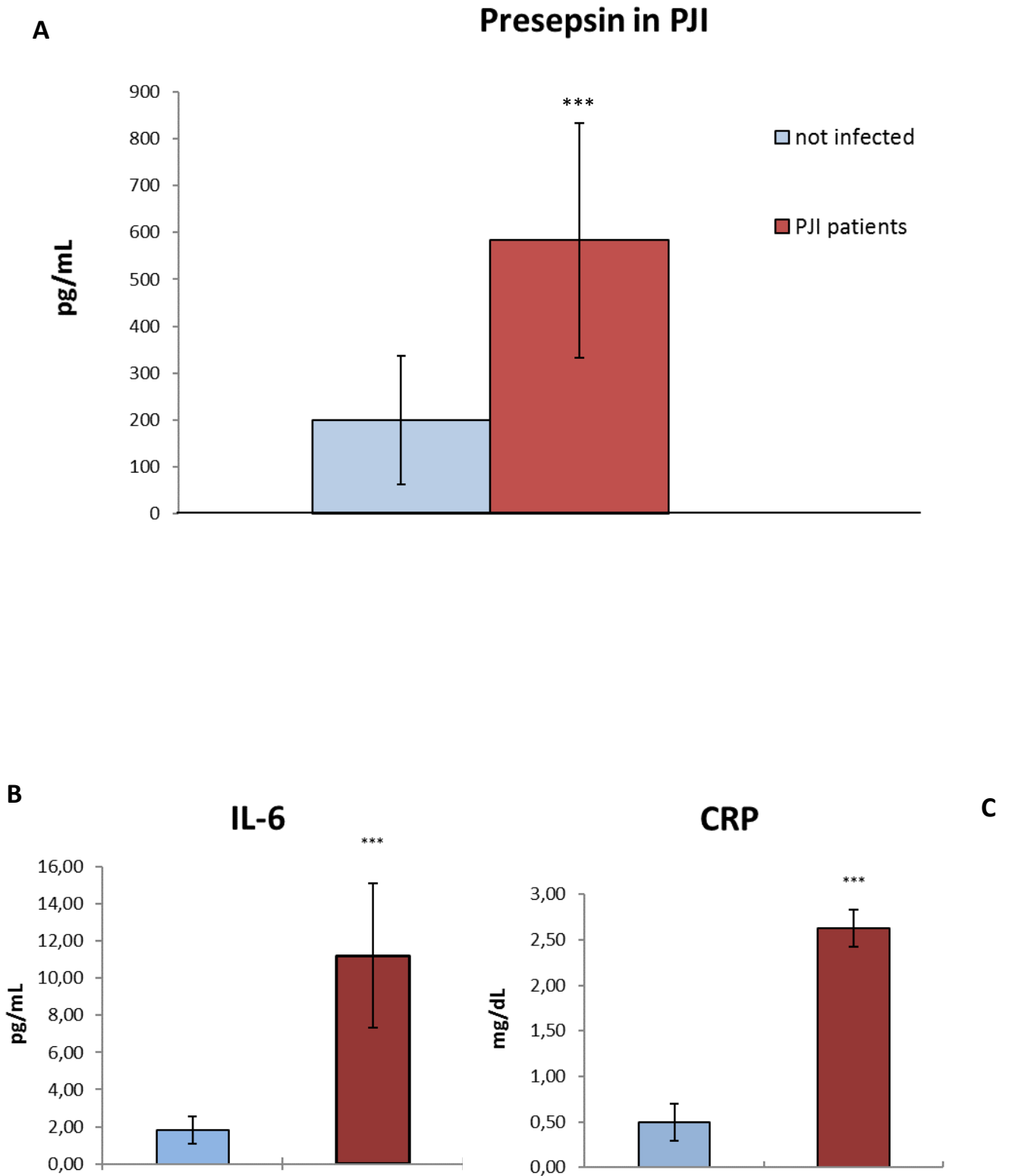


Figura 2

A

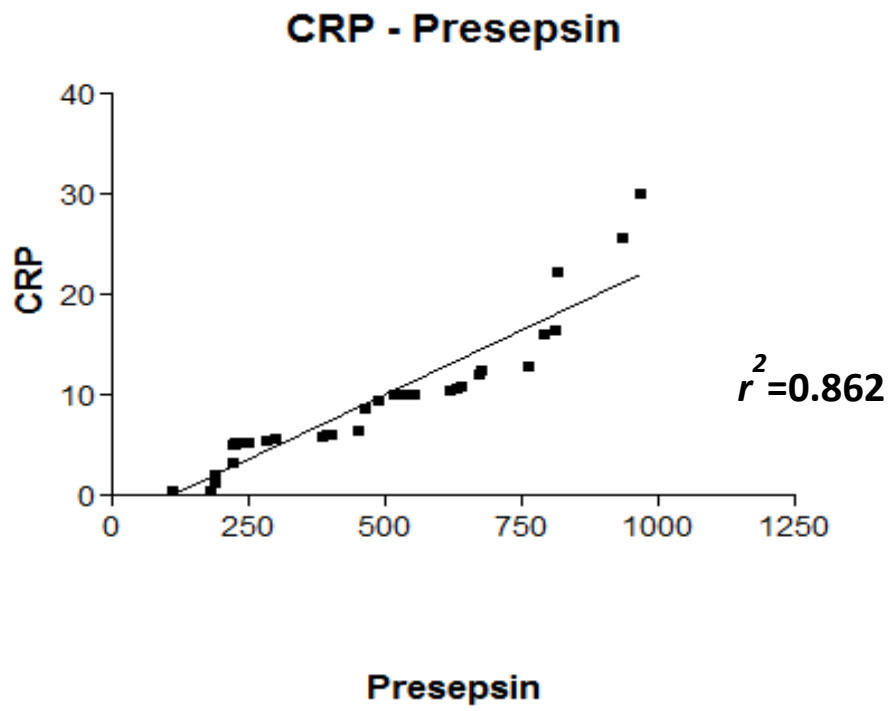
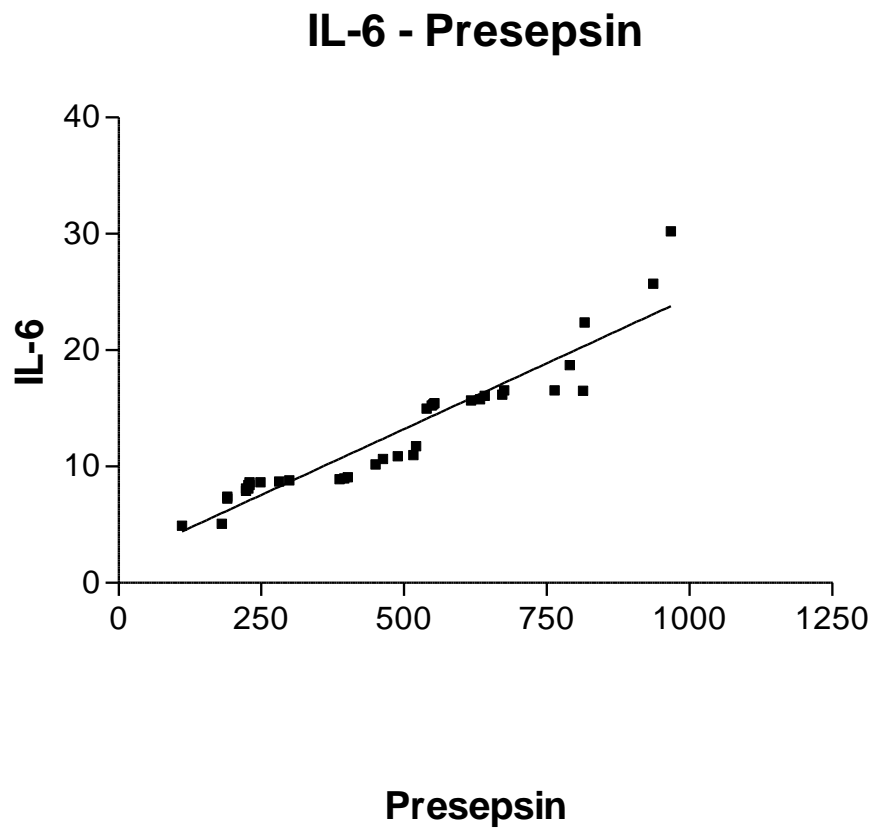


Figure 2

B



In Figure 2 Presepsin shows a very good positive linear correlation with both the inflammatory markers analyzed ( $r^2=0.862$  and  $r^2=0.887$  with CRP and IL-6, respectively), confirming its value as a marker of the inflammatory process in response to the infection.

In order to clearly compare the clinical diagnostic value of Presepsin with the two currently inflammatory markers currently used in PJI diagnosis, we calculated the ROC curve for Presepsin, IL-6 and CRP. **(FIGURE 3)**

The ROC curves can evaluate the diagnostic efficacy of a diagnostic test, by measuring the area under the ROC curve (AUC). In the clinical practice a diagnostic test is considered acceptable if its AUC is  $\geq 0.8$

In our patients AUC resulted:

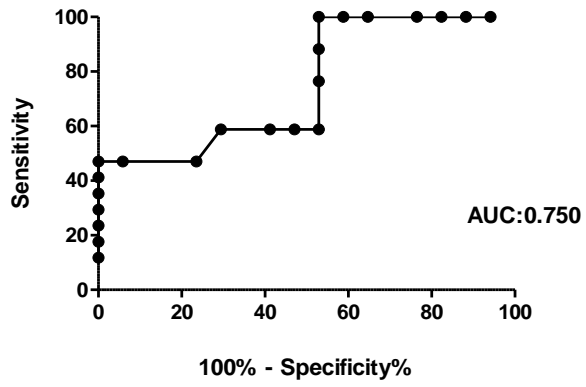
- CRP, AUC = 0.750, very low diagnostic efficacy **(FIGURE 3 - PANEL A)**,
- IL-6, AUC = 0.821, minimal diagnostic efficacy **(FIGURE 3 - PANEL B)**,
- Presepsin, AUC= 0.926, very good diagnostic efficacy **(FIGURE 3 - PANEL C)**.

These results clearly indicated that Presepsin has a greater diagnostic value than CRP and IL-6 in the diagnosis of PJI, and therefore it can be considered a better diagnostic marker that could be introduced in the clinic for the detection of PJI.

Figure 3

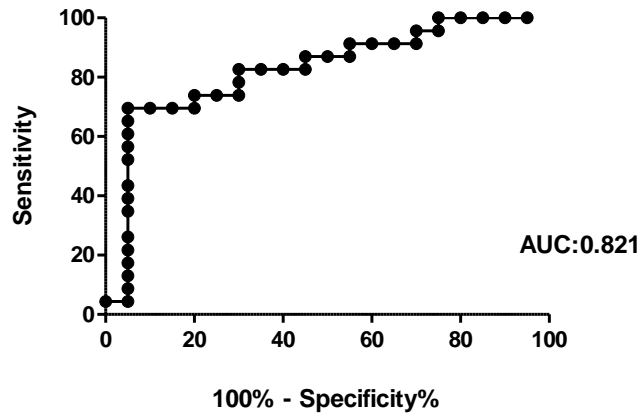
A

CRP



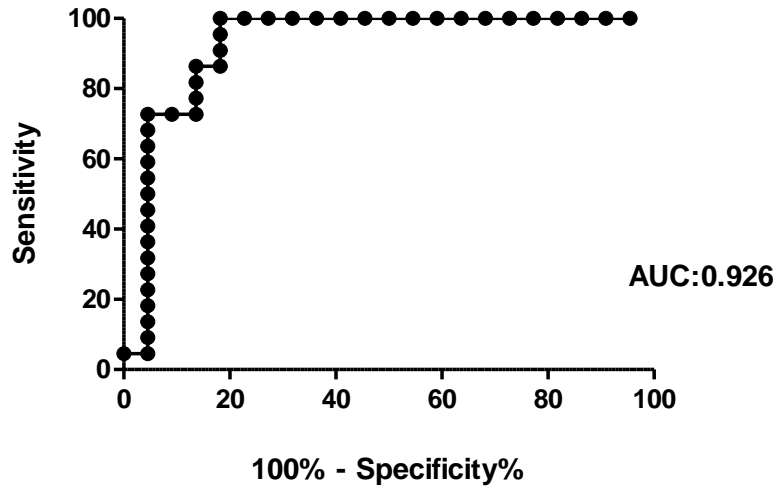
B

IL-6



C

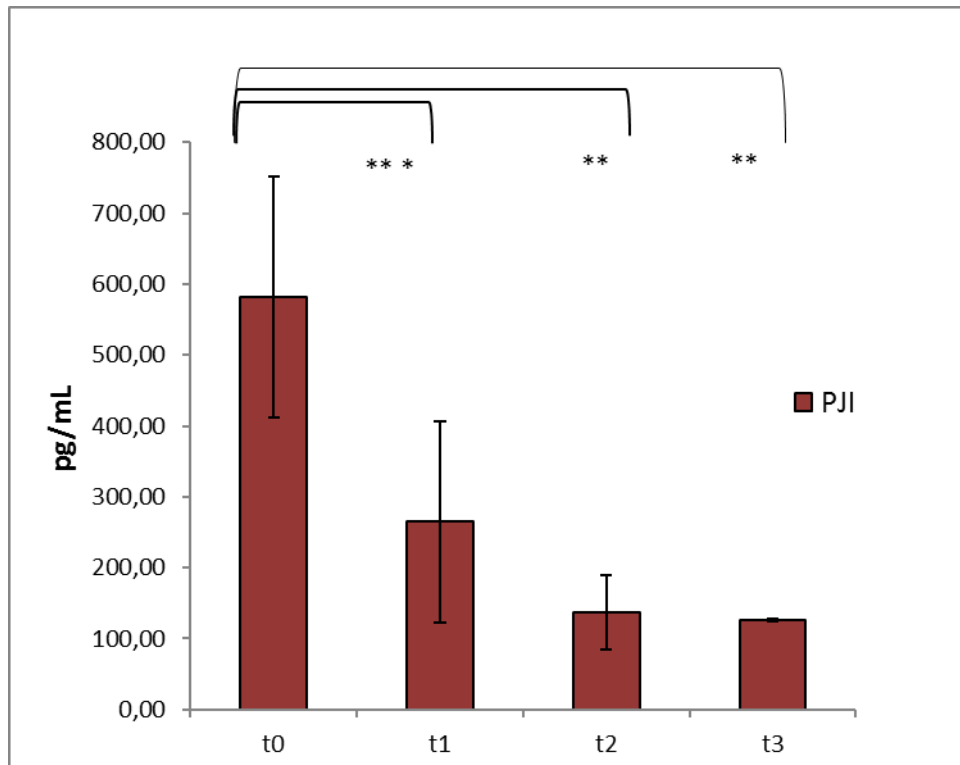
### Presepsin



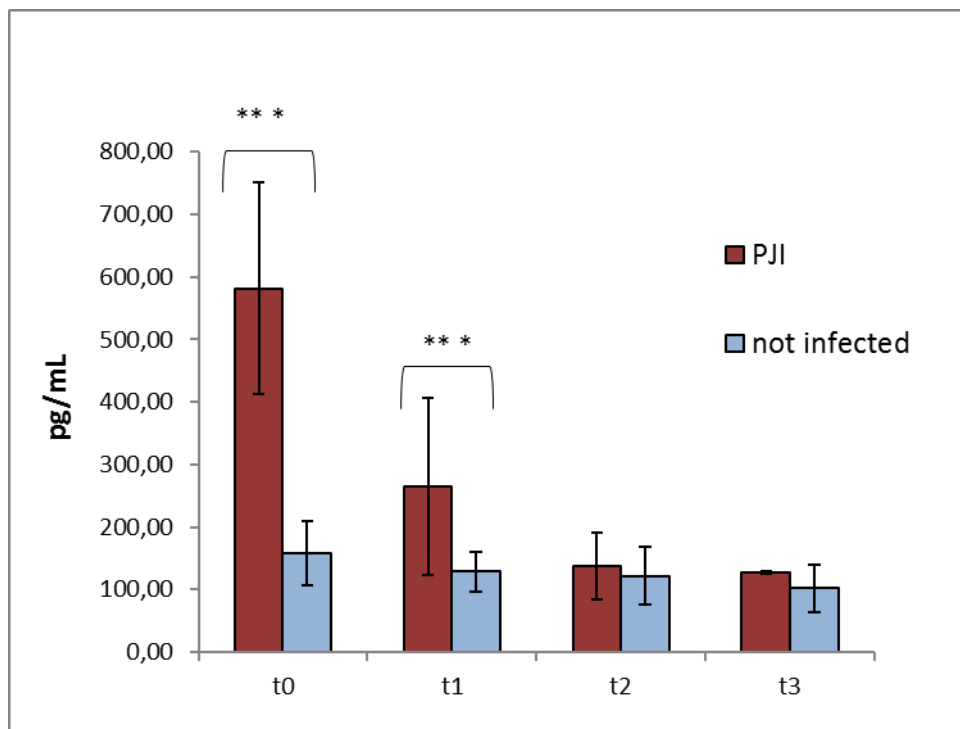
**6.4 Evaluation of prognostic values of Presepsin in PJI**

**Figure 4**

**A**



**B**



Once evaluated the diagnostic value of Presepsin in the clinical detection of PJI, we wanted to go further in the analysis of this molecule by evaluating whether Presepsin could have also a prognostic value in the clinical monitoring of the follow up of PJI after the revision surgery.

For this reason, in the second part of my PhD thesis we performed a longitudinal study by analyzing Presepsin serum values at different time points after revision surgery, both in PJI patients and in aseptic loosening of the implant (not infected subject, control group).

In figure 4 Presepsin displays a significant decrease at the increasing time of recovery after surgery in PJI patients (**FIGURE 4 PANEL A**), while it remains unaltered and significantly lower ( $p < 0.0001$ , **FIGURE 4 PANEL B**) in the patients undergone to revision surgery for aseptic loosening of the implant.

In order to complete the evaluation of Presepsin in the clinical diagnosis and monitoring of PJI, we compared Presepsin serum level with other markers of infection and inflammation, such as the chemokine CCL2, the new emerging markers of infection OPN (osteopontin), TLR2, CD-163, TREM-1 and SuPAR in the same longitudinal study.

First of all, the diagnostic value of all these markers have been evaluated by ROC AUC analysis (**FIGURE 5**).

OPN, CCL2, SuPAR and TLR2 displayed good diagnostic values in PJI, having AUC values of:

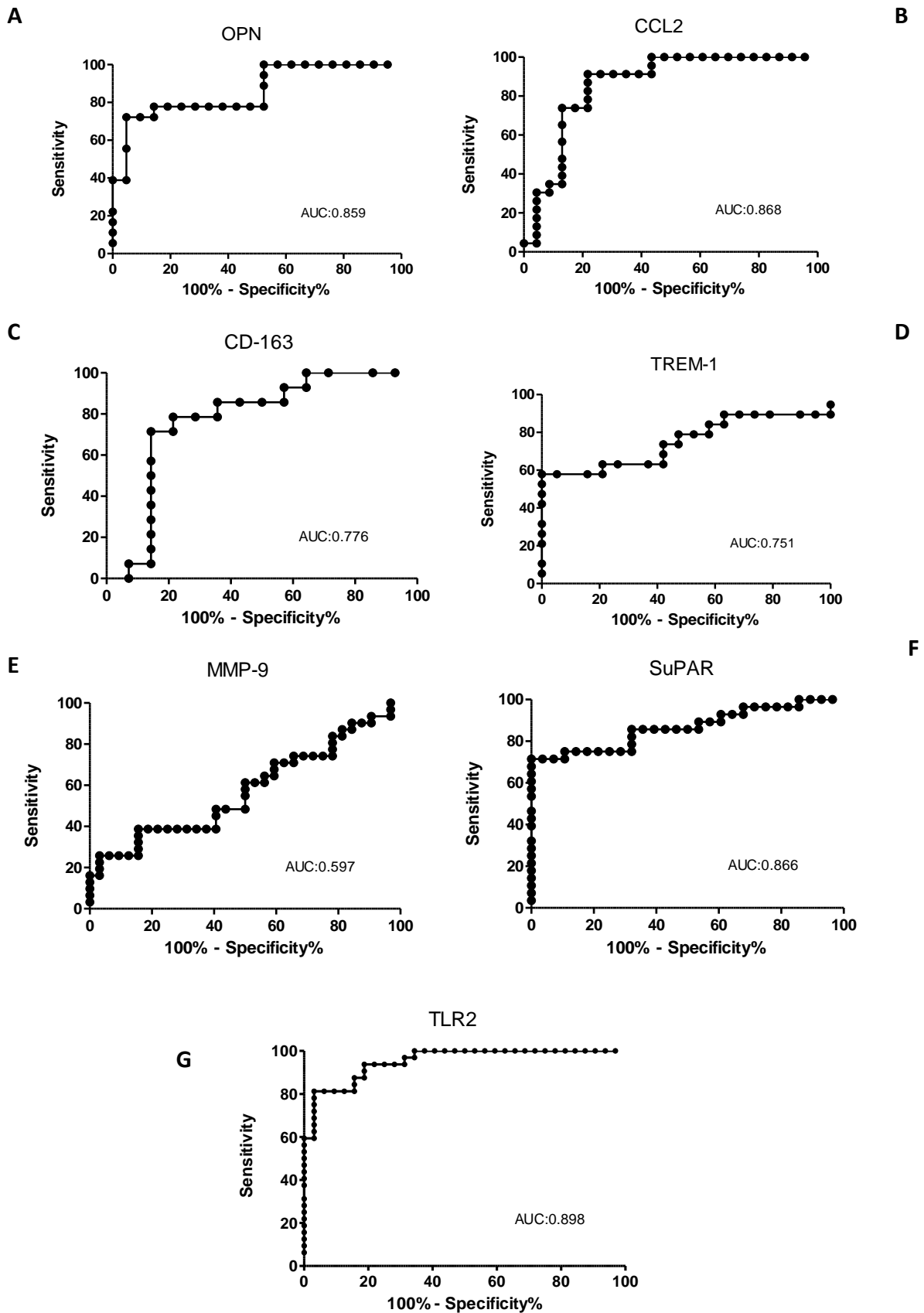
- OPN, AUC = 0.859, (**FIGURE 5 - PANEL A**),
- CCL2, AUC = 0.868, (**FIGURE 5 - PANEL B**),
- SuPAR, AUC = 0.866, (**FIGURE 5 - PANEL F**) respectively,
- TLR2, AUC = 0.898, (**FIGURE 5 - PANEL G**) respectively.

while CD163, TREM-1 and MMP-9 displayed very low diagnostic potential, showing AUC values of:

- CD163, AUC = 0.776, (**FIGURE 5 - PANEL C**),

- TREM-1, AUC = 0.751, (FIGURE 5 - PANEL D),

**Figure 5** C = 0.597, (FIGURE 5 - PANEL E) respectively.





In the longitudinal study, in order to evaluate the progressive resolution of the inflammatory process, we analyzed the primary inflammatory cytokine, IL-6.

IL-6, which is also currently used a diagnostic tool for the clinical evaluation of PJI, in **FIGURE 6** IL-6 serum level is higher in PJI patients ( $16.58 \pm 4.37$  pg/mL) and displayed a gradual decrease as long as the recovery time after surgery ( $p < 0.0001$ ), while in not infected patients IL-6 resulted undetectable at all the time points analyzed.

Since Presepsin, the soluble form of CD14 ligand, originates from mononuclear cells, we evaluated, in parallel with Presepsin, the main monocytes attractant chemokine, CCL2 (**FIGURE 7**).

As expected, the serum level of CCL2 is higher in PJI patients ( $318.58 \pm 86.23$  pg/mL) compared to control group with aseptic loosening of the implants ( $162.48 \pm 48.21$  pg/mL). At following time points, the level of the chemokine decrease rapidly, similarly to Presepsin.

Figure 6

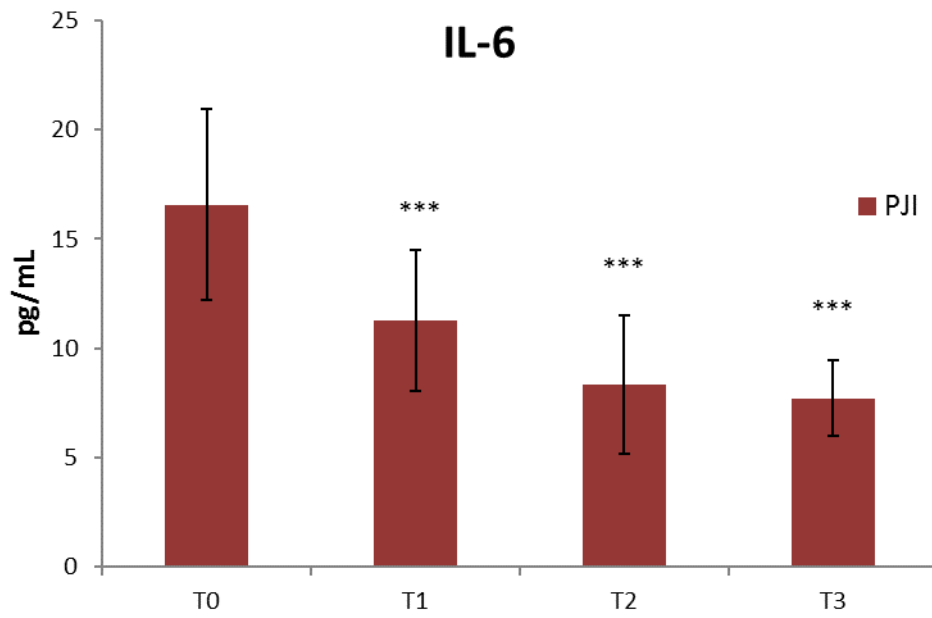
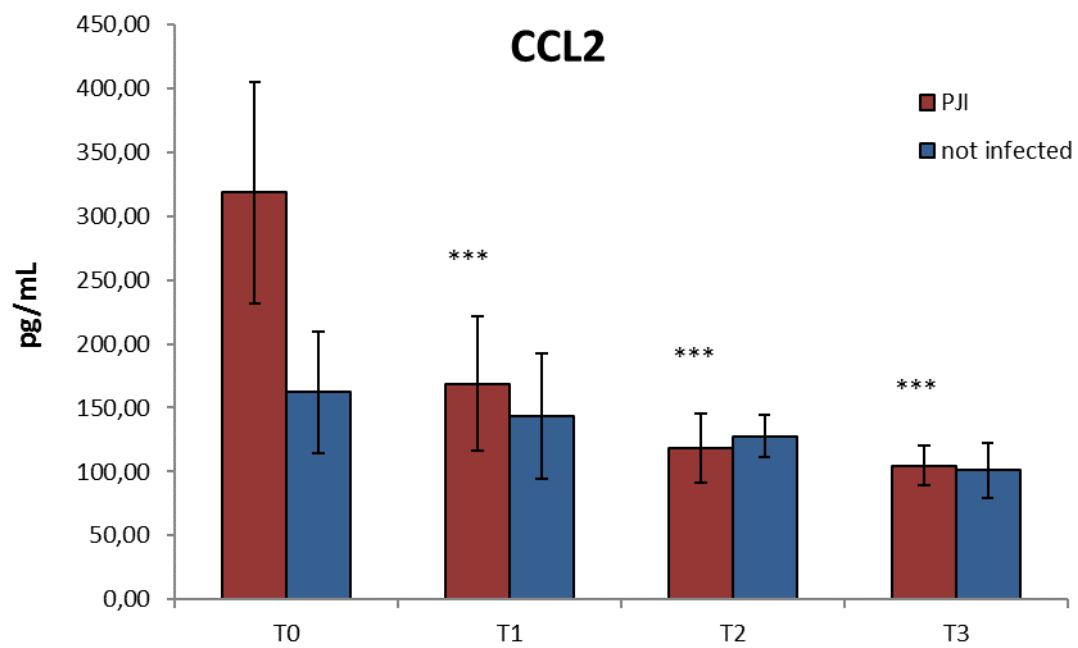
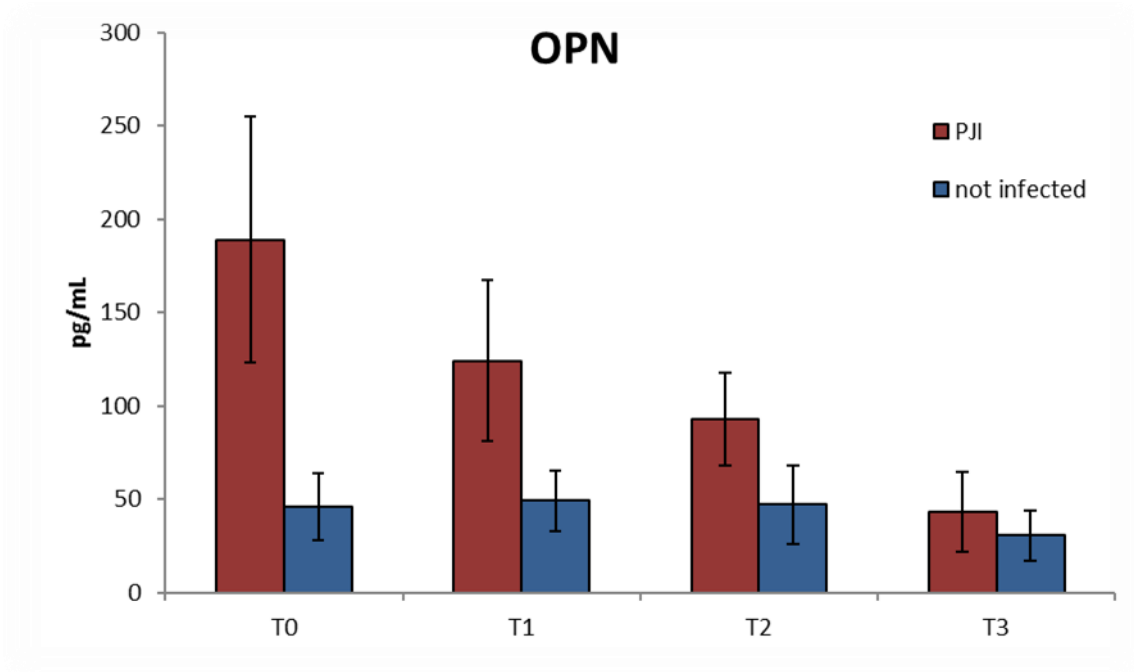


Figure 7



Besides, OPN displays a significant increase in infected patient ( $179,71 \pm 56.11$  pg/mL) compared to not infected one ( $46.18 \pm 18.02$  pg/mL, **FIGURE 8**). In addition, OPN showed the same gradual decrease observed for Presepsin, even if a significant standard deviation is observed at T0 and T1.

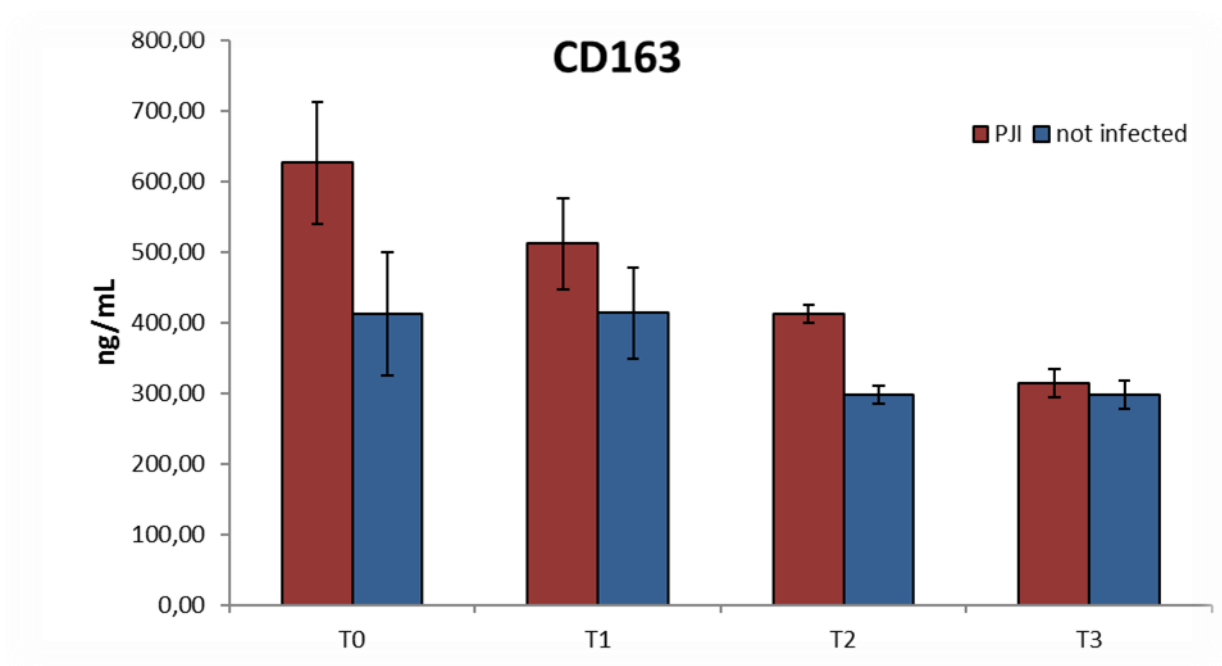
**Figure 8**



We also extend the analysis of inflammatory marker to other emerging molecules in the diagnosis of infection, such as CD163 and TREM-1.

In **FIGURE 9** CD163 displayed a gradual decrease at following time points, but it showed no significant difference between infected ( $626.28 \pm 18.02$  pg/mL) and not infected patients ( $426.8 \pm 16.23$  pg/mL). This result indicates that CD163 cannot be considered a good marker for PJI infection.

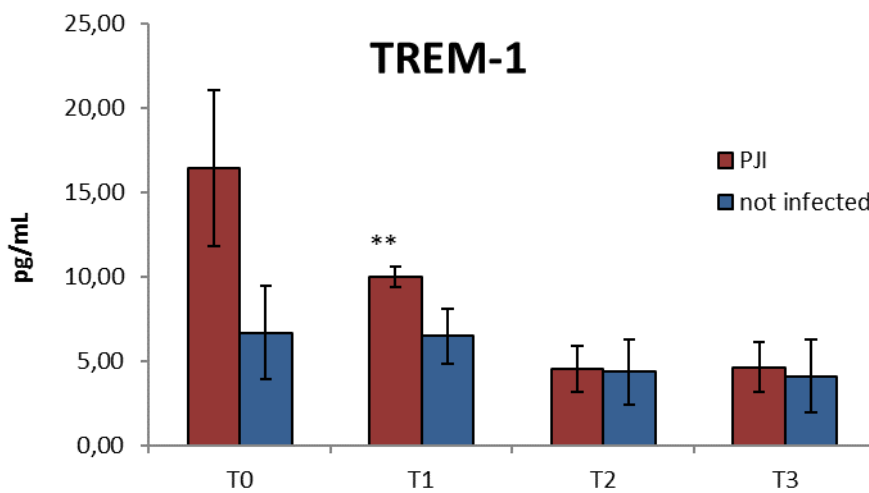
**Figure 9**



TREM-1, in **FIGURE 10** displayed a significant decrease in T1 and following time points, reaching comparable value to not infected patients.

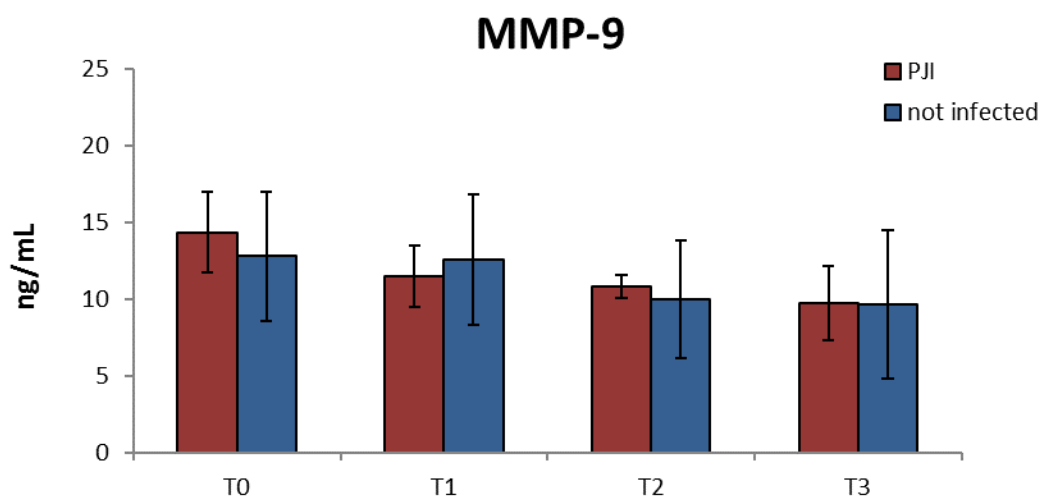
However, the absolute value of serum TREM-1, event in the highest level of T0 ( $17.07 \pm 5.01$  pg/mL), is far below the diagnostic cut off of 30 pg/mL reported in literature.

**Figure 10**



Similarly, to TREM-1, the level of MMP9 at T0 was very low ( $12.77 \pm 4.22$  ng/mL) compared to diagnostic cut off levels, **FIGURE 11**

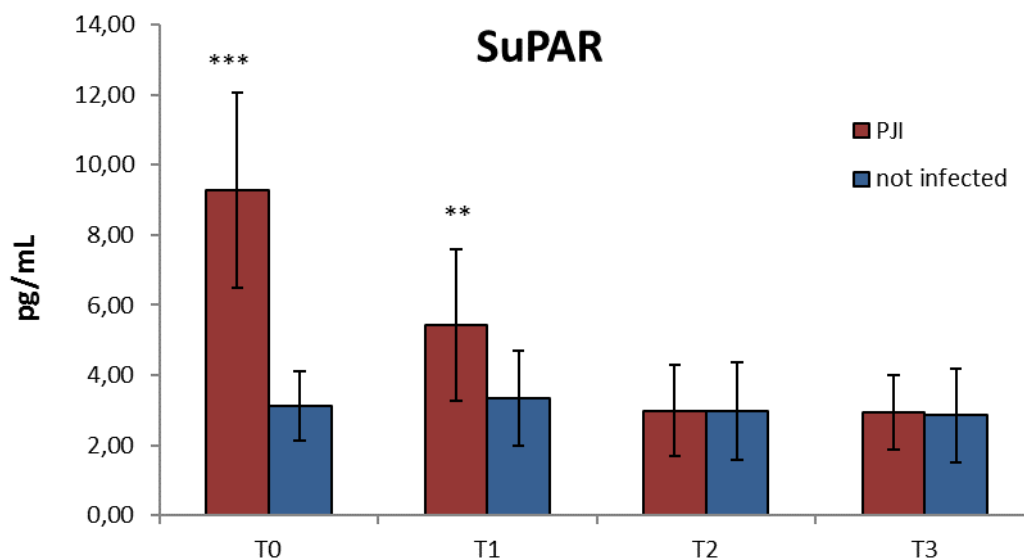
**Figure 11**



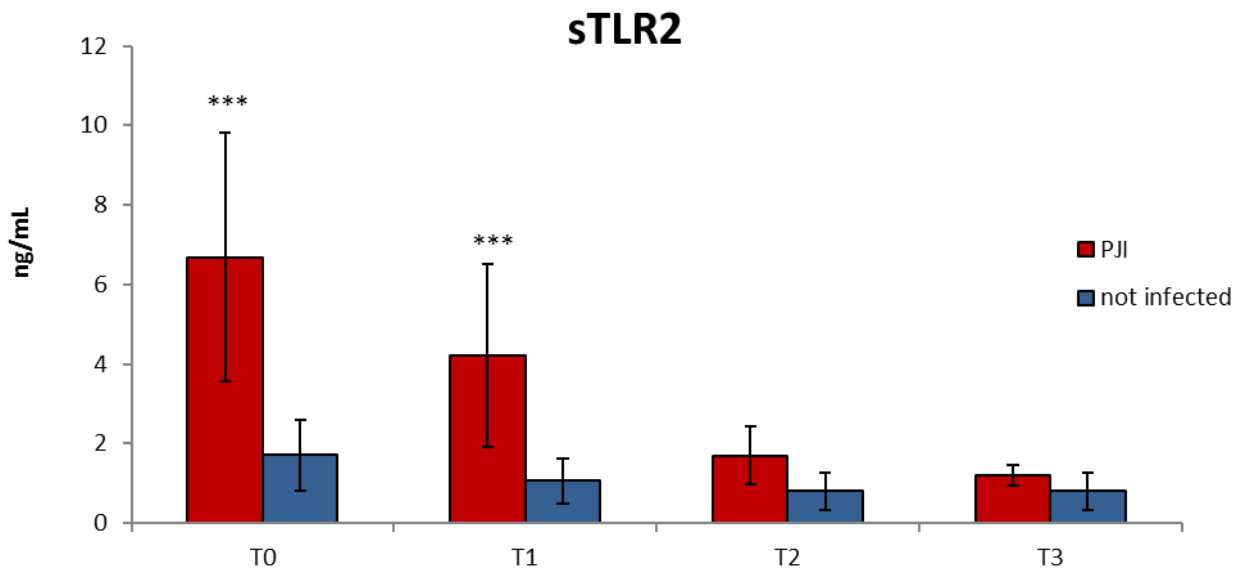
On the contrary, the inflammatory marker SuPAR (**FIGURE 12**), previously described as having a diagnostic potential role in PJI [20], displayed the same gradual decrease observed for Presepsin.

SuPAR resulted significantly higher in PJI patients compared to controls. In particular, SuPAR in PJI was  $9.27 \pm 2.78$  pg/ml, while in not infected patients resulted  $3.12 \pm 1.00$  pg/ml, with a very highly statistically significance ( $p < 0.001$ ).

**Figure 12**



**Figure 13**



Since the Prosthetic joint infection is mainly due to Gram positive bacteria, we evaluated the Gram positive specific Toll Like receptor TLR2.

TLR2, previously described as having a diagnostic potential in diagnosis of PJI and in particular confirming a Gram positive infection, displayed the same gradual decrease observed for Presepsin.

TLR2 resulted significantly higher in PJI patients compared to controls. In particular, TLR2 in PJI was  $6.68 \pm 3.13$  ng/ml, while in not infected patients resulted  $1.73 \pm 0.88$  ng/ml, with a very high statistical significance ( $p < 0.001$ ). At T1 the decrease of serum TLR2 is not statistically significant, while at following time points T2 and T3 it reaches values comparable to not infected patients.

## **7. Discussion**

Pathogen infection is one of the main adverse events of surgical procedures, which can lead to an excess of systemic inflammatory response and sepsis, causing multi organ failure and septic shock. [37,38]

In the orthopedic field, the periprosthetic joint infection is a particularly challenging complication associated with surgical procedures, especially hip and knee arthroplasty.[39]

The most common cause of failure of total joint arthroplasty is PIJ, and it even requires revision surgery.[40]

The principal cause of PIJ are Gram positive bacterial infection: the most of the time *Staphylococcus Aureus*, and more rarely Gram negative bacteria such as *Pseudomonas*. [41]

At the moment many tests are available for PIJ diagnosis, many of which can be used in combination.

Ranging from haematological markers of infection and inflammation, intraoperative culture, histology analysis. [1,3,42]

Nevertheless, there is not yet a gold standard for the diagnosis, consequently, for the treatment of PIJ. [43]

The clinical presentation is often ambiguous [40,44] and although classical inflammatory markers like C-reactive protein are helpful, they can be misleading in some cases, such as patients with chronic disease, post-operative hematomas, obesity, metabolic syndrome and insulin resistance and smokers. [45]

Infection biomarkers with fast response, high sensitivity and specificity for infection are essential for the optimization of the diagnostic process.



Biomarkers can be useful indicators for the clinical practice, since they are biological molecules that are characteristic of “normal” or “pathogenic process”. [46,47]

In this context, a previous study of our group investigated the role of Procalcitonin, C reactive protein and IL-6 as markers of post-operative orthopedics joint prosthesis infection, indicating that IL-6 and CRP were useful in the differentiation between patient with or without post-operative PJI, while C reactive protein had not a great diagnostic value in this case. [5]

Therefore, there is still the need to improve the diagnostic tools for the early detection of PJI and new emerging infection biomarkers were identified as inflammatory molecules involved in host response to bacteria, such as Toll like receptors.

Among innate immunity mediators, Toll-like receptors (TLRs) plays a crucial role in inflammation because they sense pathogen-derived molecules and initiate the inflammatory response. TLRs are also expressed in bone cells, and their activation affects osteoclasts differentiation and activity.

A recent study of our group evaluated the potential diagnostic role of TLR2 and TLR4 in the early detection of Prosthetic joint infection (PJI).

In septic patients TLR2 level is higher and correlates with inflammatory markers (IL-6 and C reactive protein), while TLR4 display no significative differences. [31]

Accordingly, IL-1beta, the main TLR2 co-player of the inflammatory response to Aureus, is higher in septic patients than in not septic patients. These results strongly suggested that TLR2 is essential in the inflammatory response to pathogen induced prosthetic joint infection.

Thus, the measure of TLR2 circulating level could be very informative in the early detection of PJI and, therefore, it could be considered potential diagnostic tool that could be associated with canonical clinical markers of inflammation in order to improve the diagnosis of prosthetic joint infection. [22,48,49]

An important role inflammatory response mediated by monocytes/ macrophages against invading bacteria, is played by SuPAR, the soluble urokinase plasminogen activating receptor.

The urokinase-type plasminogen activator receptor (uPAR) is a glycoprotein released during inflammation and infection, thereby promote the migration and adhesion of leukocytes. The soluble form of the receptor (suPAR) is obtained by proteolytic cleavage of uPAR from the cell surface, therefore suPAR can be found in blood and other organic fluids. [25]

High plasma levels of suPAR have been described to be able to predict severity of disease outcome in various infections. SuPAR could find new fields of diagnostic application, where a clear detection of the infection is still lacking, such as prosthesis joint infection. As shown paper (SUPAR), resulted clearly higher in PJI patients compared to not infected patients and showed a good positive correlation with CRP and other inflammatory markers. The measure of Serum level of SuPAR provide an extremely important benefit because it is a precise indicator of bacterial infection, and the addition of SuPAR serum level measurement to classical inflammatory markers can strongly improve the diagnosis of prosthesis joint infection. [17,19,50,51]

Among the scenario of infections diagnosis, an emerging molecule is Presepsin, the soluble fraction of CD14, recently described as a powerful diagnostic tool, able not only to detect sepsis but also to discriminate different grade of sepsis severity. [35,36]

Presepsin is a fraction of the soluble form of CD14, which is shedded from monocytes surface during inflammatory response and then released into blood. Therefore, Presepsin can be used a circulation marker of infection, but so far little is known about the mechanism of this sCD14 fraction shedding. A better understanding the mechanism of action of Presepsin in the inflammatory response, and its correlation with other inflammatory mediators could improve the diagnostic potential and clinical application of Presepsin. [38,48]

To this purpose Presepsin was compared to the two infection marker previously described (TLR2 and SuPAR) as well as new generation infection marker such as TREM-1, CD-163, OPN.

In order to confirm the ongoing inflammatory response, the IL-6 serum level was evaluated in PJI and not infected patients, as well as the chemokine CCL2, the main mediator of monocyte recruitment.

IL-6 display a significative increase, confirming the ongoing inflammatory response in PJI patients. Presepsin resulted significantly higher in PJI patients compared to not infected controls. Moreover, Presepsin has a greater diagnostic value that CRP and IL-6 in the diagnosis of PJI. Therefore, it can be considered a better diagnostic marker that could be introduced in the clinic for the detection of PJI.

Once evaluated the diagnostic value of Presepsin in the clinical detection of PJI, we wanted to go further in the analysis of this molecule by evaluating whether Presepsin could have also a prognostic value in the clinical monitoring of the follow up of PJI after the revision surgery.

For this reason, in the second part of the project we performed a longitudinal study by analyzing Presepsin serum values at different time points after revision surgery, both in PJI patients and in aseptic loosening of the implant (not infected control group).

Presepsin displays a significant decrease at the increasing time of recovery after surgery in PJI patients, while it remains unaltered and significantly lower ( $p < 0.0001$ ) in the patients undergone to revision surgery for aseptic loosening of the implant.

These results clearly indicate that Presepsin is not only a useful diagnostic tool in the diagnosis of PJI, but is also have a prognostic value, because its decrease gradually as the inflammatory process in response to the prosthetic infection is resolved.

At the first time point analyzed (T1, one week after surgery) Presepsin is nearly 50% of the value a T0, but it does not reach the value of not infected patients: this is probably due to the clearance

time of the molecule from the circulation in the early time point after surgery. At following time points after surgery, which eradicates the infection, Presepsin values in PJI patients are comparable with the ones of not infected patients. These results are crucial in the follow up of prosthetic surgery revision, because the incidence of a second infection is not low and it would lead, if not detected early, to a second revision surgery.

For this reason, a marker like Presepsin could be introduced not only in the follow up of PJI patients after the prosthetic revision surgery, in order to early detect any possible second infection, but, more interestingly, in the routine follow up after the first prosthetic surgery, in order to early detect any possible prosthetic infection at the first time.

This would allow a prompt therapeutical (antibiotic) intervention in order to avoid the revision surgery, which has more impact on the patient.

In order to complete the evaluation of Presepsin in the clinical diagnosis and monitoring of PJI, we compared Presepsin serum level with other markers of infection and inflammation, such as the chemokine CCL2, the new emerging markers of infection OPN (osteopontin), CD-163, TREM-1 and SuPAR, TREM-1, in the same longitudinal study. [50,52]

OPN, CCL2 and SuPAR displayed good diagnostic values in PJI, having AUC values 0.859, 0.868 and 0.866 respectively while CD163, TREM-1 and MMP-9 displayed very low diagnostic potential, showing AUC values of 0.776, 0.751 and 0.597 respectively. These results indicate that, among the inflammatory markers analyzed, the most relevant for PJI diagnosis are the one related to monocyte/macrophage mediated inflammatory response, such as the monocyte specific chemokine CCL2, SuPAR and OPN. In the longitudinal study in order to evaluate the progressive resolution of the inflammatory process, we analyzed the primary inflammatory cytokine IL-6, which is also currently used a diagnostic tool for the clinical evaluation of PJI.

IL-6 displayed a gradual decrease as long as the recovery time after surgery, while in not infected patients IL-6 resulted undetectable at all the time points analyzed.

On the one hand this results confirm the difference between the inflammatory response elicited by the Prosthetic infection and the aseptic loosening of the implant. On the other hand, this result is in accordance with Presepsin decrease during recovery after surgery, confirming the potential of Presepsin as a marker of infection and, in this case, of the resolution of infection.

Since Presepsin, the soluble form of CD14 ligand, originates from mononuclear cells, we evaluated, in parallel with Presepsin, the main monocytes attractant chemokine, CCL2 (Figure 6).

As expected, the serum level of CCL2 is higher in PJI patients ( $318.58 \pm 86.23$  pg/mL) compared to control group with aseptic loosening of the implants ( $162.48 \pm 48.21$  pg/mL).

At following time points, the level of the chemokine decrease rapidly, similarly to Presepsin.

CCL2 is the main chemokine regulating monocyte and macrophage activity, therefore this results are accordance with recent evidences indicating that Presepsin is released upon macrophage phagocytosis.

SuPAR, previously described as having a diagnostic potential role in PJ, displayed the same gradual decrease observed for Presepsin, confirming the potential role of Presepsin in the prognostic evaluation of PJI.

A strong correlation has been described between CD14 and Toll like receptor, in particular TLR2 and TLR4. [19,48,53]

We have previously shown that TLR2 display a good diagnostic potential in PJI, confirmed by a AUC of 0.898, therefore we evaluated TLR2 in the longitudinal study. TLR2 display a little decrease at the first time point of 48h, while at following time points it reaches values comparable to not infected patients.

These results indicate that it cannot be considered not an early marker of eradication of infection, but at the later time points it follows the same trend of decrease of Presepsin.

Osteopontin (OPN) is a multifunctional glycoprotein with pro-inflammatory properties. In severe sepsis, levels of plasma OPN are significantly higher in non-survivors than in survivors. [25]

Recent studies indicated that OPN is associated with greater inflammatory response and increased mortality, therefore we evaluated the level of OPN at following time point after surgery for infection eradication in order to analyze the prognostic potential of this marker in PJI and compare it to Presepsin. [26]

CD163 displayed a gradual decrease at following time points, but it showed no significant difference between infected. This result indicates that CD163 cannot be considered a good marker for PJI infection. This results reflect the specificity of PJI infection, where not all the infection markers, such as the canonical C-reactive protein, resulted sufficient to detect the infection. In the case of CD163, this marker is mainly reported to be a good marker the case on viral infection and chronic inflammation. [54]

Therefore, this can be the reason why it resulted not significant in the case of PJI, but the weak increase at T0 can be due only to the inflammatory response in PJI patients.

A connection between Toll like receptors, monocytes, inflammatory cytokines such as IL-1 and IL-6 and the chemokine CCL2, in the mechanism of action of Presepsin could be represented by the new infection marker TREM-1. [55]

TREM-1 is related to different aspects of the inflammatory response: TREM-1 is involved in TLR signaling, acting as an “amplifier of inflammatory response”, it is over-expressed on monocytes in parallel with CD14 in septic condition, then it is shedded from monocytes by the action of the metalloprotease MMP-9 and then it induces the inflammatory cytokines IL-1, IL-6, TNF-alpha and CCL2. In Prosthetic Joint infection other canonical infection makers, such as the C-Reactive Protein,

resulted below the diagnostic cut off limit, and this account for the difficulties in the early detection of the infection and the need of alternative diagnostic markers. In this case, TREM-1 cannot be considered a useful tool for the diagnosis and prognosis of Prosthetic joint infection.

Since the soluble form of TREM-1 is released through the action of MMP-9, we evaluated the level of MMP-9 at the same time points of TREM-1.

No significative differences was observed between PJI patients and controls at any time points, confirming that the axis MMP-9 /TREM-1 is not involved Prosthetic Joint infection.

Limitations of our study are the number of infected patients, but this is consistent with the very low frequency of Prosthetic Joint infection, which occurs in the 2.18% for hip arthroplasty and to 2.18% for knee arthroplasty [56]

To overcome this limitation, we enrolled PJI patients from different orthopedic institutes

## **8. Conclusion**

Taken together, these results indicate that Presepsin can be considered a useful tool for the diagnosis and clinical monitoring of prosthetic joint infection, and it can also be supported by a panel of new inflammatory makers involved in monocyte/macrophage mediated inflammatory response such as OPN, CCL2 and SuPAR.



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## Toll-Like Receptor 2 in Serum: a Potential Diagnostic Marker of Prosthetic Joint Infection?

Emanuela Galliera,<sup>a,b</sup> Lorenzo Drago,<sup>b,c</sup> Christian Vassena,<sup>b</sup> Carlo Romano,<sup>b</sup> Monica Gioia Marazzi,<sup>c</sup> Lucia Salcito,<sup>c</sup> Massimiliano M. Corsi Romanelli<sup>c,d</sup>

Department of Biomedical, Surgical and Oral Science, Università degli Studi di Milano, Milan, Italy<sup>a</sup>; IRCCS Galeazzi Orthopaedic Institute, Milan, Italy<sup>b</sup>; Department of Biomedical Science for Health, Università degli Studi di Milano, Milan, Italy<sup>c</sup>; IRCCS Policlinico San Donato, San Donato (Milan), Italy<sup>d</sup>

**Prosthetic joint infection (PJI) is a severe complication of arthroplasty and is still lacking diagnostic gold standards. PJI patients display high Toll-like receptor 2 (TLR2) serum levels, correlating with canonical inflammatory markers (C-reactive protein [CRP], interleukin 6 [IL-6], tumor necrosis factor alpha [TNF- $\alpha$ ], and IL-1). Therefore, TLR2 serum levels could be considered a new potential diagnostic tool in the early detection of PJI.**

Toll-like receptor (TLR) represents the first line of defense against invading pathogens (1) by recognizing the invading bacteria and activating the inflammatory response aimed to eliminate the pathogen and repair the damaged tissue. Among TLRs, TLR4 and TLR2 recognize a broad spectrum of Gram-positive and Gram-negative bacteria, respectively, and induce the main inflammatory response (2, 3). Periprosthetic joint infection (PJI) (4–8) is one of the main adverse events of orthopedic surgical procedures (9–11). Currently, a large number of tests are available for PJI diagnosis, ranging from hematological markers of infection and inflammation to intraoperative culture and histology analysis. Nevertheless, there is still a lack of gold standards for the diagnosis of PJI (12, 13), because the clinical presentation of PJI is often ambiguous (14), and classical inflammatory markers can be misleading (15–17). In order to optimize the diagnostic process, infection biomarkers with fast response and high sensitivity and specificity for infection are needed (7).

In this context, TLR could be useful for PJI diagnosis. Indeed, TLR expression has already been considered of relevance in different inflammatory conditions and infections (18), but so far the potential diagnostic use of this molecule remains unexplored. For this reason, in this study the serum levels of the two main TLRs involved in bacterial infection, TLR2 and TLR4, were measured in PJI patients and in nonseptic patients undergoing implant revision and were compared to classical inflammatory parameters, such as C-reactive protein (CRP), and with the main inflammatory cytokines, interleukin 1 (IL-1), IL-6, and tumor necrosis factor alpha (TNF- $\alpha$ ), in order to explore the potential use of TLR2 and TLR4 serum levels as novel diagnostic tools for PJI identification.

Patient population was described in Table 1. We selected 32 patients undergoing revision of total hip or total knee joint arthroplasty and displaying prosthetic chronic infection for at least 6 months, as demonstrated by clinical and laboratory signs typical of bone joint infection: swelling, erythema, joint pain, and secretion of purulent material. Diagnosis of infection was confirmed according to the criteria set forth by Spanghehl et al. (19): at least three positive results for (i) erythrocyte sedimentation rate, (ii) C-reactive protein and aspiration, (iii) frozen section, or (iv) intraoperative culture. As a control, we selected 28 noninfected patients undergoing routine orthopedic surgery without any other underlying disease or infection of inflammation and showing no

TABLE 1 Patient clinical features

Characteristic	Value	
	Controls	PJI patients
Amt of TLR (pg/ml) $\pm$ SD		
TLR4	348.81 $\pm$ 28.61	384.28 $\pm$ 43.84
TLR2	171.04 $\pm$ 34.36	504.19 $\pm$ 64.96
Amt of inflammatory marker (pg/ml) $\pm$ SD		
C-reactive protein	0.48 $\pm$ 0.27	2.63 $\pm$ 1.79
TNF- $\alpha$	1.78 $\pm$ 0.51	6.70 $\pm$ 1.94
IL-1	2.19 $\pm$ 0.52	15.79 $\pm$ 3.62
IL-6	1.822 $\pm$ 0.75	11.2 $\pm$ 3.86
No. of patients with:		
<i>Staphylococcus aureus</i> (Gram positive)		11
<i>Staphylococcus epidermidis</i> (Gram positive)		7
<i>Staphylococcus xylosum</i> (Gram positive)		1
<i>Staphylococcus warneri</i> (Gram positive)		1
<i>Staphylococcus caprae</i> (Gram positive)		1
<i>Staphylococcus aureus</i> (Gram positive)		1
<i>Streptococcus anginosus</i> (Gram positive)		1
<i>Streptococcus agalactiae</i> (Gram positive)		1
<i>Enterococcus faecalis</i> (Gram positive)		2
<i>Corynebacterium striatum</i> (Gram positive)		4
<i>Klebsiella pneumoniae</i> (Gram negative)		1
<i>Pasteurella multocida</i> (Gram negative)		1
<i>Staphylococcus aureus</i> (Gram positive)/ <i>Acinetobacter baumannii</i> (Gram negative)		1
<i>Staphylococcus aureus</i> (Gram positive)/ <i>Pseudomonas aeruginosa</i> (Gram negative)		1
No. of females and males	16 females, 12 males	16 females, 12 males
Mean age (yrs) $\pm$ SD	68 $\pm$ 22	63 $\pm$ 21

comorbid conditions that could affect the expression of TLR2 and other markers, no antibiotic therapy in progress, and no diabetes mellitus type 2 or obesity.

PJI and control patients were matched for age, sex, and severity

Received 30 September 2013 Returned for modification 29 October 2013

Accepted 6 November 2013

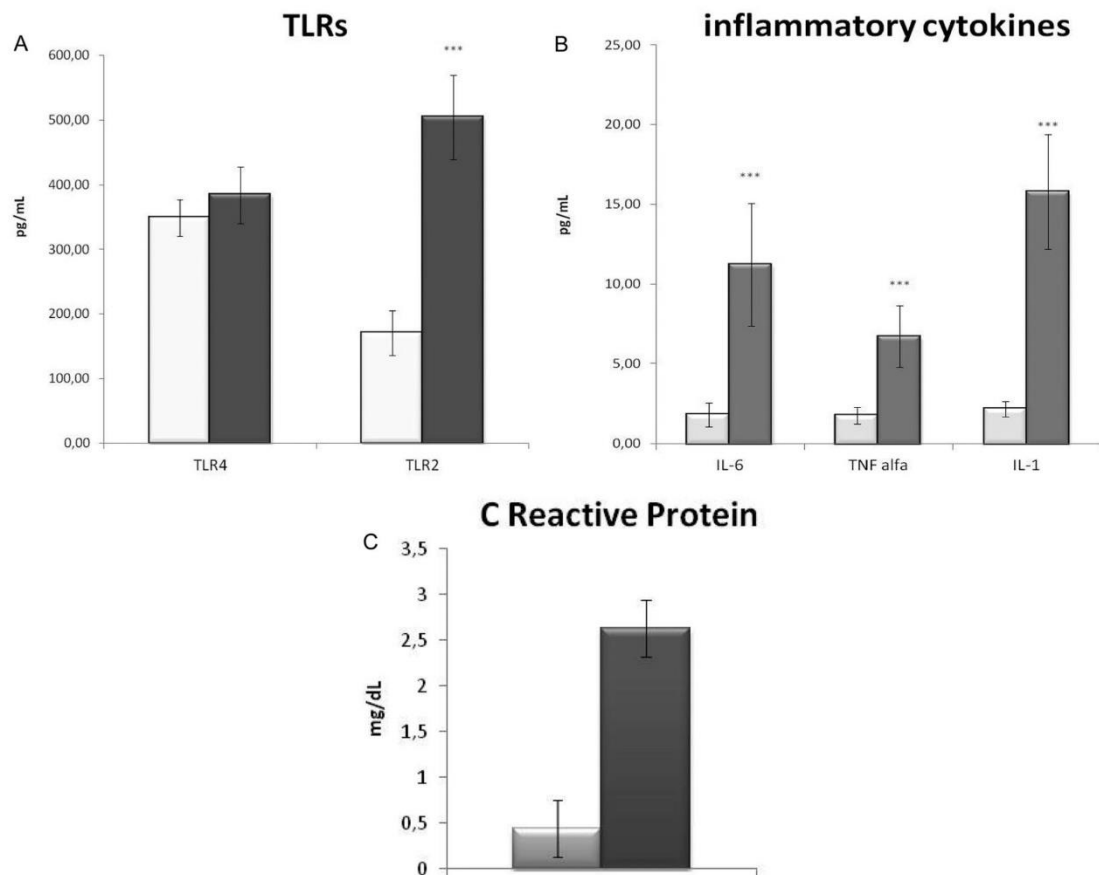
Published ahead of print 13 November 2013

Editor: G. V. Doern

Address correspondence to Emanuela Galliera, emanuela.galliera@unimi.it.

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doi:10.1128/JCM.02727-13



**FIG 1** TLRs and inflammatory mediators. (A) Serum levels of TLR2 and TLR4 in prosthetic joint infection patients (gray bars) and noninfected patients (white bars); (B) serum levels of inflammatory cytokines (IL-1, IL-6, and TNF- $\alpha$ ) in prosthetic joint infection patients (gray bars) and noninfected patients (white bars); (C) serum levels of C-reactive protein in prosthetic joint infection patients (gray bars) and noninfected patients (white bars).

of illness. Blood was drawn from all patients for serum separation, aliquoted, and stored at  $-80^{\circ}\text{C}$  until further analysis.

CRP was measured using immunoturbidimetry on an automated biochemical analyzer (CRP-Latex assay; Olympus, Central Valley, PA, USA).

Human IL-1, IL-6, and TNF- $\alpha$  and TLR2 were measured in serum using an enzyme-linked immunosorbent assay (ELISA) sandwich duo set assay, according to the manufacturer's protocols (R&D Systems, Minneapolis, MN, USA). Human TLR4 serum concentration was measured by ELISA sandwich assay according to the manufacturer's protocols (USCN LifeScience Inc., Wuhan, Hubei, People's Republic of China; catalog number E90753Hu). TLR2 ELISA kit (DY2612; R&D Systems) was optimized for the analysis of TLR2 in cell supernatants and serum.

For all the parameters analyzed, normality of distribution of the three groups was verified by the Kolmogorov-Smirnov test for normal distribution.

Statistical analysis was performed using a one-way analysis of variance (ANOVA) test, and  $P$  values of  $<0.05$  were considered

significant and  $P$  values of  $<0.005$  were considered very significant.

Linear regression analysis was performed between the different groups of data, and the 95% confidence interval of the regression line was calculated by using PRISM 3.0 software.

Surgical infection is due mainly to *Staphylococcus aureus* (20, 21), a Gram-positive bacterium recognized by TLR2 (22), while a small amount is due to Gram-negative bacteria, bound by TLR4. Accordingly, in infected patients, we observed 87.5% of Gram-positive and only 12.5% of Gram-negative infection and, as a consequence, a significant increase of TLR2 but not of TLR4 (Fig. 1). TLR2 has been described to be crucial in joint infection (20), contributing to the degenerative process and destructive arthropathy after microbial joint infection (23), indicating that TLR2 expression strictly reflects the progression of the infection in the host. So far, the alteration of TLR2 and TLR4 has been evaluated only at the gene expression level (24, 25), while the present work is the first, to our knowledge, which measures the amount of circulating protein, making it suitable for routine clinical diagnosis. In order to

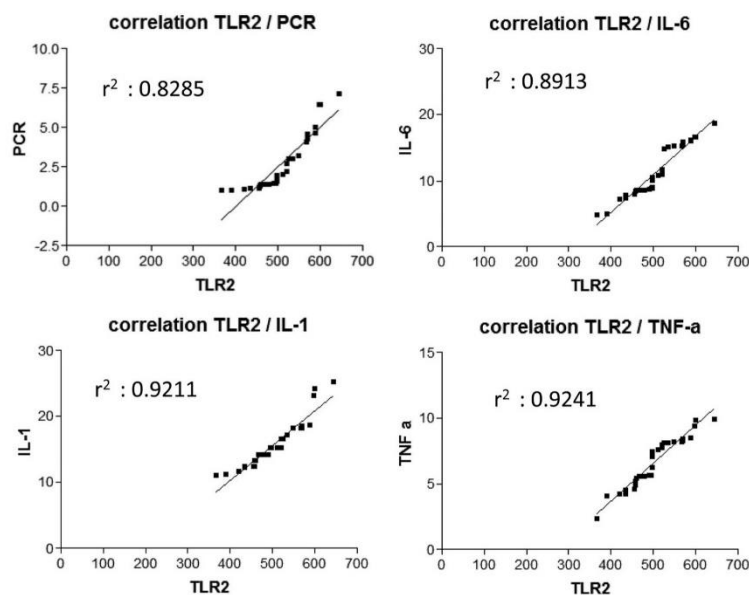


FIG 2 Correlation analysis. Linear regression analysis between TLR2 and inflammatory parameters. Top left, TLR2 and C-reactive protein (PCR); top right, TLR2 and IL-6; bottom left, TLR2 and IL-1; bottom right, TLR2 and TNF- $\alpha$  (TNF-a). For each analysis, coefficient of correlation  $r^2$  is reported.

evaluate TLR2 circulating levels as diagnostic tools, we compared them with canonical markers of infection and inflammation. TLR2 showed a strong positive correlation with CRP (Fig. 1B), the gold standard clinical marker of infection, which is increased in PJI patients, indicating that the serum TLR2 molecule is able to detect an inflammatory condition. Moreover, since TLR2 mechanism of action leads to an inflammatory response (3), we measured the circulating levels of the three main inflammatory cytokines: IL-1 $\beta$  and TNF- $\alpha$  for local inflammatory response and IL-6 for systemic response. Infected patients displayed a significant increase of all the cytokines analyzed, in particular IL-6, previously described to be a significant marker of PJI (16), and IL-1 $\beta$ . In PJI patients, TLR2 displayed a strong positive correlation with both IL-6 and IL-1 $\beta$ , which exert a protective role on the tissue in *S. aureus* infection (26), confirming the importance of TLR2 in the detection of PJI (Fig. 2). Given the small sample size of patient groups, the results of this pilot study are preliminary, but taken together, they indicate that serum TLR2 can be considered, in association with canonical parameter of inflammation, a new potential diagnostic marker of PJI.

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***File attached #2***





## Soluble urokinase-type plasminogen activator receptor (suPAR) as new biomarker of the prosthetic joint infection: Correlation with inflammatory cytokines



Emanuela Galliera<sup>a,b,\*</sup>, Lorenzo Drago<sup>b,c</sup>, Monica Gioia Marazzi<sup>c</sup>, Carlo Romanò<sup>b</sup>, Christian Vassena<sup>b</sup>, Massimiliano M. Corsi Romanelli<sup>c,d</sup>

<sup>a</sup> Department of Biomedical, Surgical and Oral Science, Università degli Studi di Milano, Milan, Italy

<sup>b</sup> IRCCS Galeazzi Orthopaedic Institute, Milan, Italy

<sup>c</sup> Department of Biomedical Sciences for Health, Università degli Studi di Milano, Milan, Italy

<sup>d</sup> U.O.C. SMEL-1 Clinical Pathology, IRCCS Policlinico San Donato, San Donato (Milan) Italy

### ARTICLE INFO

#### Article history:

Received 4 June 2014

Received in revised form 15 October 2014

Accepted 30 November 2014

Available online 9 December 2014

#### Keywords:

Prosthetic joint infection

Serum biomarker

suPAR

Diagnosis

### ABSTRACT

Post-operative prosthetic joint infection (PJI) is the most common cause of failure of total joint arthroplasty, requiring revision surgery, but a gold standard for the diagnosis and the treatment of PJI is still lacking. PJI is mainly due to Gram-positive bacteria, in particular, *Staphylococcus Aureus*, and more rarely by Gram-negative bacteria such as *Pseudomonas*. This study aimed to examine the diagnostic value of SuPAR in post-operative PJI, in order to explore the possible application of this new biomarker in the early diagnosis of PJI. The level of SuPAR has been measured in PJI patients and healthy controls, correlated with canonical inflammatory markers, such as C-reactive protein, IL-6, IL-1 and TNF $\alpha$  and the chemokine CCL2. Serum suPAR displayed a strongly significant increase in PJI patients compared to not infected controls, and a significant positive correlation with C-reactive protein, IL-6, IL-1 and TNF $\alpha$  and the chemokine CCL2. Also serum CCL2 showed statistically significant increase in PJI patients, and it displayed a strong positive correlation with serum suPAR. This study provides a clear indication of the diagnostic potential of suPAR, in association to routine inflammatory parameters such as CRP, in the diagnosis of PJI.

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### 1. Introduction

Pathogen infection is one of the main adverse events of surgical procedures, which can lead to an excess of systemic inflammatory response and sepsis, causing multi organ failure and septic shock [1]. In particular, in orthopedic field, one of the most challenging complication associated with surgical procedures, especially hip and knee arthroplasty, is periprosthetic joint infection (PJI) [2,3]. PJI is the most common cause of failure of total joint arthroplasty, requiring revision surgery [4]. PJI is mainly due to Gram-positive bacteria, in particular, *Staphylococcus Aureus*, and more rarely by Gram-negative bacteria such as *Pseudomonas*. Currently, a large number of test are available for PJI diagnosis, ranging from hematological markers of infection and inflammation, intraoperative culture and histology analysis, and many can be used in combination [5]. Nevertheless, there is still a lack of gold standard for the diagnosis and the consequent treatment of PJI [6]. The clinical presentation is often ambiguous [7,8], and although classical inflammatory markers like C-reactive protein are helpful, they can be misleading in

some cases, such as patients with chronic disease, post-operative hematomas, obesity, metabolic syndrome and insulin resistance and smokers [9]. It is extremely important to identify PJI as early as possible in order to promptly start antibiotic treatment and, in the worse case, plan surgical revision arthroplasty [10]. Moreover, it is important to identify and isolate the invading pathogen in order to provide the best fitting antibiotic treatment. Indeed, the problem of antibiotic resistance is of considerable concern since the number of PJI caused by antibiotic-resistant pathogen is increasing [11]. In order to optimize the diagnostic process, infection biomarkers with fast response and high sensitivity and specificity for infection are needed. Biomarkers are defined as biological molecules that are characteristic of normal or pathogenic process, and therefore, they can be useful indicators for the clinical practice [12]. The two most widely studied serum biomarkers that are used in the diagnosis of infection and sepsis are C-reactive protein and Procalcitonin [13,14].

In this context, a previous study of our group investigated the possibility to improve the diagnosis of PJI, by analyzing the iron status of infected patients on the one hand [15], and invested on the role of procalcitonin, C-reactive protein and IL-6 as markers of post-operative orthopedics joint prosthesis infection. Results indicated that IL-6 and CRP were useful in the differentiation of patients with or without

\* Corresponding author at: Clinical Pathology, Department of Biomedical, Surgical and Oral Science, Università degli Studi di Milano, Via Luigi Mangiagalli 31, Milano I-20133, Italy. Tel.: +39 02 5031 5354; fax: +39 02 5031 5338.

post-operative PJI, while procalcitonin had not a great diagnostic value in this case [16]. Therefore, there is still the need to improve the diagnostic tools for the early detection of PJI.

Among the scenario of infections diagnosis, an emerging molecule is suPAR, the soluble urokinase plasminogen activation receptor, recently described as a powerful diagnostic and prognostic tool, able not only to detect sepsis but also to discriminate different grade of sepsis severity [17,18]. The urokinase-type plasminogen activator receptor (uPAR) is a glycoprotein released during inflammation and infection [19]. uPAR is expressed on various cells type, including lymphocytes, neutrophils, monocytes/macrophages, endothelial and malignant cells, and it is up-regulated in response to chemotactic stimuli, such as chemokines [20]. In concert with its ligand uPA, the receptor uPAR promotes the migration and adhesion of leukocytes by binding to  $\beta$ -integrins, and it has an important role in proliferation, angiogenesis and fibrinolysis. The soluble form of the receptor (suPAR) is obtained by proteolytic cleavage of uPAR from the cell surface. Like the membrane receptor, suPAR has a pivotal role in several immune function, ranging from cell adhesion, migration, chemotaxis, immune activation, to tissue remodeling, invasion and signal transduction [21]. After cleavage from the cell surface, suPAR can be found in blood and other organic fluids (cerebrospinal, bronchoalveolar) [18]. In healthy individuals, suPAR serum levels are low and quite stable while the serum concentration increases in condition that involves immune activation [21]. Indeed, high plasma suPAR levels have been found in patients with bacteremia [22], and moreover, high plasma levels of suPAR have been described to be able to predict severity of disease outcome in various infection such as bacteremia [23,24], HIV infection [25,26], bacterial meningitis, active pulmonary tuberculosis [27], nephrotic syndrome [28] and acute chest pain. Therefore, suPAR could find new fields of diagnostic application, where a clear detection of the infection is still lacking, such as prosthesis joint infection. So far, to our knowledge, evidences about the application of suPAR to the prosthesis joint field are still lacking. Therefore this is, to our knowledge, the first study about the diagnostic potential of suPAR applied to the field of orthopedic infection, which is an emerging problem in the clinical practice, leading to an increase of morbidity, hospitalization and, in the worse cases, even mortality and therefore requiring a timely diagnosis.

The present study aimed to examine the diagnostic value of suPAR in post-operative prosthetic joint infection, in order to explore the possible application of this new biomarker in the early diagnosis of operative prosthetic joint infection. In particular, the level of suPAR has been compared and correlated with canonical inflammatory markers, such as C-reactive protein, and a panel of inflammatory and anti-inflammatory cytokines in order to evaluate diagnostic potential of suPAR in the early detection of prosthetic joint infection. Moreover, suPAR is involved in leukocyte migration and in particular monocyte recruitment, mainly mediated by the chemokine CCL2. Since a positive correlation has been recently observed between CCL2 and suPAR in atherogenesis [29], we measured the serum level of the chemokine CCL2 in PJI patients and controls, and we correlated it with suPAR in order to evaluate the role suPAR in the inflammatory mechanism in prosthetic joint infection.

## 2. Materials and methods

### 2.1. Study participants

The population of 80 selected patients was enrolled and subdivided into two groups: 45 patients undergoing revision of total hip or total knee joint arthroplasty for prosthesis infection and control group of 35 not infected patients undergoing prosthesis revision without infection. The group of infected patients display prosthetic infection showed by clinical and laboratory signs typical of bone joint infection: swelling, erythema, joint pain and secretion of purulent material, positive cultures, with isolation of the causal agent in the infectious focus. In our study (and also in orthopedics), the term "septic" is referred to the

prosthesis not to the patient. The patients included in the study did not show any clinical sign of sepsis (as usually accepted: blood infections) but they presented diagnosis of septic failure of the prosthetic implant. Among the 50 infected patients, 46 resulted positive for Gram-positive bacteria (21 *S. Aureus*, 15 *Staphylococcus epidermis*, 1 *Staphylococcus xilosus*, 1 *Staphylococcus warnerii*, 1 *Staphylococcus caprae*, 1 *Streptococcus anginosus*, 1 *Streptococcus agalactie*, 2 *Enterococcus faecalis*, 2 *Corynebacterium striatum*) and 4 resulted positive for Gram-negative bacteria (1 coinfection *S. Aureus/Pseudomonas aeruginosa*, 1 *Klebsiella pneumoniae*, 1 *Pasteurella multocida*, 1 coinfection *S. Aureus/Acinetobacter baumannii*).

Blood drawings have been performed from all patients for serum separation and  $-80^{\circ}\text{C}$  storage.

This study was approved by the Ethics Review Board, in accordance the principles expressed in the Declaration of Helsinki, and the patients have signed an informed consent.

### 2.2. Determination of suPAR and inflammatory markers

Human suPAR serum concentration was determined using commercial double monoclonal antibody sandwich immunoassay, according to manufacturer's protocol (suPARNOSTIC Standard Kit, ViroGates, Denmark). The sensitivity of the test was 0.1 ng/mL, intra- and inter-assay coefficients of variation were 3.5% and 5.1%, respectively.

Human IL-1, IL-6 and TNF alpha and CCL2 were measured using an ELISA sandwich Quantikine Assay, according to manufacturer protocol (R&D System, Minneapolis, MN, USA).

For IL-1 detection assay, the sensitivity of the test was 1 pg/mL; intra- and inter-assay coefficients of variation were 2.8% and 4.1%, respectively. For IL-6 detection assay, the sensitivity of the test was 0.7 pg/mL, intra- and inter-assay coefficients of variation were 1.7% and 2.0%, respectively. For CCL2 detection assay, the sensitivity of the test was 10 pg/mL, intra- and inter-assay coefficients of variation were 4.2% and 4.5%, respectively. For TNF alpha detection assay, the sensitivity of the test was 5.5 pg/mL, intra- and inter-assay coefficients of variation were 4.6% and 5.4%, respectively.

Human IL-10, IL-8, IL-12 and IL-1ra were measured by Luminex assay, according to Manufactures protocol (R&D System, Minneapolis, MN, USA). In particular, IL-10 was measured by Luminex Performance Assay kit and IL-8, IL-12 and IL-1ra by Screening Assay kit.

CRP was measured using immunoturbidimetry on an automated biochemical analyzer (Olympus CRP-Latex assay, Central Valley, PA, CA, USA).

### 2.3. Data analysis and statistics

For all the parameters analyzed, normality of distribution of the groups was verified by KS normality.

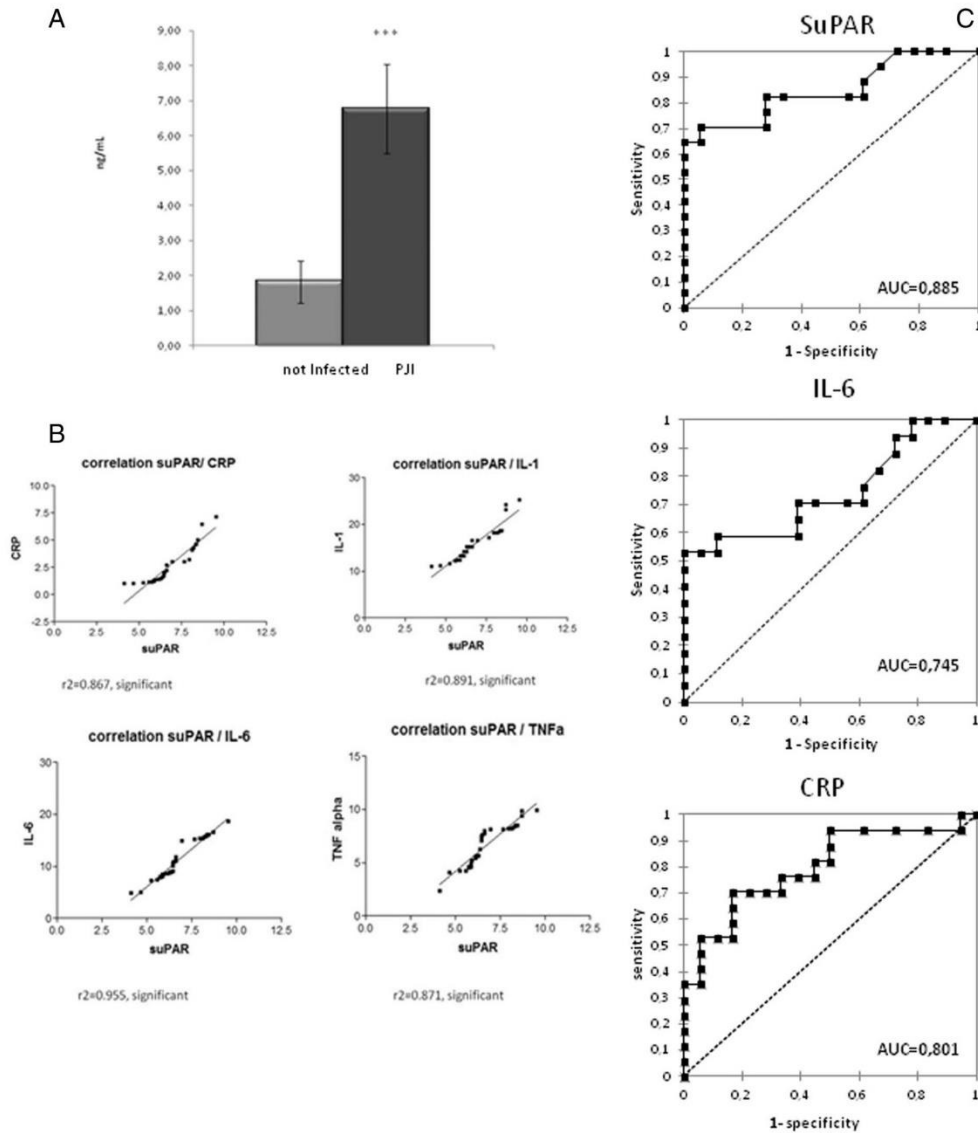
Statistical analysis was performed using a one-way ANOVA test;  $p < 0.05$  was considered significant and  $p < 0.005$  very significant. Data are expressed as the mean  $\pm$  standard deviation (SD).

Correlation analysis was measured using PRISM 3.0 software, by performing linear regression analysis between the different groups of data and by calculating the 95% confidence interval of the regression line.

Statistical analysis of receiver operating characteristic (ROC) curves and area under the curve (AUC) was performed by MedCalc 13.2.2 Software (Ostend, Belgium).

## 3. Results

The serum concentration of suPAR in PJI patients and controls is shown in Fig. 1A. In control patients, suPAR level is very low, with a mean value of  $1.813 \pm 0.114$  ng/mL, clearly below the cutoff value reported in the literature in healthy controls [30].



**Fig. 1.** suPAR serum level, correlation with other serum inflammatory markers and ROC analysis suPAR serum. Panel A: Serum levels of suPAR in prosthetic joint infection patients (dark grey bar) and not infected control patients (light grey bar). Data are expressed as the mean  $\pm$  standard deviation (SD). Panel B: suPAR serum level correlation with other serum inflammatory markers. Linear correlation between serum levels of suPAR and the inflammatory parameters CRP (C-reactive protein), IL-1 (interleukin-1), IL-6 (interleukin-6) and TNF $\alpha$  (tumor necrosis factor  $\alpha$ ). The correlation is expressed as  $r^2$  (the more  $r^2$  value are close to 1, the more are considered significant). Panel C: receiver operating characteristic (ROC) curves for SuPAR and other diagnostic marker of PJI. Statistical analysis of receiver operating characteristic (ROC) curves of SuPAR (panel A), IL-6 (panel B) and C-reactive protein (CRP, panel C).

Conversely, PJI displayed a strongly significant increase ( $p < 0.0001$  compared to not infected controls) of serum suPAR, reaching a mean value of  $6.76 \pm 0.226$  ng/mL. In order to evaluate the diagnostic value of suPAR, we compared serum level of this parameter in PJI with the serum levels of the main inflammatory and infection markers (C-reactive protein, IL-1, IL-6 and TNF- $\alpha$ ). Fig. 1B describes the results of linear regression analysis of serum suPAR with C-reactive protein, IL-1, IL-6 and TNF- $\alpha$ . In all the cases, suPAR displayed a positive and strongly significant correlation ( $p < 0.0001$  in all the cases) with all the parameters analyzed ( $r^2 = 0.0867, 0.891, 0.955$  and  $0.871$  with C-reactive protein,

IL-1, IL-6 and TNF- $\alpha$ , respectively). In order to complete the inflammatory status evaluation, Luminex assay were performed on the cytokines IL-10, IL-12, IL-8 and IL-1ra on serum sample of PJI patients and non-infected controls (Supplementary material). IL-12 and IL-10 display little, if none, difference between PJI patients and non-infected controls (Fig. 1A and B), while IL-8 displayed a strongly significant increase in PJI patients compared to not infected controls (Fig. 1C). IL-1ra showed quite high levels in both the groups, with a significant lower level in PJI patients (Fig. 1D). For these two cytokines displaying statistically significant differences between PJI patients and non-infected controls, a

correlation analysis with serum SuPAR was performed. As shown in Fig. 1E, serum SuPAR displayed a strong positive correlation with IL-8 ( $r^2 = 0.907$ ), while it showed a strong negative correlation with IL-1ra ( $r^2 = 0.892$ , Fig. 1F).

In order to compare the diagnostic potential of SuPAR to the inflammatory markers currently used in the diagnosis of PJI (CRP and IL-6), receiver operating characteristic (ROC) was performed, as shown in Fig. 1A, B and C, respectively, and the area under the curve resulted in 0.745, 0.801, 0.885 for CRP, IL-6 and SuPAR, respectively.

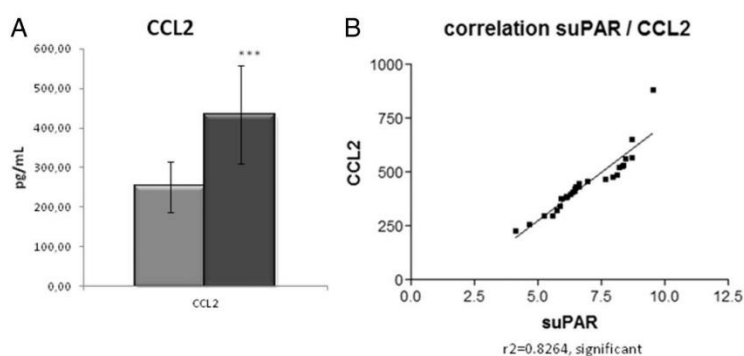
In order to further evaluate the suPAR regulatory mechanism in the host response to the infection, we analyzed the role of the main chemokine involved in monocyte/macrophage action, CCL2. As shown in Fig. 2, the serum level of CCL2 showed a non-dramatic but statistically significant increase in PJI patients ( $251.61 \pm 12.26$  pg/mL and  $433.51 \pm 22.04$  pg/mL in controls and PJI patients, respectively). Moreover, it displayed a strong positive correlation (Fig. 2B) with serum suPAR ( $r^2 = 0.864$ ).

#### 4. Discussion

In the present study, in order to evaluate the diagnostic potential of serum concentration of suPAR in prosthetic joint infection, SuPAR serum level were measured in patients with PJI and patients undergoing prosthesis revision without infection. On the one hand, SuPAR level did not reach the high level measured in severe sepsis [12]; on the other hand, it resulted clearly higher in PJI patients compared to not infected patients. Recently, a plasmatic level of 9.25 ng/mL was defined as the cutoff for mortality risk, while a cutoff value of 6 ng/mL has only 63% sensitivity and 60% specificity predicting ICU mortality [30]. Our PJI patients displayed a value of suPAR, with a mean value of  $6.76 \pm 28$  ng/mL, which account for a clear infection but low risk of mortality. This result can be explained by the condition of the patients analyzed, where the infection is at the initial stage, and it has not reached the stadium of severe sepsis, ultimately leading to the risk of mortality. So far, indeed, their main clinical utility of SuPAR was referred to the prognostic level, as various studies defined the high plasmatic level of suPAR as a powerful biomarker for the prognosis of mortality in sepsis, severe infection [22,31] and critically ill patients [32], but the diagnostic potential of this markers still lacks a precise definition. In recent studies, higher plasmatic levels of suPAR have been described in bacteremia and endotoxemia [33] and critically ill patients [30], defining a cutoff value of 5.5 ng/mL with a sensitivity of 75% and a specificity of 72% for diagnosis sepsis. In these studies, suPAR plasmatic concentrations were closely related to other sepsis markers (CRP, PCT and TNF $\alpha$ ), even with a lower diagnostic accuracy compared to these canonical sepsis markers [18].

In our study, we wanted to explore the diagnostic potential of SuPAR in the detection of infection even at early stage, when the prompt diagnosis is extremely important to start the pharmacological therapy or the surgical revision, in order to prevent further complication for the patient. In particular, we compared the diagnostic potential of SuPAR with canonical inflammatory biomarkers, such as C-reactive protein (CRP), and the main inflammatory cytokines: IL-6, TNF alpha and IL-1, already measured in the two groups of patients in a previous study of our group [34]. All these molecules showed statistically significant increase in PJI compared to not infected patients, in particular, CRP and the cytokine IL-6, as demonstrated by a previous study of our group. In addition, our previous study showed that PJI patients displayed an statistically significant increase of IL-1 and TNF alpha, thus confirming the ongoing inflammatory reaction [34]. On the one hand, the analysis of this panel of inflammatory biomarkers can be very useful in the diagnosis of prosthesis joint infection; on the other hand, they still need to be improved because they still lack a reliable cutoff value. PCT and CRP are currently used on a routine basis for diagnosis and monitoring of treatment of sepsis in critically ill patients, but they still present some limits. Recent findings indicated that PCT levels may be low or indeterminate in the early stage of disease [35,36], or it can be high in not infection condition [37]. Indeed, in a previous study of our group on PJI patients, PCT did not show a significant difference between infected and not infected patients [16]. Similarly, CRP has been reported to have no differential capacity with regard to sepsis, severe sepsis, septic shock or septic complication in patients with trauma, in the post-traumatic period [38]. These finding showed that PCT and CRP can usefully be used in bacteremia diagnosis but are insufficient in terms of differential diagnosis, and these parameters cannot be used as prognostic marker. In particular, in our PJI patients, the level of plasmatic CRP was not so high to be a clear and univocal indicator of bacterial infection. This further enhances the diagnostic importance of suPAR [39]. To this purpose, in order to clearly show the diagnostic improvement given by of SuPAR in our condition of PJI diagnosis, we compare the AUC of ROC curve of serum SuPAR with the two main inflammatory markers currently used in PJI diagnosis, IL-6 and CRP. As showed in Fig. 1, CRP did not display an efficient AUC in detecting PJI patient, and IL-6 AUC was slightly higher but not very efficient. On the contrary, SuPAR displayed a very good AUC, significantly higher than CRP and IL-6 AUC. This result clearly shows that the measure of serum level of SuPAR provides an extremely important benefit because it is a precise indicator of bacterial infection, and the addition of SuPAR serum level measurement to classical inflammatory markers can strongly improve the diagnosis of prosthesis joint infection.

The diagnostic and prognostic value of suPAR was measured so far in condition of severe sepsis in order to predict the risk of mortality. In this



**Fig. 2.** CCL2 serum level and correlation with serum suPAR. Panel A: serum concentration of chemokine CCL2, prosthetic joint infection patients (dark grey bar) and not infected control patients (light grey bar). Data are expressed as the mean  $\pm$  standard deviation (SD). Panel B: linear correlation between serum levels of CCL2 and serum levels of suPAR. The correlation is expressed as  $r^2$  (the more  $r^2$  value are close to 1, the more are considered significant).

context, suPAR displayed a weak correlation with canonical inflammatory markers, such as IL-6 and CRP [12]. In our case, suPAR displayed a positive correlation with all the inflammatory markers analyzed, suggesting that this biomarker can be very suitable to the diagnosis of prosthetic joint infection. In order to complete the inflammatory status of PJI patients, the serum levels of the main inflammatory and anti-inflammatory cytokine were measured in serum. No significative differences were observed for the anti-inflammatory cytokines IL-12 and IL-10, while a strong significative increase of IL-8, the main mediator of acute inflammation, was observed in PJI patients, confirming the ongoing inflammatory response. The inflammatory response is under the control of negative regulators, mainly the anti-inflammatory cytokine IL-1ra (IL-1 receptor antagonist). In our patients, IL-1ra resulted quite high in both the groups, compared to physiological range, probably due to surgery stress, but it clearly resulted in lower in PJI patients, suggesting that in these patients the inflammatory balance is shifted toward an inflammatory response against the ongoing infection. Accordingly, SuPAR serum level showed a strong positive and a strong negative correlation with IL-8 and IL-1ra, respectively, clearly indicating that SuPAR strictly reflects the ongoing inflammatory response to the invading pathogen. Therefore, it can be considered a good marker bacterial infection.

At present, it is still unclear whether plasmatic suPAR actually exerts a proinflammatory role or it just reflects an inflammatory condition. Further studies are still needed to clarify the regulatory mechanism of suPAR in the host response to infection. Being involved in leukocyte migration, a positive association between SuPAR and CC chemokines has been recently described, in particular, CCL2, a CC chemokines involved in monocyte/macrophage action in the inflammatory response [40]. In order to study the regulatory mechanism of suPAR in prosthetic joint infection, we investigated the possible involvement of chemokine CCL2 in suPAR regulated inflammatory response in PJI. We analyzed the serum level of CCL2 in PJI and, even if it did not display a strong increase compared to control patients, it is statistically significant and, more interestingly, it strongly correlates with SuPAR levels, underlining the diagnostic potential of this biomarker in PJI.

This study analyzed for the first time, to our knowledge, the diagnostic potential of suPAR in the orthopedic field, in particular, in the context of prosthetic infection, which still lacks clear diagnostic golden standard. To our knowledge, this is the first study correlating the serum levels of SuPAR to a panel of inflammatory and anti-inflammatory cytokine and, moreover, comparing the ROC curve of SuPAR to CRP and IL-6 in the diagnosis of PJI in order to define the best diagnostic value.

Being a pilot study, a limit of this study can be represented by the low number of patients, but it provides a clear indication of the diagnostic potential of suPAR, in association to routine inflammatory parameters such as CRP, in the diagnosis of prosthetic joint infection.

#### Acknowledgment

The authors acknowledge ViroGates, Denmark, and B.S.N, Castelleone (CR) Italy for providing the suPAR/NOSTIC Standard Kit.

The authors also acknowledge the Italian Ministero dell' Istruzione, Università e Ricerca (MIUR) and Italian Ministero della Salute for providing funds for this research project.

#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.cca.2014.11.029>.

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