Dissertation

Novel Biomarkers in Joint Infection Diagnosis

submitted by

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for the Academic Degree of

Doctor of Medical Science
Dr. scient. med.

at the
Medical University of Graz

Department of Orthopaedics and Trauma

under the Supervision of

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Submitted in 2022

Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the "Guidelines of the Medical University of Graz on Good Scientific Practice".

Graz, 26.6.2022

Disclosures

Part of this thesis has been published in S. M. Klim, F. Amerstorfer, G. Glehr, G. Hauer, M. A. Smolle, L. Leitner, A. Leithner, M. Glehr. „Combined serum biomarker analysis shows no benefit in the diagnosis of periprosthetic joint infection" (2020; International Orthopaedics (1)). All co-authors have agreed to the inclusion of the published data in the dissertation, and permission to reproduce illustrations and figures from own or third-party publications has been granted.

Figure 1 - EBJIS criteria for the diagnosis of clinically suspected periprosthetic joint infection. Reproduced from McNally et al. (BJJ 2020 (2)) with permission of publisher "The British Editorial Society of Bone & Joint Surgery".

Table 1 – PJI definition scoring system. According to the ICM (MSIS) PJI criteria proposal of 2018. Reproduced from Parvizi et al. The 2018 Definition of Periprosthetic Hip and Knee Infection: An Evidence-Based and Validated Criteria. (J Arthroplasty. 2018 (3)) with permission of publisher "Elsevier".

Figure 17 - ROC curves depicting the performance of the calculated multi-biomarker model. Reproduced from Klim et al. (Int Orthop. 2020 (1)) with permission of publisher "Springer".

Acknowledgements

Doctoral student S.M.K. received consumable cost funding from the Medical University of Graz through the Doctoral School Bones, Muscles, Joints.

I would like to express my sincere thanks to my thesis supervisors Prof. Martin Stradner and his team, and Dr. Heinz Winkler for their help in the conceptualisation of the thesis and its implementation.

Foreword

Conducting a prospective study and successfully writing a dissertation initially requires a motivated dissertation candidate but can only succeed with the right environment of supervisors and mentors.

Therefore, I would like to take this opportunity to thank Prof. Mathias Glehr, the chair of my dissertation committee, for his guidance and support in both scientific and clinical matters. Together with Dr. Amerstorfer, whom I would like to thank in particular, we have successfully published several scientific papers in recent years, starting with my diploma thesis. I was also able to greatly learn from them in the course of my clinical work, which made us not only colleagues but also friends.

Furthermore, I would like to thank Prof. Tobias Madl and Doz. Jürgen Prattes for their cooperation in this joint project.

My thanks and appreciation to all colleagues who were involved in the process of patient recruitment for the present study.

Thanks are also due to Prof. Leithner, the head of the University Clinic for Orthopaedics and Traumatology, who always strives to create and maintain a supportive and appreciative environment for young clinical scientists.

Finally, I would like to thank my family, friends, and Stefanie for their unconditional support and motivation, and who made it all worthwhile.

Table of Contents

1. Introduction **1 – 25**

1.1 Epidemiology and Risk Factors 1 – 4

1.1.1 Septic Arthritis 1 – 2

1.1.2 Aseptic Arthritis 2 – 3

1.1.3 Periprosthetic Joint Infection (PJI) 3 – 4

1.2 Microbiology 4 – 6

1.2.1 Septic Arthritis 4 – 5

1.2.2 Periprosthetic Joint Infection 5 – 6

1.3 Diagnosis 6 – 22

1.3.1 Septic Arthritis 6 – 10

1.3.1.1 Clinical Features 7

1.3.1.2 Laboratory and Microbiology 8

1.3.1.3 Synovial Biomarkers 8 – 10

1.3.2 Periprosthetic Joint Infection 10 – 21

1.3.2.1 Clinical Features and PJI Definition(s) 10 – 15

1.3.2.2 Serum Biomarkers 15 – 18

1.3.2.3 Synovial Biomarkers 18 – 21

1.3.3 Novel Biomarkers 21 – 22

1.4 Therapy 22 – 25

1.4.1 Native Arthritis 22 – 23

1.4.2 Periprosthetic Joint Infection 23 – 25

1.5 Study Questions 25

2. Methods **26 – 29**

2.1 Novel Methods in Synovial Analysis 26 – 28

2.2 Multi Biomarker Model 29

3. Results **30 – 47**
3.1 Metabolomic Profiling via NMR 31 – 40
3.1.1 Diagnostic Performance of NMR in PJI 31 – 33
3.1.2 Diagnostic Performance of NMR in SA 34 – 36
3.1.3 Diagnostic Performance of NMR in the Total Study Population 37 – 40
3.2 Soluble urokinase plasminogen activator receptor (suPAR) 41 – 46
3.2.1 Diagnostic Performance of suPAR in PJI 41 – 43
3.2.2 Diagnostic Performance of suPAR in SA 44 – 46
3.3 Multi Biomarker Model 47

4. Discussion **48 – 58**
4.1 Metabolomic Profiling via NMR 48 – 51
4.1.1 Context of the Current Literature 48 – 50
4.1.2 Implications and Outlook 51
4.2 Soluble Urokinase Plasminogen Activator Receptor (suPAR) 51 – 54
4.1.1 Context of the Current Literature 51 – 53
4.1.2 Implications and Outlook 54
4.3 Multi Biomarker Model 54 – 56
4.1.1 Context of the Current Literature 54 – 56
4.1.2 Implications and Outlook 56
4.4 Strengths and Limitations 57
4.5 Conclusion 58

5. References **59 – 73**

6. Appendix **74**

Figures and Tables

Figure 1 - EBJIS criteria for the diagnosis of clinically suspected periprosthetic joint infection. Reproduced from McNally et al. (BJJ 2020 (2)) with permission of publisher "The British Editorial Society of Bone & Joint Surgery" 12

Figure 2 – Up- (red) and down- (blue) regulation of specific metabolites in patients with PJI measured via NMR spectroscopy (not significant = grey). 31

Figure 3 - Orthogonal partial least squares discriminant analysis (OPLS-DA) regression model of aseptic ("2"; red) and septic (PJI) cases ("4", green). T score 18.8%, orthogonal T score 20.3%. 32

Figure 4 - ROC curves including the true positive rate, the false positive rate and the standard deviation of the best performing metabolite ratios in the diagnosis of PJI. 33

Figure 5 – Up- (red) and down- (blue) regulation of specific metabolites in patients with SA measured via NMR spectroscopy (not significant = grey). 34

Figure 6 - Orthogonal partial least squares discriminant analysis (OPLS-DA) regression model of aseptic ("1"; red) and septic (native joint) cases ("3", green). T score 17.8%, orthogonal T score 19.6%. 35

Figure 7 - ROC curves including the true positive rate, the false positive rate, and the standard deviation of the best performing metabolites and metabolite ratios in the diagnosis of SA. ... 36

Figure 8 - sparse partial least squares discriminant analysis (sPLS-DA) model of septic ("1"; red) aseptic ("2", green), gout ("3", purple), and CPPD arthritis ("5", blue). 37

Figure 9 - The individual metabolites and the strength of their expression in the respective groups (features). 1= septic, 2= aseptic, 3= gout, 5= CPPD arthritis. 38

Figure 10 - Prediction value = Q^2 quantifying the algorithms discrimination power between different groups on an independent test set (sPLS-DA). 1= septic, 2= aseptic, 3= gout, 5= CPPD arthritis. 39

Figure 11 - Mannose ($p<0.0001$) and Glucose ($p<0.0001$) as the two best performing single metabolites in sepsis diagnosis (total study cohort). 39

Figure 12 - one-way ANOVA calculation of differences among eight metabolites. The boxes extend from the 25th to 75th percentile, the bars represent 2.5-to-97.5 percentiles. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 40

Figure 13 - ROC curves including the AUC, sensitivity, and specificity of the synovial (above) and plasma (below) suPAR measurements in the prosthesis group. 42

Figure 14 - ROC curves including the AUC, sensitivity, and specificity of the serum leucocytes (WBC, above) and C-reactive protein (CRP, below) measurements in the prosthesis group. 43

Figure 15 - ROC curves including the AUC, sensitivity, and specificity of the plasma (above) and synovial (below) suPAR measurements in the native joint group. 45

Figure 16 - ROC curves including the AUC, sensitivity, and specificity of the serum leucocytes (WBC, above) and C-reactive protein (CRP, below) measurements in the native joint group. 46

Figure 17 - ROC curves depicting the performance of the calculated multi-biomarker model (AUC 0.95, specificity 91%, sensitivity 72%; continuous line) in PJI diagnosis. CRP (AUC 0.91, specificity 67%, sensitivity 90%; small dotted line) and fibrinogen (AUC 0.93, specificity 73%, sensitivity 94%; big dotted line) for comparison. Reproduced from Klim et al. (Int Orthop. 2020 (1)) with permission of publisher “Springer”. 47

Table 1 - PJI definition scoring system according to the ICM (MSIS) PJI criteria proposal of 2018. Leukocyte esterase (LE); (a) for patients with inconclusive minor criteria, operative criteria can also be used to fulfil definition for PJI. (b) Consider further molecular diagnostics such as next-generation sequencing. Reproduced from Parvizi et al. The 2018 Definition of Periprosthetic Hip and Knee Infection: An Evidence-Based and Validated Criteria. (J Arthroplasty. 2018 (3)) with permission of publisher "Elsevier"……………………………13

Table 2 – Result overview. 1 best performing metabolite combination; 2 best performing single metabolite; *evaluated on a different data set; NMR= Nuclear Magnetic Resonance; suPAR= Soluble urokinase plasminogen activator receptor; CRP= C-reactive protein; WBC= white blood cell (leucocyte) count; AUC= area-under-the-curve……………………………..30

Abbreviations and Definitions

AA	aseptic arthritis
SA	septic arthritis
PJI	periprosthetic joint infection
CPPD	calcium pyrophosphate deposition disease
TKA	total knee arthroplasty
THA	total hip arthroplasty
RR	risk ratio
LR	likelihood ratio
MRSA	methicillin-resistant Staphylococcus aureus
MSSA	methicillin-sensitive Staphylococcus aureus
CI	confidence interval
ESR	erythrocyte sedimentation rate
WBC	white blood cell count
CRP	c-reactive protein
IL-6	interleukin-6
PMN %	polymorphonuclear cell percentage
CT	computed tomography
AUC	area-under-the-curve
mPCR	multiplex polymerase chain reaction
EBJIS	European Bone and Joint Infection Society
MSIS	Musculoskeletal Infection Society
ICM	international consensus meeting
IDSA	Infectious Diseases Society of America
LE	leukocyte esterase
PCT	procalcitonin
ELISA	enzyme-linked immunosorbent assay
NPV	negative predictive value

PPV	positive predictive value
NMR	nuclear magnetic resonance
suPAR	soluble urokinase plasminogen activator receptor
DAIR	debridement, antibiotics and implant retention
PMMA	polymethylmethacrylate
EDTA	Ethylenediaminetetraacetate
IF-alpha	Interferon alpha
ROC curve	receiver operating characteristic curve
OPLS-DA	Orthogonal partial least squares discriminant analysis
sPLS-DA	sparse partial least squares discriminant analysis
ANOVA	analysis of variance
NLR	neutrophil–lymphocyte ratio
PLR	platelet count–lymphocyte ratio

Zusammenfassung

Einleitung Die rasche und genaue Diagnose von septischen Arthritiden (SA) und periprothetischen Gelenksinfektionen (PJI) ist von zentraler Bedeutung für das Therapieoutcome dieser Erkrankungen. Der Einsatz von Biomarkern brachte deutliche Fortschritte in der Diagnostik, jedoch haben die aktuellen Biomarker nicht die diagnostische Leistungsfähigkeit um als alleiniges Entscheidungskriterium herangezogen zu werden. Daher ist das Ziel dieser Dissertation und der zugrundeliegenden Studie die Untersuchung der Eigenschaften der Metabolomanalyse mittels Kernspinresonanz (NMR), des löslichen Urokinase-Plasminogen-Aktivator-Rezeptor (suPAR) als potentiell neue Analysemethoden sowie der Kombination verschiedener Biomarker in einem Multi-Biomarker Modell in der Diagnostik von SA und PJI.

Methoden

Patienten mit klinischem Verdacht auf PJI oder SA, bei denen in unserer Einrichtung innerhalb eines Zeitraums von 32 Monaten eine diagnostische Arthrozentese durchgeführt wurde, wurden prospektiv bezüglich des Studieneinschlusses evaluiert. Die Gruppenzuordnung (septisch/aseptisch) erfolgte nach den EBJIS-Kriterien (PJI) und den modifizierten Newman-Kriterien (SA). Nach der Entnahme von Synovialflüssigkeit und Blutplasma wurden die Proben bei -80°C gelagert. Der SuPAR-Spiegel wurde mit einem kommerziellen Sandwich-Immunoassay (suPARnostic®, ViroGates A/S, Dänemark) bestimmt. Die NMR-Messungen wurden bei 310 K mit einem AVANCE™ NeoBruker Ultrashield 600 MHz Spektrometer durchgeführt. Die Bruker Topspin Version 4.0.2 wurde für die NMR-Datenerfassung verwendet. Insgesamt wurden 37 Metaboliten analysiert. Das Multi-Biomarker-Modell wurde anhand eines Datensatzes berechnet, der 124 Knie- bzw. Hüftrevisionoperationen umfasste. Für die Biomarker und alle ihre Verhältnisse wurde eine logistische Regression verwendet. Nach einer Kreuzvalidierung unter Verwendung eines Trainingsdatensatzes (75% aller Fälle) zur Ermittlung der besten diagnostischen Aussagekraft wurde das endgültige Modell an einem separaten Satz (25%) getestet.

Ergebnisse

Insgesamt wurden 182 Proben inkludiert. Die diagnostischen Eigenschaften wurden getrennt für SA- und PJI-Fälle berechnet. Plasma suPAR (AUC 0,74 bei SA und 0,40 bei PJI) und synoviale suPAR (AUC 0,87 bei SA und 0,76 bei PJI) konnten Serum CRP (AUC 0,81 bei SA und 0,79 in PJI Fällen) nicht übertreffen. Die NMR zeigte die besten diagnostischen Ergebnisse, wenn Metaboliten-Verhältnisse berechnet wurden: Isobuttersäure/Methionin (AUC 0,9462) bei SA und Glucose/Glykogen (AUC 0,9073) bei PJI. Die besten Einzelmetaboliten waren Mannose (AUC 0,8558) bei SA und Taurin (AUC 0,8558) bei PJI. Das errechnete Multi-Biomarker-Modell für PJI umfasste Fibrinogen, CRP, das Verhältnis von Fibrinogen zu CRP und das Verhältnis von Serumthrombozyten zu CRP (AUC 0,92; Spezifität 0,92 und Sensitivität 0,68).

Zusammenfassung

Die Untersuchung neuer Biomarker für die Diagnose von Gelenkinfektionen erbrachte sowohl positive als auch negative Ergebnisse. Die Ergebnisse zeigen, dass die NMR-Metabolomanalyse eine vielversprechende Methode für die Diagnose sowohl der septischen Arthritis als auch der PJI ist. Diese Analysemethode zeigte durchweg die beste diagnostische Leistung. Eine Einschränkung ist die derzeit begrenzte Verfügbarkeit in der klinischen Praxis. Der bereits kommerziell erhältliche suPAR-ELISA-Test lieferte keine signifikante diagnostische Verbesserung, die die im Vergleich zur venösen Blutentnahme invasivere Probenentnahme einer Gelenkpunktion rechtfertigen würde. Das Multi-Biomarker-Modell zeigte eine gute diagnostische Leistung. Es muss jedoch berücksichtigt werden, dass dieses Modell keine signifikante Verbesserung im Vergleich zu den Ergebnissen der einzelnen Biomarker (CRP, Fibrinogen) auf dem verwendeten Datensatz darstellt.

Abstract

Introduction The rapid and accurate diagnosis of septic arthritis (SA) and periprosthetic joint infection (PJI) is of central importance for the therapeutic outcome of these diseases. The use of biomarkers has brought significant advances in diagnosis, but current biomarkers do not have the diagnostic power to be used as the sole decision criterion. Therefore, the aim of this dissertation and the underlying study is to investigate the properties of nuclear magnetic resonance (NMR) metabolomics, soluble urokinase plasminogen activator receptor (suPAR) as potential new biomarkers, and the combination of different biomarkers in a multi-biomarker model in the diagnosis of SA and PJI.

Methods

All patients with a clinical suspicion of PJI or SA who underwent a diagnostic arthrocentesis at our institution within a 32-month period were prospectively evaluated for study enrolment. Group assignment (septic vs. aseptic) was performed according to the EBJIS criteria (PJI) and modified Newman criteria (SA). After synovial fluid and blood plasma collection, the samples were stored at -80°C. SuPAR levels were determined using a commercially available sandwich immunoassay (suPARnostic®, ViroGates A/S, Denmark). The NMR experiments were performed at 310 K on an AVANCE™ NeoBruker Ultrashield 600 MHz spectrometer. Bruker Topspin version 4.0.2 was used for NMR data acquisition. In total, 37 metabolites were analysed using a volume of 200 µl per synovial sample. The multi-biomarker model was calculated on a dataset including 124 knee or hip revision arthroplasty procedures. Logistic regression was used for the biomarkers and all their ratios. After a cross-validation using a training sample set (75% of all samples) for best performance estimates, we evaluated the final model on a separate set (25%).

Results

In total, 182 cases were included. Diagnostic properties were determined separately for SA and PJI cases. Plasma suPAR (AUC 0.74 in SA and 0.40 in PJI cases) and synovial suPAR (AUC 0.87 for SA and 0.76 for PJI cases) could not outperform serum CRP (AUC 0.81 in SA and 0.79) in PJI cases. NMR has shown the best diagnostic results when metabolite ratios were calculated: Isobutyric acid/Methionine (AUC 0.9462) in SA and Glucose/Glycogen (AUC 0.9073) in PJI. The best single metabolites were Mannose (AUC 0.8558) in SA and Taurine

(AUC 0.8558) in PJI. The final multi-biomarker model for PJI included Fibrinogen, CRP, the ratio of Fibrinogen to CRP, and the ratio of serum Thrombocytes to CRP (AUC 0.92, accuracy 0.77, specificity 0.92, sensitivity 0.68).

Conclusion

The investigation of new biomarkers in the diagnosis of joint infections yielded both positive and negative results. Results indicate that NMR metabolome analysis is a promising method for diagnosing both septic arthritis and PJI. This analysis method consistently showed the best diagnostic performance. A limitation is the currently limited availability in clinical practice. The already commercially available synovial suPAR ELISA test did not provide any significant diagnostic value that would justify the more invasive sample collection of a joint puncture compared to venous blood sampling. The multi-biomarker model showed a good diagnostic performance. However, it must be considered that this model does not represent a significant improvement compared to the results of the individual biomarkers (CRP, fibrinogen) on the used dataset.

1. Introduction

Arthritis represents an inflammatory condition of a joint due to different causes. Currently, there are over 100 different arthritis types described (4). A useful subdivision is done into primary and secondary (reactive) arthritis. In primary arthritis, the joint pain and the local symptoms are predominant. In secondary arthritis, the joint problems occur as a concomitant symptom within the framework of the primary disease and other organ systems are frequently affected. However, the most important distinction in clinical work is done between septic (SA, bacterially caused) and aseptic arthritis (AA). This distinction is essential for an optimal medical treatment and a quick recovery of the patient. The same applies to the diagnostic process following total joint arthroplasty. Although native septic arthritis and periprosthetic joint infection (PJI) differ in terms of pathogenesis and surgical therapy, the presence or absence of bacteria remains decisive for diagnosis. For this reason, the present thesis is divided into two parts. When necessary and appropriate, native joints (and the target pathology, septic arthritis (SA)) and joints following arthroplasty (PJI) are considered and discussed separately. In the methodological part of the thesis, however, this division is obsolete in view of the identical procedure regarding patient recruitment, sample collection, and analysis.

The following introduction will give an overview on the epidemiological aspects of both pathologies as well as the known risk factors, and the causative microorganisms. The subsequent part will outline and discuss the current standard procedure in clinical, serum, and synovial diagnostics in SA and PJI. Finally, the last section will address the medical and surgical therapy options, to underline why the differentiation between septic and aseptic joint inflammation is crucial and a fast diagnosis is mandatory in both native joints and joints after arthroplasty.

1.1 Epidemiology and Risk Factors

1.1.1 Septic Arthritis

Due to the rarity of SA, the heterogenous inclusion criteria, and the mostly small, retrospective study cohort, an exact epidemiological survey is difficult. The incidence in Western Europe’s general population is estimated at four to ten cases per 100,000 patient-years (5, 6). Studies

show an increased risk of SA in children, especially under two years of age, as well as in older patients, men, and patients with low socioeconomic status (7, 8). While no additional risk factor for the occurrence of SA can be found in about 22% of patients (5), there are some other risk factors that are worth mentioning: Preexisting joint pathologies, diabetes, intravenous drug use as well as advanced liver and kidney damage, and skin diseases such as ulcers, psoriasis, and eczema (6, 9-11). Several medical procedures have also shown an increased risk of postinterventional SA. Among these are arthroscopy, where studies report a SA rate of 14 per 10.000 interventions, and intraarticular corticosteroid injection with 4 cases per 10.000 interventions (12).

1.1.2 Aseptic Arthritis

As already mentioned, this disease group consists of a very heterogeneous field of various pathologies. What they all have in common is the primary or secondary inflammation of one or more joints, leading to a variety of symptoms ranging from mild to most severe. In acute recurrent or chronic courses, the picture of increasing joint destruction up to mutilation can be observed. An epidemiological description of all AAs would go beyond the scope of this thesis. Therefore, only the most frequent ones are mentioned here, as they are potential differential diagnoses for SA and thus represent study relevant entities.

Osteoarthritis is affecting over 40 million people in Europe (13). Risk factors include age, genetics, diet, obesity, muscle weakness, and joint laxity (14). Based on chronic osteoarthritis, an activated osteoarthritis can develop, showing the clinical symptoms of acute arthritis. A complex cascade of cytokines and other inflammatory mediators is activated leading to synovitis and accelerated joint destruction (15).

Rheumatoid arthritis is a chronic autoimmune disorder affecting 0,5 to 1% of all adults in the developed world, with a decreasing incidence from north to south and from cities to countryside (16). Smoking, a lower socioeconomic status, as well as positive family history/genetic factors (3-5 times) increase the risk for rheumatoid arthritis (17, 18). Inheritance of rheumatoid arthritis seems likely but with different frequencies depending on the type (seropositive 40-65%, seronegative ~20% (19)).

Gout usually occurs in flares caused by the accumulation of monosodium urate crystals in a joint, with high blood serum concentrations of urate (hyperuricemia) being the most important risk factor. Besides the typical arthritis symptoms, which have been already described, a rapid onset of symptoms and stabbing pain in the affected joint are typical for gout. The prevalence of gout has risen in the 20th century. With an incidence ranging between 0,6 and 2,9 per 1000 person years and prevalence ranging between 0,68 and 3,9% in adults, a high variability is reported over different regions and ethnic groups suggesting an influence of food and genetics (20-23) .

Pseudogout or calcium pyrophosphate deposition disease (CPPD) is responsible for another type of crystal caused arthritis and a potential differential diagnosis of SA. Risk factors include hyperparathyroidism (odds ratio 4,87), osteoarthritis (odds ratio 2,91), chronic renal failure (odds ratio 2,29), and the use of loop diuretic drugs (odds ratio 1,35) (24). Data on the incidence of CPPD arthritis is very rare. One study has published data on the prevalence of knee chondrocalcinosis, comparing a Chinese population (1.8% in men, 2.7% in women) with a Caucasian population (6.2% in men, 7.7% in women) (25).

1.1.3 Periprosthetic Joint Infection (PJI)

Whit a significantly rising number in total joint arthroplasty procedures in the past and the continuing of this trend in future projections across the world, the incidence of PJI and the resulting costs are increasing (26-29). In total knee arthroplasty (TKA), the reported risk of revision surgery after 15 years is 2,0% for PJI and 1,2% for aseptic loosening, being the two most common causes for revision. About half of the PJI revision surgeries were performed within two years following the index TKA (30). The PJI rate following total hip (THA) and shoulder arthroplasty is usually reported lower than in TKA (about 1% (31)). Elbow prosthesis have shown PJI rates ranging from zero to 12%. This is mainly on account of the thin surrounding soft tissue and the primary arthroplasty indications (post-traumatic osteoarthritis and rheumatic disorders) (32, 33). The negative impact of a PJI is also underlined by the significantly higher one-year (10,6% vs. 2%) and five-year-mortality-rates (25.9% vs. 12,9%) of patients undergoing revision surgery due to infection compared to revision surgery due to aseptic causes (34). Additionally, a trend in the United States suggests increasing PJI cases between 2001 and 2009 (1.99 to 2.18% for THA and from 2.05 to 2.18% for TKA) (26). This

was also reported for Scandinavian countries, where the periods 1995 to 1999 (0.46%) and 2005 to 2009 (0.71%) for septic hip revision had been compared (35) .

A recent joint registry study on PJI risk factors including 3659 PJI cases (out of a total of 679 010) was performed in England and Wales. The statistically significant PJI risk factors for patients after TKA include male sex (rate ratio (RR) 1,8), elevated body mass index (BMI; RR for BMI ≥30 kg/m2 vs <25 kg/m2 1,5), surgery for trauma (RR 1,9), a history of septic arthritis (RR 4,9), constrained condylar prostheses (RR 3,5), and age (RR for age ≥80 years *vs* <60 years 0,5) (36). Not all these risk factors can be actively influenced by the surgeon or patient, but it does allow giving the patient more case specific information regarding the expected risks after TKA. Additional PJI risk factors after THA include diabetes (RR 1,4), dementia (RR 3,8), and surgery due to femoral neck fracture (RR 1,8) (37).

1.2 Microbiology

1.2.1 Septic Arthritis

The most common germ in septic native joint infection is staphylococcus aureus with a reported frequency ranging from 37 to ~65% (11, 38). Considering cases with a history of orthopedic surgery, resistant germs such as *methicillin-resistant Staphylococcus aureus* (MRSA) have been reported more frequently in elderly patients and intravenous drug abusers (39). The frequency of SA due to MRSA in the general population remains stationary over the last three decades (38).

Group B streptococci are frequently found in elderly patients with comorbidities, such as diabetes cancer and heart failure, and are also causing osteomyelitis (40). A mortality of 9% and polyarticular infestation in 32% of cases were reported (41). Streptococcus pneumoniae is a relatively rare cause of SA, which is responsible for approximately 3% of cases, however with a high mortality rate of 19% (42) (43). SA cases due to gram-negative rods, such as Pseudomonas aeruginosa and Escherichia coli, are generally rare in the healthy population. However, higher frequencies of gram-negative SA are reported in young children, immunocompromised adults, and geriatric patients (15% of SAs). Though the number of recent studies focusing on these patients is low (44) (9).

Polymicrobial SA is a rare entity accounting for 5% of all SA cases (9). A recent study reported *coagulase-negative Staphylococci* (31%), *methicillin-sensitive Staphylococcus aureus* (MSSA, 29%), and *Enterococci* (24%) to be the most frequent pathogens (45). Compared to patients with one causative microorganism, patients who acquire SA with multiple causative microorganism tend to have more severe symptoms (14.9% vs. 5.5%), require transfers to intensive care units more often ((39.1% vs. 18%), and have longer hospital stays (16.1 vs. 10.9 days) (45).

1.2.2 Periprosthetic Joint Infection

A main causative microorganism for PJI is staphylococcus aureus, being responsible for 27% of hip and knee PJIs in total, and 38% of early onset hip and knee PJIs (46). On one hand, this seems to be due to its ubiquitous occurrence, and on the other hand to the high virulence of the pathogen. This is further increased by patient-related risk factors, such as (temporarily or permanently) implanted foreign material, such as endoprostheses and catheters. Pre-existing conditions such as diabetes, rheumatoid arthritis, and kidney failure with ongoing hemodialysis should also be mentioned (47) (48). When comparing MRSA and methicillin-susceptible S. aureus regarding the treatment outcome (infection eradication), both microorganisms were equal. Further, the use of the biofilm active antibiotic drug rifampin was associated with higher rates of infection eradication (49).

A group of staphylococci, generally referred to as coagulase-negative Staphylococcus species is responsible for another 27% of PJI after THA and TKA. These bacteria can cause early, delayed, and late onset PJIs, with different symptoms such as prolonged wound secretion, local swelling, and/or pain (46). Most of them are found on human skin, with staphylococcus epidermidis being the most frequent germ (50). This also explains one of the most frequent modes of occurrence, namely intraoperative infection or iatrogenic spread through intra-articular injections. Attention must also be paid to possible delayed diagnosis in cases where the detected germ is thought to be a contamination. Although not a typical high virulent pathogen or explicitly difficult to treat, staphylococcus epidermidis is a challenging foe in PJI therapy due to its biofilm production (51). One of the few non β-lactamase producing

microorganisms in the group of coagulase-negative Staphylococcus species is staphylococcus lugdunensis, which explains it susceptibility to penicillin (52) (53).

Polymicrobial PJIs are rather frequent in early infections with 56% of all polymicrobial PJIs occurring within the first three months after implantation in contrast to 29% of monomicrobial PJIs (54). When comparing the factors associated with mono- and polymicrobial PJI occurrence, surgical wound dehiscence (odds ratio (OR) 5.9), drainage (OR 5.0), and age >65 years (OR 2.8) were most frequently observed (54). PJI caused by fungi is among the most serious complications following total joint arthroplasty with an infection eradication rate of 50% reported after a two stage prosthesis exchange and a longtime oral antifungal therapy (55). Caused by *Candida species* in around 80% of cases, fungi are responsible for less than 1% of PJI cases, often following revision arthroplasty due to prior bacterial PJI in patients with one or more systemic pathology (56) (46).

If the definition criteria for PJI are fulfilled but no microbiological identification of the causative pathogen is possible, a culture negative PJI is diagnosed. The frequency of culture negative PJI described in literature ranges from 5% to 42% (57). This is either possible due to a misclassification (the symptoms are not caused by PJI) or the current microbiological methods are not able to detect the pathogen (58). This might be the case if an antimicrobial therapy is administered prior to germ detection (joint puncture, intraoperative sampling) (46). In selected cases, the use of polymerase chain reaction in combination with next generation sequencing and mass spectrometry might be utilized to identify the causative pathogen (57).

1.3 Diagnosis

1.3.1 Septic Arthritis

The differentiation between AA and SA is occasionally difficult since the two entities share similar clinical features. Therefore, the distinction between these two pathologies cannot be done by clinical and laboratory examination alone. Many biomarkers, laboratory parameters, and radiologic imaging techniques have been developed to support the attending physician in the diagnostic process. Due to the huge variety of aseptic arthritis entities and this thesis

focusing on the diagnosis of bacterial joint inflammation, the clinical features and diagnostic methods regarding SA are described in the following paragraphs.

Diagnostic criteria

In the year 1976, J.H. Newman described four main criteria in the diagnosis of septic arthritis (59). These include:

(a) an organism isolated from synovial fluid aspiration

(b) clinical signs of joint inflammation and an organism isolated from elsewhere

(c) no organism isolated but

(i) histological or radiological evidence of infection

or

(ii) turbid fluid aspirated from the joint (in the case of previous antibiotic therapy)

To take more recent scientific findings into account, these criteria can be modified, as previously described by Sigmund et al. (60). Post-mortem or pathological features of septic arthritis, synovial leucocyte count > 50 000/µl, and synovial polymorphonuclear cell percentage (PMN%) > 90% were added to the Newman criteria.

1.3.1.1 Clinical Features

The most observed symptoms include joint pain and subsequently reduced function and range of motion, swelling, redness, warmth, and stiffness (61). However, none of these symptoms is pathognomonic and one or more symptoms may be absent or subacute (62). In the case of septic arthritis (SA) high-grade fever can be observed in 58% of patients and low-grade fever in 90% (63). Joint pain is present in 85% (95% CI, 78%-90%) of patients and local swelling in 78% (95% CI, 71%-85%) of patients (62). Regarding the localization of SA, large joints (knee and hip, followed by shoulder) are most frequently affected (64). However, any joint can be affected, especially after (animal or human) bites or penetration trauma in the vicinity of a joint. Infections of the iliosacral and sternoclavicular joints as well as the pubic symphysis are atypical in the healthy population but occur more frequently in intravenous drug users and in patients performing certain sports (10, 39, 65) .

1.3.1.2 Laboratory and Microbiology

The current laboratory standard in SA diagnostics includes the erythrocyte sedimentation rate (ESR, sensitivity 98% at cutoff >10 mm/h), the C-reactive protein (CRP, sensitivity 92% at cutoff ≥20 mg/L), and the white blood cell count (WBC, sensitivity 48% at cutoff >11,000 cells/ mm3). Although these tests in combination with the synovial leucocyte count are extremely sensitive with values approaching 100%, the specificity is low at 24% (66, 67). Furthermore, non-elevated ESR, CRP, and serum leukocyte count do not exclude septic arthritis (8). Nevertheless, the measurement of these widely available laboratory parameters is recommended to support the diagnostic process and to serve as a marker for the therapeutic response (39). Serum procalcitonin has shown promising results in SA diagnosis regarding sensitivity 59,3–100% and specificity 46-86% depending on the chosen cutoffs (0,1–0,25 ng/ml) (68, 69). Serum interleukin 6 (IL-6) has shown a poor diagnostic performance and was unable to provide valuable information in the differentiation between SA (mean 11.6 ±2.8 pg/μL) and AA (mean 11.9 ±3.3 pg/μL; p= 0.5) (70).

Although a negative blood culture does not rule out SA, cultures are recommended in all cases of suspected SA prior to the initiation of antibiotic therapy as 24% to 30% of patients develop associated bacteriaemia (5, 9). Gram staining is a fast method to detect bacterial contamination via staining followed by microscopy. In a small series, gram staining has shown a rather low sensitivity of 45% but a specificity of 100% in SA diagnosis. It was considered an unreliable diagnostic method in early decision making by the authors (71). Another study has shown a similar diagnostic profile of gram staining in SA and PJI with a sensitivity of 0,37% and 0,33% respectively at 99% specificity (72). Another possibility for improving microbiological diagnostics is the cultivation of synovial fluid in blood culture bottles with studies reporting superior germ detection with reduced contamination (73).

1.3.1.2 Synovial Biomarkers

Synovial fluid white blood cell count (WBC), Polymorphonuclear Cell percentage (PMN%), Joint puncture, and collection of synovial fluid (ultrasound-, CT-targeted or unaided) under sterile conditions are essential for an accurate diagnosis in arthritis cases of unclear cause. Ideally, this should be done before starting antibiotic therapy in order to increase the chance of

detecting the causative microorganisms. Synovial fluid WBC, usually in combination with the PMN%, represents a well-established diagnostic tool in the differential diagnosis of unspecific arthritis. Studies have shown the increasing likelihood of septic arthritis with increasing cell count. Empirical antibiotics are recommended for cell counts above 50,000 cells/µL (62). SA was diagnosed in 77% of patients with synovial WBC above 100.000/µL. If the synovial WBC was between 50.000 and 100.000 cells/µL, SA was diagnosed in 47% of cases, and with a cell count below 50000/µL in 5% of cases (74). PMN values of 90% or more indicate a significantly increased likelihood of SA (LR, 3.4; 95% CI, 2.8-4.2), whereas PMN values below 90% are associated with a significant reduction in likelihood (LR, 0.34; 95% CI, 0.25-0.47) (62).

Synovial lactate has shown a moderate diagnostic performance (sensitivity and specificity of 0.55 and 0.76 at a cutoff of ≥ 5 mmol/L) in the diagnosis of SA, however, its value could lie in its quick and easy determinability (75). A recent study reported the synovial lactate to glucose ratio to be a very specific indicator (98,1%) with acceptable sensitivity (52%) and an area-under the curve of 0.859, outperforming the diagnostic accuracy of the individual biomarkers (76). Lenski and Scherer conducted a retrospective study including 82 participants suffering from either gout or SA (77). They were able to show that the concentration of synovial fluid lactate is significantly higher in SA compared to gout (11.7 (range 0.2– 48.0) vs. 3.5 (1.5–7.9); $p<0.05$; AUC 0.901).

When comparing synovial procalcitonin (sensitivity 24% and specificity 96%) to serum procalcitonin (68% and 80% at cutoff > 0.5 ng/ml) and other easy-to-obtain serum biomarkers, such as CRP (92% and 30% at cutoff >18mg/L) and ESR (100% and 26% at cutoff > 17 mm/h for men and > 25 mm/h for women), the study authors reported acceptable results for all biomarkers. Yet, they concluded that synovial procalcitonin was not superior to the tested serum biomarkers, with the drawback of the necessary arthrocentesis during sample collection (70).

Synovial glucose levels have also shown a good diagnostic performance in the differentiation between SA and gout with levels of 41 mg/dL (range 1.0–203) and 100 mg/dL (range 47–262) respectively ($p<0.05$). Urid acid concentrations in serum and synovial fluid were consistently and significantly higher in gout cases (serum: 8.7 mg/dL (3.4–12.5) $p<0.05$; synovial fluid: 8.33 mg/dL (range 3.0–11.5) $p<0.05$) compared to SA cases (serum: 6.0 mg/dL (2.6–12.6) $p<0.05$; synovial fluid: 5.31mg/dL (range 0.5–19.2) $p<0.05$) respectively (77). This study covered several interesting biomarkers, but the retrospective design and the inclusion of only gout cases,

while also excluding large parts of the group of aseptic arthritis causes in the process, must be seen as major limitations.

Sigmund et al. have investigated the synovial fluid of 72 patients, by testing the diagnostic properties of the multiplex polymerase chain reaction system mPCR compared to the current gold standard, which is microbiologic culture. They report a similar diagnostic performance with a sensitivity of 38% and a specificity of 100% for mPCR, and a sensitivity of 29% and specificity of 100% for the synovial fluid culture. The combination of both tests led to an improved sensitivity of 43% at 100% specificity. Tissue culture reached similar diagnostic levels with a sensitivity of 40% and a specificity of 100% (60).

1.3.2 Periprosthetic Joint Infection

A PJI is divided into an acute and chronic type depending on the symptom duration. An acute infection (high grade PJI) is defined by a duration of symptoms up to three to four weeks; in this case, an immature biofilm is to be expected. The symptoms are primarily triggered by bacteria freely present in the joint (planktonic life form). The cause are usually highly virulent organisms such as *staph. aureus* and *gram-negative bacteria*. A chronic infection exists with a symptom duration of more than three to four weeks, in which case a mature biofilm is to be expected. The symptoms are primarily triggered by bacteria occurring in biofilm. A chronic (low grade PJI) is usually caused by low virulence germs such as *staph. epidermidis* and *propionibacterium acnes* (78).

Further, depending on the time between the index operation and the first onset of symptoms, there is also the distinction between an early postoperative and a late infection. By definition, an early postoperative infection occurs within the first three months after prosthesis implantation, a late infection thereafter (up to several years postoperatively) (78).

1.3.2.1 Clinical Features and PJI Definition(s)

Clinical symptoms may vary greatly depending on whether an acute or a chronic PJI is present. An acute PJI often features acute joint pain, fever and/or shivering, as well as a red, hot, and swollen joint. Usually there are clearly elevated blood serum inflammation parameters present.

Chronic PJI, on the other hand, often presents itself with chronic pain, clinical and radiological signs of prosthesis loosening, and, in some cases, fistula formation.

To simplify clinical and scientific work (for better study comparability, collaboration, and data interpretation), different PJI definition criteria have been postulated by various professional societies. In the following paragraphs, the most important and widely used PJI definition criteria will be outlined. The presentation of three different PJI definitions at this point is intended to illustrate not only the complexity of the topic but also the great research activity in this field.

PJI definition criteria of the European Bone and Joint Infection Society (EBJIS)

The EBJIS criteria for PJI diagnosis (Figure 1) were published in the year 2020. The aim was to provide a practical guide for clinicians in determining whether a PJI is present or not. According to the authors, special focus was put on the detection and diagnosis of low grade PJI, in order to avoid the delayed or missed diagnosis of such cases (2).

	Infection Unlikely (all findings negative)	Infection Likely (two positive findings)[a]	Infection Confirmed (any positive finding)
Clinical and blood workup			
Clinical features	Clear alternative reason for implant dysfunction (e.g. fracture, implant breakage, malposition, tumour)	1) Radiological signs of loosening within the first five years after implantation 2) Previous wound healing problems 3) History of recent fever or bacteraemia 4) Purulence around the prosthesis[b]	Sinus tract with evidence of communication to the joint or visualization of the prosthesis
C-reactive protein		> 10 mg/l (1 mg/dl)[c]	
Synovial fluid cytological analysis[d]			
Leukocyte count[c] (cells/μl)	≤ 1,500	> 1,500	>3,000
PMN (%)[c]	≤ 65%	> 65%	> 80%
Synovial fluid biomarkers			
Alpha-defensin[e]			Positive immunoassay or lateral-flow assay[e]
Microbiology[f]			
Aspiration fluid		Positive culture	
Intraoperative (fluid and tissue)	All cultures negative	Single positive culture[g]	≥ two positive samples with the same microorganism
Sonication[h] (CFU/ml)	No growth	> 1 CFU/ml of any organism[g]	> 50 CFU/ml of any organism
Histology[c,i]			
High-power field (400x magnification)	Negative	Presence of ≥ five neutrophils in a single HPF	Presence of ≥ five neutrophils in ≥ five HPF
			Presence of visible microorganisms
Others			
Nuclear imaging	Negative three-phase isotope bone scan[c]	Positive WBC scintigraphy[j]	

Summary Key

a. Infection is only likely if there is a positive clinical feature or raised serum C-reactive protein (CRP), together with another positive test (synovial fluid, microbiology, histology or nuclear imaging).

b. Except in adverse local tissue reaction (ALTR) and crystal arthropathy cases.

c. Should be interpreted with caution when other possible causes of inflammation are present: gout or other crystal arthropathy, metallosis, active inflammatory joint disease (e.g. rheumatoid arthritis), periprosthetic fracture, or the early postoperative period.

d. These values are valid for hips and knee periprosthetic joint infection (PJI). Parameters are only valid when clear fluid is obtained and no lavage has been performed. Volume for the analysis should be > 250 μL, ideally 1 ml, collected in an EDTA containing tube and analyzed in <1h, preferentially using automated techniques. For viscous samples, pre-treatment with hyaluronidase improves the accuracy of optical or automated techniques. In case of bloody samples, the adjusted synovial WBC= synovial $WBC_{observed}$ – [WBC_{blood} / RBC blood x $RBC_{synovial\ fluid}$] should be used.

e. Not valid in cases of ALTR, haematomas, or acute inflammatory arthritis or gout.

f. If antibiotic treatment has been given (not simple prophylaxis), the results of microbiological analysis may be compromised. In these cases, molecular techniques may have a place. Results of culture may be obtained from preoperative synovial aspiration, preoperative synovial biopsies or (preferred) from intraoperative tissue samples.

g. Interpretation of single positive culture (or < 50 UFC/ml in sonication fluid) must be cautious and taken together with other evidence. If a preoperative aspiration identified the same microorganism, they should be considered as two positive confirmatory samples. Uncommon contaminants or virulent organisms (e.g. *Staphylococcus aureus* or Gram negative rods) are more likely to represent infection than common contaminants (such as coagulase-negative staphylococci, micrococci, or *Cutibacterium acnes*).

h. If centrifugation is applied, then the suggested cut-off is 200 CFU/ml to confirm infection. If other variations to the protocol are used, the published cut-offs for each protocol must be applied.

i. Histological analysis may be from preoperative biopsy, intraoperative tissue samples with either paraffin, or frozen section preparation.

j. WBC scintigraphy is regarded as positive if the uptake is increased at the 20-hour scan, compared to the earlier scans (especially when combined with complementary bone marrow scan).

Figure 1 - EBJIS criteria for the diagnosis of clinically suspected periprosthetic joint infection. Reproduced from McNally et al. (BJJ 2020 (2)) with permission of publisher "The British Editorial Society of Bone & Joint Surgery"

The PJI definition criteria of the United-States dominated MSIS were first introduced in 2011 and are currently the world's most widely used PJI criteria (79). Despite the criticism sometimes voiced, that while the MSIS criteria have a high specificity for PJI, but by using these, low-grade infections are more often missed than with other definitions, these criteria have been a milestone in PJI treatment and research. They were updated following an international consensus meeting (ICM) in 2013 (80) and revised again in 2018 (3) (as depicted in table 1).

Major criteria (at least one of the following)	Decision
Two positive cultures of the same organism	Infected
Sinus tract with evidence of communication to the joint or visualization of the prosthesis	

		Minor Criteria	Score	Decision
Preoperative Diagnosis	Serum	Elevated CRP *or* D-Dimer	2	≥6 Infected **2-5 Possibly Infected [a]** 0-1 Not Infected
		Elevated ESR	1	
	Synovial	Elevated synovial *WBC count or LE*	3	
		Positive alpha-defensin	3	
		Elevated synovial PMN (%)	2	
		Elevated synovial CRP	1	

	Inconclusive pre-op score *or* dry tap [a]	Score	Decision
Intraoperative Diagnosis	Preoperative score	-	≥6 Infected **4-5 Inconclusive [b]** ≤3 Not Infected
	Positive histology	3	
	Positive purulence	3	
	Single positive culture	2	

Table 1 – PJI definition scoring system according to the ICM (MSIS) PJI criteria proposal of 2018. Leukocyte esterase (LE); (a) for patients with inconclusive minor criteria, operative criteria can also be used to fulfil definition for PJI. (b) Consider further molecular diagnostics such as next-generation sequencing. Reproduced from Parvizi et al. The 2018 Definition of Periprosthetic Hip and Knee Infection: An Evidence-Based and Validated Criteria. (J Arthroplasty. 2018 (3)) with permission of publisher "Elsevier"

Clinical guidelines for PJI Diagnosis by the Infectious Diseases Society of America (IDSA)

The IDSA took a different approach to the topic by combining evidence-based and opinion-based recommendations into a set of guidelines published in 2013 (81). According to the IDSA, the following diagnostic PJI criteria/statements are backed by moderate supportive evidence:

- The presence of a sinus tract communicating with the prosthesis is definitive evidence of PJI
- The presence of acute (histopathologic) inflammation of periprosthetic tissue at the time of surgical debridement or prosthesis removal is highly suggestive evidence of PJI
- The presence of purulence surrounding the prosthesis is definitive evidence of PJI (without another known etiology)
- Two or more intraoperative cultures, or preoperative aspiration (or a combination) that yield the same organism may be considered definitive evidence of PJI
- Presence of one virulent microorganism in a single specimen of a tissue biopsy or synovial fluid may also represent PJI
- One of multiple tissue cultures or a single aspiration culture that yields an organism and is a common contaminant, should not be considered definitive evidence of PJI (further evaluation necessary)
- Even if the above criteria are not met, PJI is still possible; Clinical judgement is necessary for a final decision

In PJI diagnosis, two major biomarker groups can be distinguished. These are (a) the serum biomarkers, which are collected by means of a venous blood sample. The advantages of serum biomarkers include the ease of sample collection and thus the rapid and simple repeatability of the measurement. The risk of iatrogenic infection is also inherently lower compared to intraarticular puncture. However, serum biomarkers are significantly more susceptible to bias than (b) synovial biomarkers when there are underlying inflammatory or chronic diseases present, such as chronic liver disease, kidney disease, obesity, smoking, or malignancies (82-84). In view of the PJI definitions described in the previous paragraph, serum and synovial biomarkers are particularly important in preoperative diagnostics and screening since implant sonication and pathological and microbiological tissue diagnostics require an invasive

procedure. In the following paragraphs, the most promising biomarkers of both groups will be presented and discussed.

1.3.2.2 Serum Biomarkers

C-reactive protein (CRP) is an acute phase protein and one of the most important inflammatory parameters in clinical practice. It can be elevated in a variety of underlying conditions and must be interpreted in the context of local symptoms as well as the patient's general health and comorbidities. Nevertheless, the use of CRP is recommended in most PJI definitions and diagnostic guidelines (3, 81, 85). Wide ranges of sensitivity (62% to 100%) and specificity (64% to 96%) regarding the diagnostic performance of CRP are reported in the cited literature (86-89). When looking at the two main PJI entities, acute and chronic infection, the diagnostic properties of CRP differ significantly. This is also reflected in the PJI definitions: While the EBJIS recommends a cut-off of >10mg/L, the ICM (MSIS) criteria of 2018 proposed a distinction between acute and chronic PJI with individual CRP cut-offs (>100mg/L (acute) and >10mg/L (chronic)) (3, 85). As previously mentioned, high virulent organisms frequently cause acute PJI (with planktonic bacteria), while low virulent organisms tend to cause chronic infections (bacteria residing in biofilm producing form). Studies indicate that these different PJI types significantly differ regarding the CRP levels (17.6 and 12 mg/L (chronic) versus 49.2 and 35 mg/L (acute)) (90, 91). Another factor which complicates the correct CRP interpretation are the elevated CRP levels following tissue damage. This can lead to increased serum CRP levels for 30 to 60 days postoperatively with an infection being present (92, 93).

White blood cell count (WBC) is a frequently used infection-biomarker with rather poor sensitivity (21-42%) but high specificity (89-94%) in PJI cases (1, 86, 94, 95). The aforementioned studies used cut-off levels between 8 and 10 G/L. When aiming for a higher sensitivity and applying a cut-off of 5.48, a sensitivity of 91% was reached at a specificity of 34% (88). These low cut-off values pose a problem in clinical application. Most physicians, surgeons, and laboratories only consider the WBC to be elevated from about 11 G/L onwards. This “cut-off problem” is underlined by different study results: A study including 57 PJI and 99 non-PJI cases reported WBC levels of 7.8 G/L and 6.4 G/L respectively ($p < 0.001$) (96). Another paper reported similar results when comparing PJI cases (8.2 G/L) with aseptic cases (6.1 G/L, ($p = 0.0024$) (95). A recent review article concluded that WBC should only have a

limited role in routine clinical workup of PJI cases due to the low diagnostic performance reported in literature (86).

The erythrocyte sedimentation rate (ESR) is another rather unspecific inflammation parameter with a widespread application in clinical practice. While the EBJIS criteria for PJI do not include ESR, the MSIS criteria recommend the usage of ESR as a minor criterion for PJI (3, 85). A meta-analysis investigated 3,370 patients in 25 studies and found pooled sensitivity and specificity values of 75% and 70% for ESR regarding PJI (92). Studies report sensitivities ranging from 33% to 95% and specificities from 60% to 100% (89, 97) (98). To combine the diagnostic strengths of both CRP and ESR, some authors tried to combine the biomarkers. While some papers report improved results (99), others indicate that in order to improve sensitivity, specificity drops and vice versa (100). Alijanipour et al. even reported a decline of both sensitivity (86% vs. 95%) and specificity (61% vs. 71%) when CRP and ESR was combined compared to ESR alone (97). Looking at these data on ESR, it is reasonable to conclude that, although it is suitable as a screening test due to its ubiquitous availability, ease of implementation, and low cost, it can by no means be considered a confirmatory test for PJI.

Fibrinogen, a glycoprotein synthesized in liver cells, plays a main role in the coagulation cascade and inflammatory mechanisms. Studies have shown a correlation between fibrinogen and bacterial infections such as sepsis, appendicitis, as well as PJI (101-103). A study conducted by our research group first reported a sensitivity of 90% and specificity of 66% at a cut-off level of 519 mg/dl (103). Follow up studies around the world reported sensitivities of 69% to 94% and specificities of 73% to 95% at different cut-offs ranging from 360 to 515mg/dL (1, 86, 104). When comparing fibrinogen (AUC range from 0.785 to 0.928) with other serum PJI biomarkers, it showed satisfying results, being essentially on the same level with CRP (AUC range from 0.785 to 0.951) regarding the area-under-the-curve (AUC) and performing better than D-dimer, ESR, WBC, IL-6, and PCT (1, 86, 104-106). Although fibrinogen has shown a high diagnostic performance for a single serum PJI biomarker comparable to CRP, the results are not allowing a definitive decision on whether a PJI is confirmed or excluded. Nevertheless, due to its cost effective and simple measurement methods, fibrinogen holds its value in PJI diagnostics. D-Dimer, another PJI biomarker protein which has its origin in the coagulation cascade, has recently drawn attention with different results regarding the diagnostic performance in seven published studies since 2017. It has even made its way to the MSIS PJI definition of 2018 as a minor criterion (3). Studies suggest cut-off values between 410 and 1170

ng/mL, and report sensitivities ranging from 60% to 96% and specificities from 32% to 93% (105) (106) (107) (108). While Shahi et al. concluded that, based on their results, D-Dimer seemed more accurate than CRP and ESR in PJI diagnosis, all the other authors of the mentioned studies showed inferior diagnostic properties. Furthermore, there is a risk of false-positive bias in cases where a D-Dimer elevation is known to occur, for example in recent surgery and in patients with thromboses or embolisms. Considering these evidence, D-Dimer cannot be recommended as a serum biomarker for PJI at this time.

Interleukin-6 (IL-6) is a pro-inflammatory cytokine released by many different cells triggering the production of CRP. Its half-life of about 15h allows fast serum level changes and thus a rapid reaction to (bacterial) inflammation or tissue damage (109). The diagnostic properties of IL-6 as a biomarker for PJI has been extensively investigated over the last decade. Some studies report very good results for IL-6, with a sensitivity (100% vs. 82% vs. 100%) and specificity (91% vs. 83% vs. 89%) superior to ESR and CRP respectively (110), and AUC of 0.814 (IL-6) vs. 0.793 (CRP) vs. 0.744 (ESR) (95). Bottner et al. investigated 78 patients including 21 PJIs and reported equal sensitivity and superior specificity for CRP (95% and 96%) compared to IL-6 (95% and 87%), indicating good accuracies for IL-6 (87). However, on a closer look, the cited studies have some methodological drawbacks. Bottner et al. might have underdiagnosed PJI, as they only used intraoperative cultures and histopathology as the diagnostic gold standard. Elgeidi et al. included only a small number of patients (n=40 with only 11 PJI cases) and again used non-standardized diagnostic criteria for PJI. Furthermore, the microbiological cultures were only incubated for 7 days (110). High sensitivity of 94% of IL-6 for PJI was also shown by Glehr et al., with a rather low specificity of 53% at a cut-off of 2.55 pg/mL (88). Randau et al. have shown either low sensitivity (79% at 2.6 pg/mL and 49% at 6.6 pg/mL) or low specificity (58% at 2.6 pg/mL and 88% at 6.6 pg/mL) when applying two different thresholds (94).

The idea of procalcitonin (PCT) as a biomarker for PJI has its roots in the remarkable diagnostic and prognostic accuracy of PCT in bacterial inflammation and sepsis (111, 112). The main disadvantage of PCT in PJI diagnostics seems to be the strong gradient of sensitivity and specificity depending on the cut-off used. This is indicated by the available studies reporting sensitivities ranging from 13% to 90% and specificities ranging from 28% to 100% (88, 91, 94). While results show that PCT is not feasible as a screening parameter due to its low

sensitivity, it seems to have its justification as a confirmatory test due to its high specificity, as most authors conclude (86, 88, 94).

1.3.2.3 Synovial Biomarkers

The synovial leukocyte count is a reliable standard test for PJI with a high sensitivity and specificity. This fact is also reflected in the current PJI definition criteria of EBJIS and ICM/MSIS. While the EBJIS distinguishes between two cut-off values (<1500 cells/µL = infection unlikely, between 1500 and 3000 cells/µL = infection likely, >3000 cells/µL = infection confirmed), the MSIS sets the cut-off for a fulfilled minor criterion for PJI at 3000 cells/µL (2, 3). While there is disagreement about the interpretation of values below 3000 cells/L, there currently seems to be a consensus about the cut-off at 3000 cells/µL (with the exception that the EBJIS assumes a confirmed infection at values above 3000 cells/µL, which is not the case in the MSIS definition). A recent systematic meta-analysis found a pooled sensitivity of 0.89 (0.86-0.91), a specificity of 0.86 (0.80-0.90), and an AUC of 0.91 (113). One source of error is the postoperative increase in synovial cell count due to inflammation and hemorrhage. This assumption is supported by data from a study which found an average of 4,200 cells (aseptic) and 92,600/µL (septic) in joint aspirate 17 days postoperatively (114). This must be considered when deciding on reparation for suspected early infection. A study of 571 patients showed a slow decrease in elevated synovial leukocytes over one to two years after primary total knee arthroplasty. Average values of 2533.2 cells/µL during the first 45 days postoperatively, 269.5 cells/µL from three months to one year, and 240.8 cells/µL from one to two years were reported (115). However, these results must be interpreted with caution in terms of cut-offs and specificity, as a large proportion of the studies excluded patients with aseptic joint inflammation. In the regular clinical setting, a reduced specificity of the synovial leukocyte count can therefore be expected (46).

The PMN% is often combined with the synovial leukocyte count and a well-researched biomarker for PJI. It has shown high sensitivity of 0.89 (0.82-0.93) and specificity of 0.86 (0.77-0.92) with an AUC surpassing that of synovial leukocyte count (0.93) (113). Various cut-offs are reported with different diagnostic performance. Two studies including 562 patients recommended a cut-off of 64% and 65%, with a sensitivity of 95% and 97% and a specificity of 90% respectively (116) (117). While the EBJIS PJI criteria suggest two cut-offs (>65% infection likely; >80% infection confirmed), the MSIS recommends a cut-off of 80% in order

to be considered a minor criterion for PJI (2, 3). In view of these results, further data and studies seem to be necessary to decide on the optimal cut-off for PMN%.

Leukocyte esterase, an enzyme produced by neutrophils if activated by a bacterial infection, can be detected by a low-cost colorimetric strip test and is therefore a quick and convenient diagnostic method in PJI detection (118). A recent meta-analysis including 12 studies, conducted between 2011 and 2018, has shown a pooled sensitivity of 79% (95% CI 75–82%) and specificity of 96% (95% CI 95–97%) (119). However, a problem, which occurred with this test, is the risk of non-usability in cases of blood in the synovial fluid. This, in combination with unreadability due to debris and undetermined results, presents a major limitation leading to the rejection of 29% of LE-tests in one study (120). The interpretation of the test results poses another controversial aspect. Some studies considered the test to be positive at "+", "++", as well as "+++", however, the MSIS definition recommends at least "++" for a positive test result (3, 120).

Alpha-defensins are members of the defensin family and produced in response to microbial products and/or proinflammatory cytokines (121). It can be tested via an alpha-defensin test kit (lateral flow) on site or in a laboratory using an enzyme-linked immunosorbent assay (ELISA) test. Alpha-defensin has shown impressive test results, resulting in a pooled sensitivity of 0.97 (95% CI: 0.93-0.99) and a specificity of 0.96 (95% CI: 0.94-0.98), while the AUC was at 0.99 (113). A recent study has shown a sensitivity of 89.5% (95% CI: 78.5% to 96.0%) and a specificity of 94.8% (95% CI: 91.2% to 97.2%) for the alpha-defensin lateral flow test in a prospective cohort. When excluding 17 patients with strong blood admixture, the sensitivity increased to 94.3% (95% CI: 84.3% to 98.8%) (122). The authors further investigated the diagnostic performance of the alpha-defensin lateral flow test compared to the ELISA test but found no statistical significant difference in neither sensitivity nor specificity (122). However, a more recent meta-analysis concluded the opposite, namely that the alpha-defensin ELISA test is superior to the lateral flow test kit in terms of diagnostic performance (123). Another study, investigating a qualitative alpha-defensin test including 50 patients with 13 cases of PJI and 36 aseptic cases (one inconclusive case, MSIS criteria), reported a sensitivity of 69% and a specificity of 94% (124). In summary, alpha defensin in its various measurement methods is an extremely promising PJI biomarker with a strong diagnostic performance. The disadvantage is the price of the commercially available test kit. Currently, alpha defensin is recommended as a confirmatory test in PJI diagnostics with varying sensitivity but consistently high specificity

(125). Still, alpha-defensin was included in both ICM/MSIS and EBJIS definitions for PJI (2, 3).

Synovial fluid CRP is a rather frequently investigated biomarker for PJI with different cut-off levels reported. The diagnostic properties are acceptable with a pooled sensitivity of 0.85 (95% CI: 0.78-0.90) and specificity of 0.88 (95% CI: 0.78-0.94). An AUC of 0.90 was reported by a metanalysis including 10 studies (113). Another recent study reported a synovial CRP cut-off of 7.26 mg/l leading to a mean sensitivity of 84.62% (95% CI 69.5 to 94.1) and a mean specificity of 93.10% (95% CI 83.3 to 98.1%). The authors tried to improve the diagnostic performance by using two combined models (model I: Serum CRP > 10.2 mg/l OR Synovial CRP > 7.26 mg/l; model II: not OR, but AND), including serum CRP and synovial CRP at different cut-offs, leading to either a high negative predictive value (NPV) of 96.67% with a low positive predictive value (PPV) of 56.72% (model I), or a NPV of 84.06% and a PPV of 100% (model II) (126).

Synovial fluid interleukins (IL-6, IL-8, IL-1β) have shown different sensitivities (69% to 100%) at high specificities (93% to 100%) in the past (127) (128) (129). One of those studies reported a sensitivity and specificity of 100% for both, IL-1β and IL-6, including 51 patients (14 PJI and 37 aseptic cases) (128). These results clearly exceed those of all other available studies and must therefore be interpreted critically. More recent studies were able to confirm the satisfying results on the diagnostic properties with an overall pooled sensitivity of 0.81 (95% CI 0.70-0.89) and a specificity of 0.94 (95% CI 0.88-0.97) for IL-6. IL-8 has shown an even better diagnostic performance with a pooled sensitivity of 0.87 (95% CI 0.67-0.96) and a specificity of 0.94 (95% CI 0.88-0.97). The AUCs were 0.95 and 0.96, and the diagnostic odds ratio 4.38 (95% CI 2.86-5.89) and 4.92 (95% CI 2.84-7.00) respectively (113).

Few studies have been published on the diagnostic properties of synovial PCT in PJI diagnostics. Saeed et al. measured synovial PCT and aimed to differentiate septic from aseptic arthritis in both native and prosthetic joints (total n= 76, septic arthritis n= 26 (including 8 prostheses and 18 native joints), aseptic arthritis n= 50 (including 6 prostheses and 44 native joints)). They reported varying sensitivity and specificity at different cut-offs: at a cut-off of 0.5 µg/L the sensitivity was 0.88 (95% CI: 0.69-0.97) and the specificity 0.57 (95% CI: 0.42-0.71); at a cut-off of 4.5 µg/L the sensitivity was 0.54 (95% CI: 0.34-0.73) and the specificity 0.94 (95% CI: 0.82-0.98) (130). A prospective pilot study included 32 patients, with 20 patients

in the PJI group and 12 in the aseptic group based on the MSIS 2013 criteria for PJI (131). The authors compared the diagnostic performance of serum PCT (cut-off level 0.5 ng/mL, AUC 0.70, sensitivity 40.0%, specificity 100.0%) and synovial PCT (cut-off level 0.08 ng/mL, AUC 0.87, sensitivity 90.0%, specificity 83.3%) (132).

1.3.3 Novel Biomarkers

Metabolomic Profiling via Nuclear Magnetic Resonance (NMR)

Metabolomic profiling is used for the identification and quantification of metabolites in a biologic sample. The entire spectrum of metabolites such as carbohydrates, amino acids, oligopeptides, and lipids is called the metabolome. The synovial fluid metabolome has a dynamic composition depending on surrounding tissues and possible joint pathologies. Studies report altered metabolomic profiles for osteoarthritis, rheumatoid arthritis, and different stages of disease progression (133, 134). Further, metabolomic research offers new possibilities regarding the identification of biomarkers for diagnostic, prognostic, and therapeutic use in a variety of pathologies (135). Proton-based nuclear magnetic resonance (1H-NMR) allows a comprehensive profiling of complex metabolomes with high technical reproducibility and has become a frequently used research technique (135-138). Possible metabolomic patterns in human synovial fluid, which distinguish between different causes of aseptic arthritis, septic arthritis, and PJI, have been described in small cohort and pilot studies, and indicate different metabolic patterns for each pathology (139-141). Larger series are recommended to validate the results and expand the relatively young field of research.

Soluble Urokinase Plasminogen Activator Receptor (suPAR)

The urokinase plasminogen activator plays a vital role in the proteolytic activation and degradation of plasminogen to plasmin in pericellular matrix during tissue remodeling (142). This effect is modulated by a cell bound receptor (uPAR), which is expressed by many cells including cancer cells and cells of the immune system. UPAR also occurs in a soluble form (suPAR; a 55-60 kDa glycoprotein) in the blood plasma as well as in the cerebrospinal and synovial fluid of both the healthy population and patients with cancer or inflammatory diseases (143) but with stable plasma concentrations under circadian changes and fasting (144, 145). Besides being a plasminogen activator with an important role in coagulation and fibrinolysis, it

has also been shown that suPAR has a proinflammatory effect in inflammatory diseases (146, 147). SuPAR levels are measured via ELISA tests and have been in the focus of recently published papers investigating plasma biomarkers for sepsis, cardiac disease, pneumonia, and autoimmune disorders (148) (149) (150) (151) (152). This could also be a future opportunity for the development of new drug therapies for inflammatory diseases such as rheumatoid arthritis (153). Currently, the published data from an Italian group indicating good diagnostic performance of plasma suPAR in PJI diagnostic (AUC 0.885; n total=80, n PJI=45) are the only available evidence regarding this topic (154).

1.4 Therapy

Adequate antibiotic therapy is necessary for successful treatment of both pathologies SA and PJI. If possible, combined drug and surgical therapy should be used in both cases. The antibiotic therapy should be pathogen-specific (if the pathogen is known) for a minimal duration of four to six weeks. If there is foreign material in the joint (e.g. screws, ligament allografts or prostheses) on which biofilm might form, this must be taken into account in the choice of antibiotic therapy (46, 155). With the aim of an interdisciplinary approach, it has become common to consult an infectious disease specialist for the determination of antibiotic therapy. The following paragraphs will give an overview of the surgical therapy options in SA and PJI and will emphasize the significance of a rapid and correct diagnosis.

1.4.1 Native Arthritis

While AA rarely requires acute surgical intervention and is often even contraindicated, verified SA usually requires a fast and determined surgical therapy. An exception is gonococcal arthritis, which is typically treated with specific antibiotics and percutaneous drainage in case of purulent effusions (156). On the contrary, patients with SA greatly benefit from a timely surgical intervention to reduce the bacterial load and additional antibiotic therapy. If a surgical intervention is not performed in time, the result can be the destruction of the joint, sepsis, and even death of the patient. Even with surgical intervention, the reported mortality rate of SA ranges from 11% to 16% in monoarticular cases and up to 50% in polyarticular cases (8, 157). A permanent loss of joint function is described in up to 40% of cases (6).

Regarding the type of invasive therapy for septic arthritis, there is consensus on the need for rapid drainage of putrid synovial fluid. This improves local blood circulation (which allows antibiotic agents to be more effective due to better availability), reduces local pain symptoms by relaxing the joint capsule, and removes microorganisms as well as their metabolic products and toxins (9, 155). However, there is still no international consensus on whether this drainage should primarily be achieved by repeated arthrocentesis or arthroscopic irrigation and intra-articular drainage tube insertion. In our clinic, arthroscopic irrigation, including synovectomy of the affected joint, is performed first (with repetition if necessary), and if this does not achieve the desired effect, then open synovectomy, irrigation, and drainage is indicated.

1.4.2 Periprosthetic Joint Infection

The aim of PJI treatment is the eradication of infection and the achievement of stable prosthesis fixation with satisfactory function of the joint. The selection of the optimal surgical therapy method, in combination with the choice of the appropriate antibiotic (determined by an infectious disease specialist), requires experience of the attending surgeon and depends on various factors. In addition to the general and local condition of the patient, the time of diagnosis is particularly decisive for the success of the therapy. A quick and reliable diagnosis is therefore important for the choice of therapy and its success rate, and to prevent unnecessary interventions. In the following paragraphs, the most frequent therapy options are briefly described, including indications and success rates.

Debridement, Antibiotics, and Implant Retention (DAIR)

A DAIR-procedure can be recommended to patients with early postoperative and acute hematogenous infections, and a duration of symptoms of up to three to four weeks (46). The implant must have a stable fixation. A sinus tract is associated with a worse outcome (158) (159). The surgical procedure includes the replacement of all mobile (not bone-anchored) parts. In the case of a knee prosthesis, this would be the polyethylene-inlay, and in the case of a hip prosthesis, the head and cup-inlay. Thorough debridement as well as extensive irrigation and collection of germs and tissue samples (especially if no responsible germ has been found yet) is necessary. Reported success rates vary largely in literature, however, it is usually stated at around 50% (160, 161).

One-Stage Prosthesis Exchange

If a DAIR procedure is not possible or promising for the reasons mentioned above, the prosthesis must be replaced. This can be done in a one-stage procedure, where the prosthesis is explanted, and a new prosthesis is implanted in a single surgery following debridement and irrigation. With appropriate patient selection, the one-stage exchange is comparable to the two-stage exchange with infection eradication rates of around 90% (162, 163). Appropriate bone-stock reserves and soft tissue conditions are necessary (164). In addition, some authors required a preoperative determination of the responsible microorganism, which is ideally susceptible to antibiotics available orally and in PMMA (81, 165).

Two- (or Multiple-) Stage Prosthesis Exchange

As the name implies, a two-stage exchange is a prosthesis exchange performed in at least two operations. This is considered to be the gold-standard, especially in Anglo-American countries (46). In rare cases, spacer changes are necessary in between, resulting in a multiple-stage exchange. The first procedure involves removal of the prosthesis, extensive debridement and irrigation, and implantation of a PMMA spacer (with added antibiotics). The second procedure is performed approximately four to six weeks after the first procedure, although there is no consensus among experts concerning the optimal timing (164). The cement spacer is removed, and the revision prosthesis is implanted. As already mentioned, one-stage and two-stage exchanges have similar outcomes regarding infection eradication in standard cases. However, there are special circumstances in which a two-stage exchange is recommended: Patients with signs of sepsis, no germ or a difficult-to-treat organism (multi-resistant or fungi) is identified, patients with a sinus tract, or bad soft tissue coverage (164).

Salvage Procedures

In cases where a curative approach with infection eradication and preservation of the joint function is not feasible, salvage procedures are intended to ensure that the patient has as good a function and quality of life as possible. Among these salvage procedures are resection arthroplasties (i.e. Girdlestone), chronic sinus tract, arthrodesis, and amputation. In comparison to a girdlestone resection arthroplasty following a total hip arthroplasty, advantages of a chronic fistulation were brought forth by a recent study. Those include, for example, the risk reduction in revision surgeries and hospitalizations (166). Further, currently unpublished data from a

multicenter study, including contribution from our institution, indicate patients with chronic sinus tract treatment to have a good quality of life. Although amputation is rare after total knee arthroplasty (0.1%), amputation following a failed two-stage revision total knee arthroplasty is reported in 14 to 25% of cases (46).

1.5 Study Questions

A single confirmatory test for the presence of bacteria in synovial fluid is one of the main research topics for orthopedic surgeons, rheumatologists, and infectious disease specialists. It would lead to a faster and more accurate diagnosis as well as a more effective treatment. Since such a gold standard test is currently not available, physicians must improvise using more or less accurate definition criteria for the diagnosis of SA and PJI. Synovial and serum biomarkers have shown good diagnostic performance in SA and PJI, yet a single biomarker with high reliability in terms of specificity, sensitivity, and AUC, which is easy to handle in daily routine, is missing.

Therefore, the aim of this thesis and the underlying research is to address and answer the following questions:

1. What are the diagnostic properties of metabolomic profiling via nuclear magnetic resonance (NMR) in the diagnosis of SA and PJI?

2. What are the diagnostic properties of soluble urokinase plasminogen activator receptor (suPAR) in blood plasma and synovial fluid in the diagnosis of SA and PJI?

3. Is it possible to combine serum biomarkers for PJI into an algorithm based multi-biomarker model to improve their diagnostic performance?

2. Methods

2.1 Novel Methods in Synovial Analysis

This study was approved by the ethical review board of the Medical University of Graz (31-086 ex 18/19). All patients of full age who have undergone a diagnostic joint puncture due to suspected arthritis of any cause (native or after arthroplasty treatment) at the University Department of Orthopaedics and Traumatology or the Department of Immunology and Rheumatology Graz in the study period from 1.1.2019 - 31.10.2021 were eligible for inclusion. After giving their informed consent, general patient data was collected prospectively from the hospital digital medical records and stored pseudonymized. Patients from protected groups were excluded from study participation (guardianship, underage). The minimum amount of synovial fluid needed per patient were 200 µL for NMR metabolomic analysis and 500 µL for the suPAR analysis. This minimum amount of synovial fluid must be reached after the required quantities for the routine analyses have been collected (microbiological incubation in brain-hearth infusion and synovial fluid cytological analysis), otherwise a study inclusion is not possible. A blood sample was taken as part of the routine diagnostic process for arthritis. For study purposes, an excess of Ethylenediaminetetraacetate (EDTA) blood plasma not used in routine operation was collected (10ml).

The synovial as well as the EDTA blood sample was stored at -80°C until study analyses were performed. In cases where immediate cooling to -80°C was not possible (e.g. punctures outside normal working hours), the blood samples were, as a first step, immediately cooled to -15°C and permanently stored at -80°C on the next working day. Diagnosis assignment (septic (PJI) or aseptic) in the prosthesis group was conducted according to the previously described EBJIS criteria (85). Diagnosis assignment in the native joint group (aseptic and septic arthritis) was carried out based on the previously described modified Newman criteria for septic arthritis (59, 60). The results of the routinely performed synovial analysis via microscopy were used to further divide the aseptic group into gout and CPPD arthropathy depending on whether positive or negative birefringent crystals were found.

2.1.1 Metabolomic Profiling via Nuclear Magnetic Resonance (NMR)

In total, 37 metabolites are analysed: 2-Hydroxy-3-methylbutyric acid, L-Leucine, L-Isoleucine, L-Valine, Isobutyric acid, 3-Methyl-2-oxovaleric acid, 3-Hydroxybutyric acid, L-Lactic acid, L-Alanine, L-Arginine, Acetic acid, Acetone, L-Glutamic acid, Succinic acid, L-Glutamine, Citric acid, L-Methionine, L-Lysine, Creatine, Dimethyl sulfone, Choline, Phosphorylcholine, Glycerophosphocholine, Taurine, Glycine, L-Threonine, D-Mannose, D-Glucose, Glycogen, Nucleotides (AXP,IMP), L-Tyrosine, L-Histidine, L-Phenylalanine, L-Tryptophan, Formic acid, Inosinic acid, and Adenosine monophosphate.

Reagents

Anhydrous disodium phosphate (Na2HPO4), sodium azide (NaN3), hydrochloric acid (HCl), sodium hydroxide (NaOH), and methanol were obtained from Roth (Karlsruhe, Germany). Deuterium oxide (D2O) and 3-(trimethylsilyl) propionic acid-2,2,3,3-d4 sodium salt (TSP) were bought from Eurisotop (Saint-Aubin, France). “NMR buffer” was freshly prepared of 5.56 g of Na2HPO4, 0.4 g TSP, 0.2 g NaN3, and dissolved in 500 ml D2O after adjusting it to pH 7.4 with either 1 M NaOH or 1 M HCl.

Sample Preparation for NMR Measurement

Patient samples of synovial fluid were stored at -80°C until further processing. When thawed on ice, the viscous liquids were spun down for 1 min at 10.000 x g to remove any solids (mucous membrane remnants, clots). Consequently, the samples were processed as described previously (166-168). In short, 200 µl of sample were pipetted into 1.5 ml tubes andmixed with 400 µl of ice-cold methanol to precipitate proteins and inactivate any enzymatic activity. After storage at -20°C for 30 min, the samples were centrifuged at 10.000 x g for 30 min at 4°C, and the supernatant was collected into new 1.5 ml tubes and stored until further processing. Finally, all samples were lyophilized overnight using a SpeedVac System (Thermo Scientific, Vienna, Austria), and the remaining pellets were resuspended in 530 µl NMR buffer, with 500 µl thereof filled into 5 mm NMR tubes.

Quantification of Metabolites Using NMR Spectroscopy

All NMR experiments were performed at 310 K on an AVANCE™ NeoBruker Ultrashield 600 MHz spectrometer equipped with a TXI probe head. The 1D CPMG (Carr-

Purcell_Meiboom_Gill) pulse sequence (cpmgpr1d, 512 scans, 73,728 points in F1, 11,904.76 HZ spectral width, 512 transients, recycle delays 4 s) with water suppression using pre-saturation was used for 1H 1D-NMR experiments. Bruker Topspin version 4.0.2 (Bruker GmbH, Rheinstetten, Germany) was used for NMR data acquisition. The spectra for all samples were automatically processed (exponential line broadening of 0.3 Hz), phased, and referenced using TSP at 0.0 ppm. Spectra processing and data analysis was carried out using Matlab® scripts provided by the group of Prof. Jeremy Nicholson at Imperials College London and MetaboAnalyst 5.0 (169). NMR data were imported to Matlab® vR2014a (Mathworks, Natick, MA, USA), regions around the water, TSP, and remaining methanol signals excluded, and probabilistic quotient normalization (170) was performed to correct for sample metabolite dilution as previously described (171). Reported concentrations corresponded to normalized arbitrary units of spectral intensities.

2.1.2 Soluble Urokinase Plasminogen Activator Receptor (suPAR)

We determined concentrations of suPAR in blood plasma and synovial fluid samples using an established and commercially available sandwich immunoassay (suPARnostic® AUTO Flex ELISA, manufactured by ViroGates A/S, Birkeroed, Denmark). Samples had been thawed out until they reached room temperature, then homogenized, and lastly centrifuged. All sample supernatants were analyzed according to the manufacturer's instructions. Since we expected the suPAR levels in the synovial fluid specimen to exceed the linear range of the assay (0.4 - 14.2 ng/mL), we evaluated the most suitable dilution in a preliminary investigation. In alignment with those results, all samples where a-priori diluted 1:5 with diluent provided by the manufacturer. When the measured values still exceeded the linear range, further dilution steps (up to 1:40) were performed. The final results were calculated by multiplying the values with the respective dilution factor.

2.2 Multi Biomarker Model

The multi biomarker model was calculated on a previously published dataset including 124 surgical procedures due to suspected PJI (78 were PJI positive and 46 were PJI negative) (88). These 124 cases were recruited within a 27-month period at the Department of Orthopaedics and Trauma of the Medical University of Graz. We investigated the serum leukocyte count, CRP, IL-6, PCT, IF-alpha, and fibrinogen as potential biomarkers for PJI. The diagnosis assignment (PJI or aseptic) was carried out by a blinded researcher according to the MSIS 2011 criteria for PJI (79). In order to answer our research question of combining multiple biomarkers into a multi-biomarker model, the dataset was split into a training (75% of cases) and a test (25% of cases) set. Optimal cutoffs were calculated using the Youden Index and univariate logistic regression (172). These calculated cutoff values were then applied to the test set and the performances recorded in the form of the AUC from the ROC curves and p-values of the logistic regression. The possible ratios of the biomarkers were calculated and included in the model calculation process. By repeating the holdout method multiple times to receive more valid performance estimates (cross-validation), a logistic regression with lasso-regularization was conducted on the training samples (75% of total cases). Lastly, the final model was used to calculate a cutoff, and both were applied to the test set (25%). The software R, Version 3.6.1 (R Core Team, R Foundation for Statistical Computing,Vienna, Austria) was used for statistical analyses.

3. Results

The results section is divided into three parts including the respective sub-analyses according to the three diagnostical methods investigated. By including native and arthroplasty-provided joints, the methods "Metabolomic profiling via NMR" and "Soluble urokinase plasminogen activator receptor (suPAR)" were statistically evaluated separately regarding the subgroups "diagnostic performance in PJI" and "diagnostic performance in SA". In total, 182 cases in 173 patients were included in this part of the study. For "Metabolomic profiling via NMR", the total study population was also evaluated. This division was chosen to consider both the basic research aspect of detecting metabolic products as evidence for the presence of bacteria (total study population) and the clinical application of the research results (PJI and SA). Table 3 gives an overview of the results.

Method of Analysis	Joint Type	AUC
NMR[1]	Prosthesis	**0.91**
NMR[2]	Prosthesis	0.86
suPAR (synovia)	Prosthesis	0.76
suPAR (plasma)	Prosthesis	0.40
CRP (serum)	Prosthesis	0.79
WBC (serum)	Prosthesis	0.57
Multi biomarker*	Prosthesis	0.95
NMR[1]	Native Joint	**0.95**
NMR[2]	Native Joint	0.86
suPAR (synovia)	Native Joint	0.87
suPAR (plasma)	Native Joint	0.74
CRP (serum)	Native Joint	0.81
WBC (serum)	Native Joint	0.65

Table 2 – Result overview. [1] best performing metabolite combination; [2] best performing single metabolite; *evaluated on a different data set; NMR= Nuclear Magnetic Resonance; suPAR= Soluble urokinase plasminogen activator receptor; CRP= C-reactive protein; WBC= white blood cell (leucocyte) count; AUC= area-under-the-curve;

3.1 Metabolomic Profiling via NMR

3.1.1 Diagnostic Performance of NMR in PJI

In total, 76 samples were included in this final analysis, among those are 48 PJI cases and 28 aseptic cases. At the day of arthrocentesis, the PJI group had a mean leukocyte count of 10.5 G/L (±6.0), a mean CRP of 101.7 mg/dL (± 81.6), and a mean synovial WBC of 38751 cells/µL (± 41924). In the aseptic group, mean leukocyte count was 10.12 G/L (±5.7), mean CRP 35.3 mg/dL (± 40.5), and the mean synovial WBC 1360 cells/µL (± 4716). The most frequent microorganisms were *staphylococcus aureus* (n= 13; 27%) and *staphylococcus epidermidis* (n= 7; 15%). In 14 cases (29%), it was not possible to isolate a microorganism.

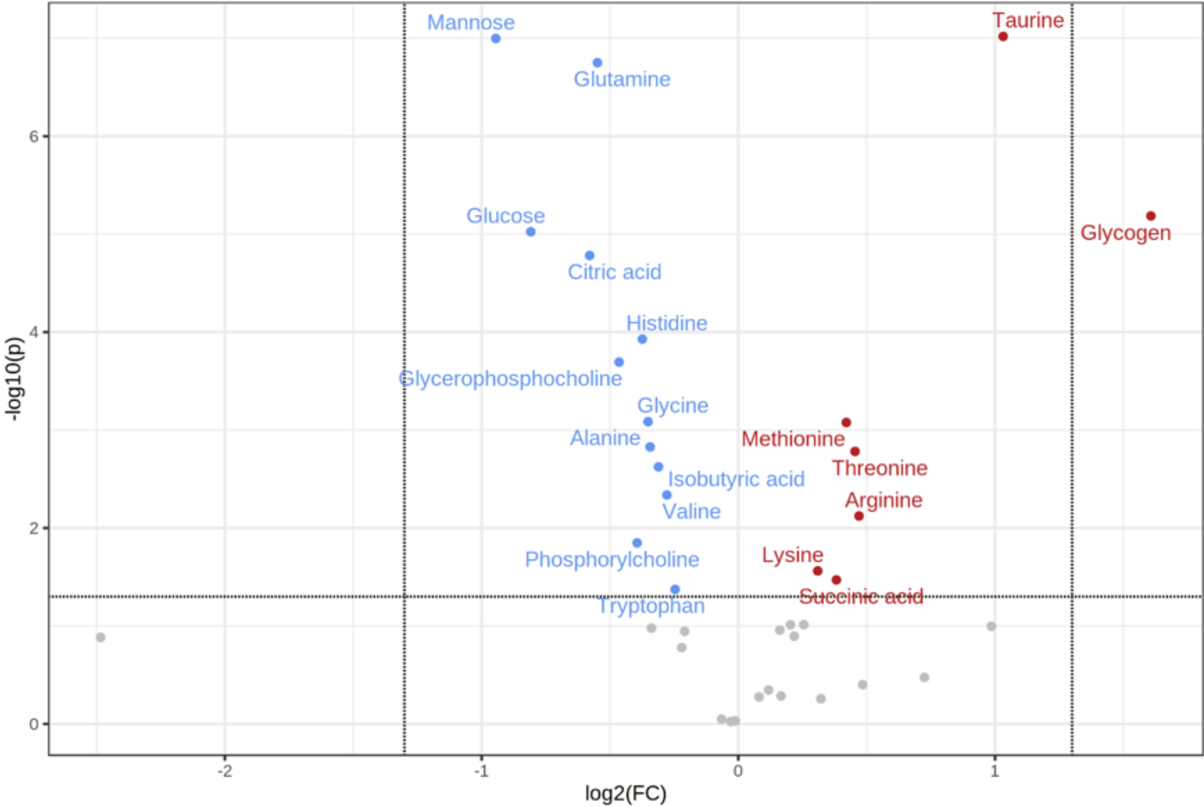


Figure 2 – Up- (red) and down- (blue) regulation of specific metabolites in patients with PJI measured via NMR spectroscopy (not significant = grey).

Figure 2 shows the metabolites analyzed, depicting the significant up- or down-regulation in PJI patients on a logarithmic scale. The Orthogonal partial least squares discriminant analysis (OPLS-DA) is depicted in figure 3.

Figure 3 - Orthogonal partial least squares discriminant analysis (OPLS-DA) regression model of aseptic ("2"; red) and septic (PJI) cases ("4", green). T score 18.8%, orthogonal T score 20.3%.

Five single metabolites have shown an AUC exceeding 0.8: Taurine (AUC 0.8558, p<0.0001), Glutamine (AUC 0.8333, p<0.0001), Mannose (AUC 0.8326, p<0.0001), Glycogen (AUC 0.8275, p<0.0001), and Methionine (0.8043, p<0.0001). Moreover, the combination of two metabolites as a ratio showed even better diagnostic performance: Glucose/Glycogen (AUC 0.9073, p<0.0001), Taurine/Mannose (AUC 0.9073, p<0.0001), Mannose/Glycogen (AUC 0.8992, p<0.0001), and Taurine/Glucose (AUC 0.8956, p<0.0001). The ROC curves, including

the true positive rate, the false positive rate and the standard deviation of the best performing ratios, are depicted in figure 4.

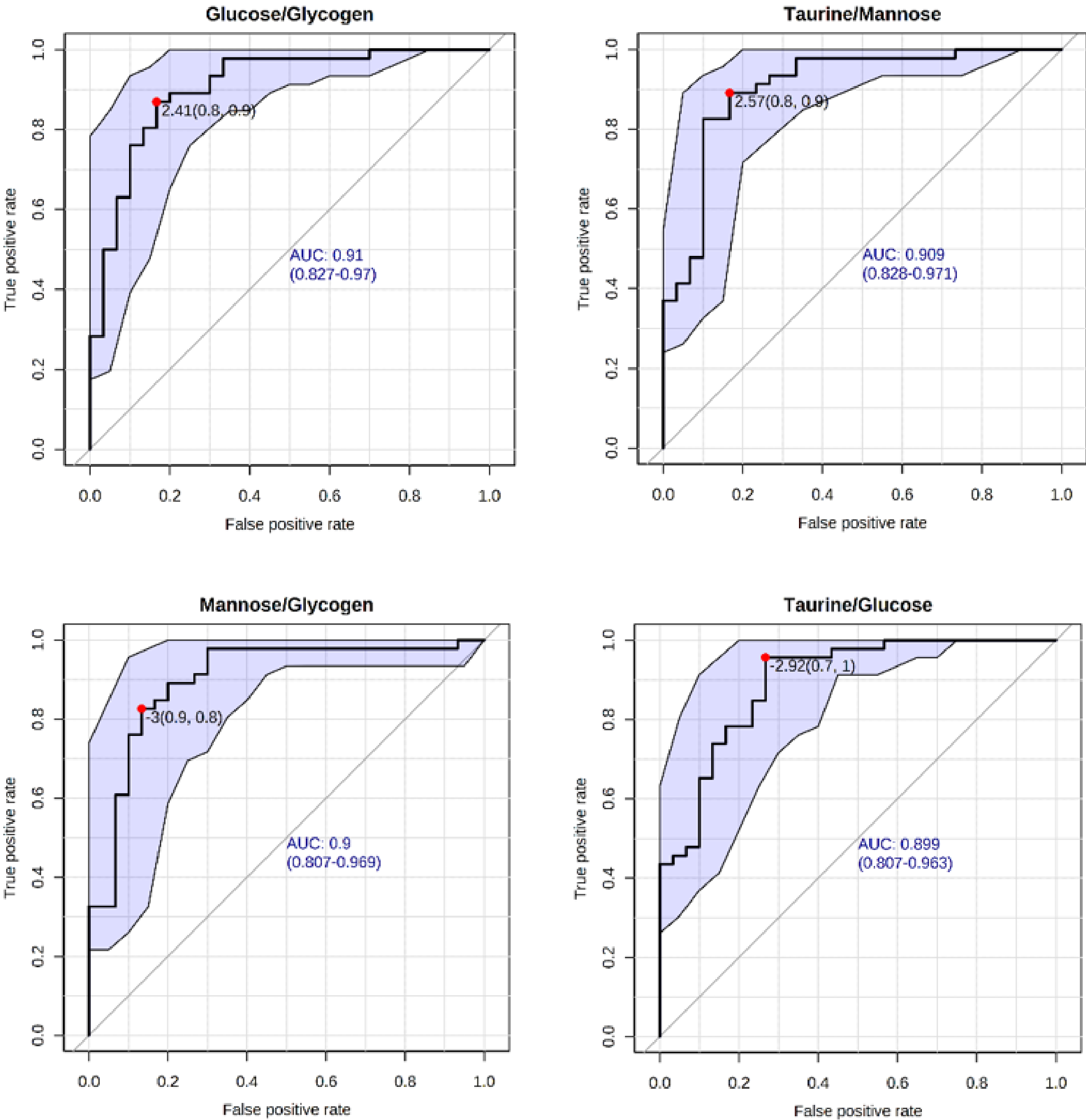


Figure 4 - ROC curves including the true positive rate, the false positive rate and the standard deviation of the best performing metabolite ratios in the diagnosis of PJI.

3.1.2 Diagnostic Performance of NMR in SA

In total, 103 samples were included in this final analysis, among those are13 SA cases and 90 aseptic cases. The aseptic cases included 27 cases of gout and 25 cases of calcium pyrophosphate deposition disease (CPPD, pseudogout), and were counted as standard aseptic arthritis cases in the statistical analysis. At the day of arthrocentesis, the SA group had a mean leukocyte count of 12.43 G/L (±4.43), a mean CRP of 208.9 mg/dL (± 102), and a mean synovial WBC of 43617 cells/µL (± 47925). In the aseptic group, mean leukocyte count was 10.17 G/L (±2.92), mean CRP 94.2 mg/dL (± 86.1), and the mean synovial WBC 14434 cells/µL (± 14150). The most frequent microorganisms were *staphylococcus aureus* (n= 3; 23%) and *streptococcus agalacticae* (n= 3; 23%). It was not possible to isolate microorganism in seven cases (54%). In three cases, a microorganism was counted as contamination due to a lack of further evidence for septic arthritis (*staph. epidermidis, cutibacterium acnes,* and *micrococcus luteus*).

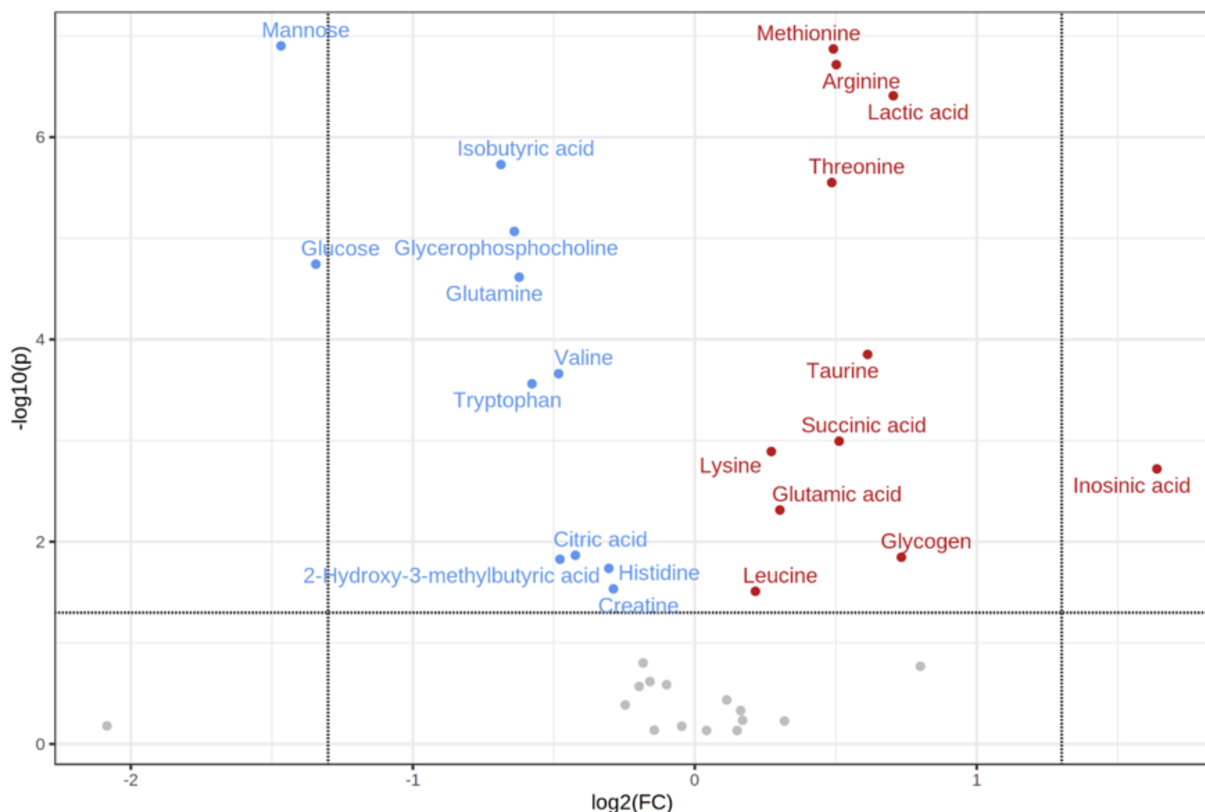


Figure 5 – Up- (red) and down- (blue) regulation of specific metabolites in patients with SA measured via NMR spectroscopy (not significant = grey).

Figure 5 shows the metabolites analyzed, depicting the significant up- or down-regulation in SA patients on a logarithmic scale. The Orthogonal partial least squares discriminant analysis (OPLS-DA) is depicted in figure 6.

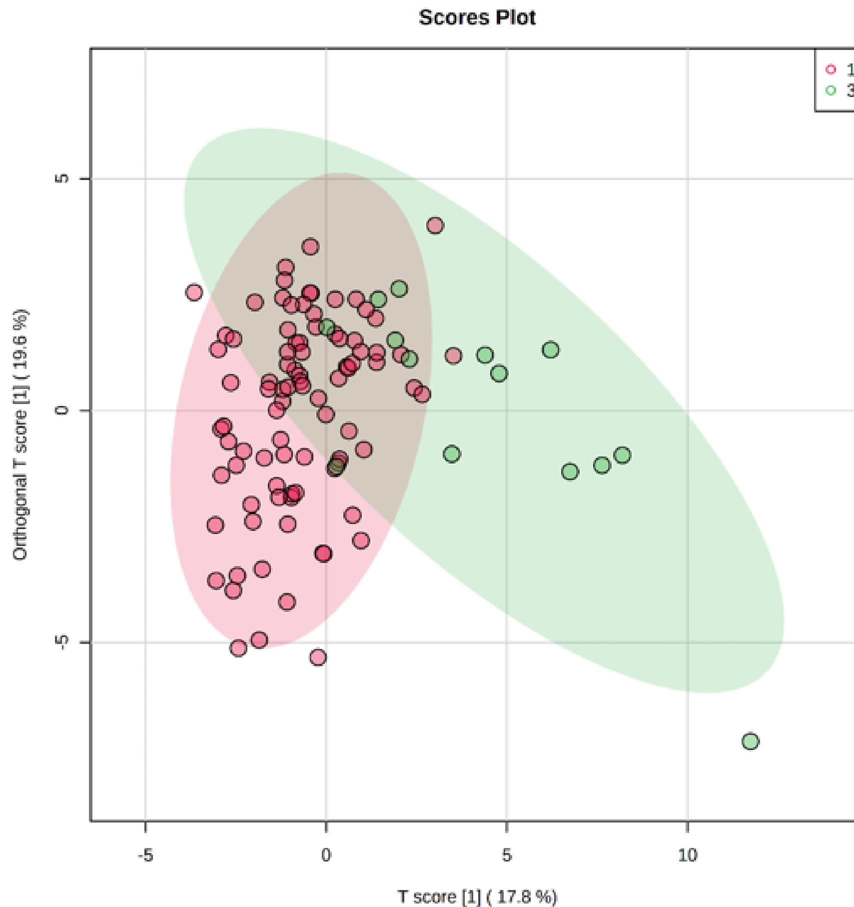


Figure 6 - Orthogonal partial least squares discriminant analysis (OPLS-DA) regression model of aseptic ("1"; red) and septic (native joint) cases ("3", green). T score 17.8%, orthogonal T score 19.6%.

Three single metabolites have shown an AUC exceeding 0.9: Mannose (AUC 0.8558, $p<0.0001$), Glutamine (AUC 0.8333, $p<0.0001$), Mannose (AUC 0.9366, $p<0.0001$), Glucose (AUC 0.9221, $p<0.0001$), and isobutyric acid (0.9133, $p<0.0001$). Further, the combination of two metabolites as a ratio showed even better diagnostic performance: Isobutyric acid/Methionine (AUC 0.9462, $p<0.0001$), Arginine/Glucose (AUC 0.9374, $p<0.0001$), Methionine/Mannose (AUC 0.9366, $p<0.0001$), and Taurine/Glucose (AUC 0.9358,

p<0.0001). The ROC curves, including the true positive rate, the false positive rate, and the standard deviation of the best performing single metabolite and the respective ratios are depicted in figure 7.

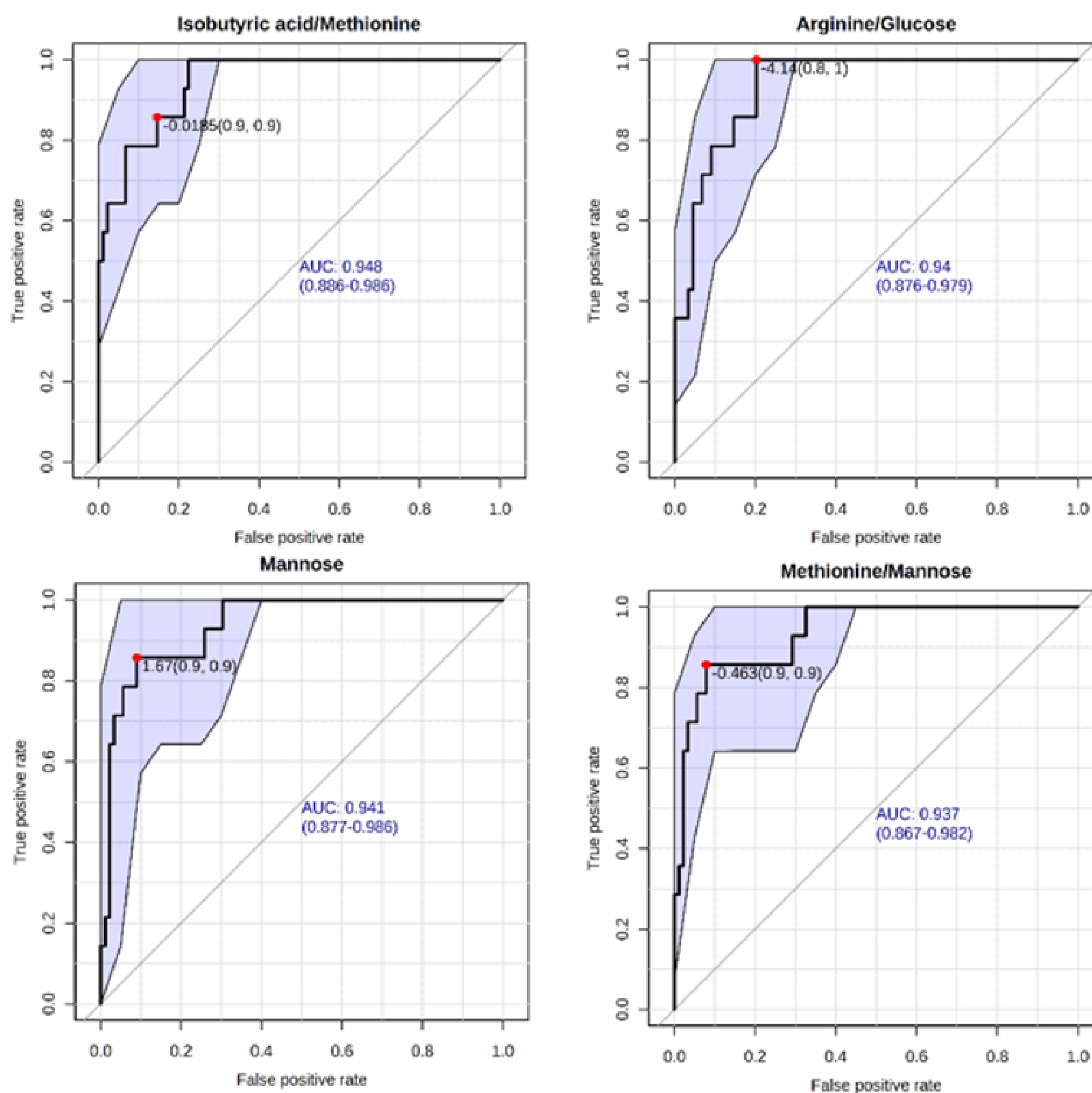


Figure 7 - ROC curves including the true positive rate, the false positive rate, and the standard deviation of the best performing metabolites and metabolite ratios in the diagnosis of SA.

3.1.3 Diagnostic Performance of NMR in the Total Study Population

179 samples were included in the statistical analysis of the total study population. These are divided into 61 septic cases (SA or PJI), 64 cases of non-crystal aseptic arthritis, 27 cases of gout, and 27 cases of CPPD crystal arthropathy. At the day of arthrocentesis, the septic group had a mean leukocyte count of 10.94 G/L (±5.7), a mean CRP of 125.3 mg/dL (± 97.3), and a mean synovial WBC of 39823 cells/µL (± 43365). The non-crystal aseptic arthritis group had a mean leukocyte count of 9.63 G/L (±4.6), a mean CRP of 49.6 mg/dL (± 56.7), and a mean synovial WBC of 4174 cells/µL (± 7140). The gout group has shown a mean leukocyte count of 10.28 G/L (±3.03), a mean CRP of 113.2 mg/dL (± 92.3), and a mean synovial WBC of 16917 cells/µL (± 12607). The CPPD group had a mean leukocyte count of 10.09 G/L (±2.64), a mean CRP of 97.7 mg/dL (± 83.3), and a mean synovial WBC of 18070 cells/µL (± 16833).

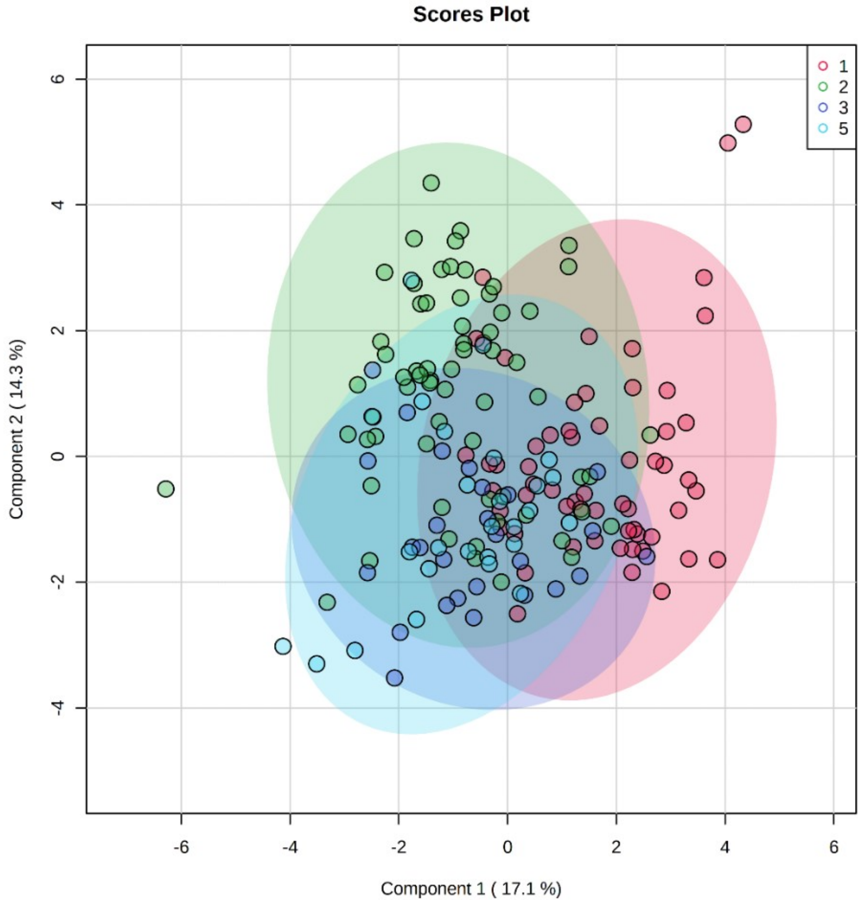


Figure 8 - sparse partial least squares discriminant analysis (sPLS-DA) model of septic (“1”; red) aseptic (“2”, green), gout (“3”, purple), and CPPD arthritis (“5”, blue).

Figure 8 shows the sparse partial least squares discriminant analysis (sPLS-DA) model for the whole study cohort, distinguishing between septic, aseptic, gout, and pseudogout cases. The individual metabolites and the strength of their expression in the respective groups are shown in figure 9.

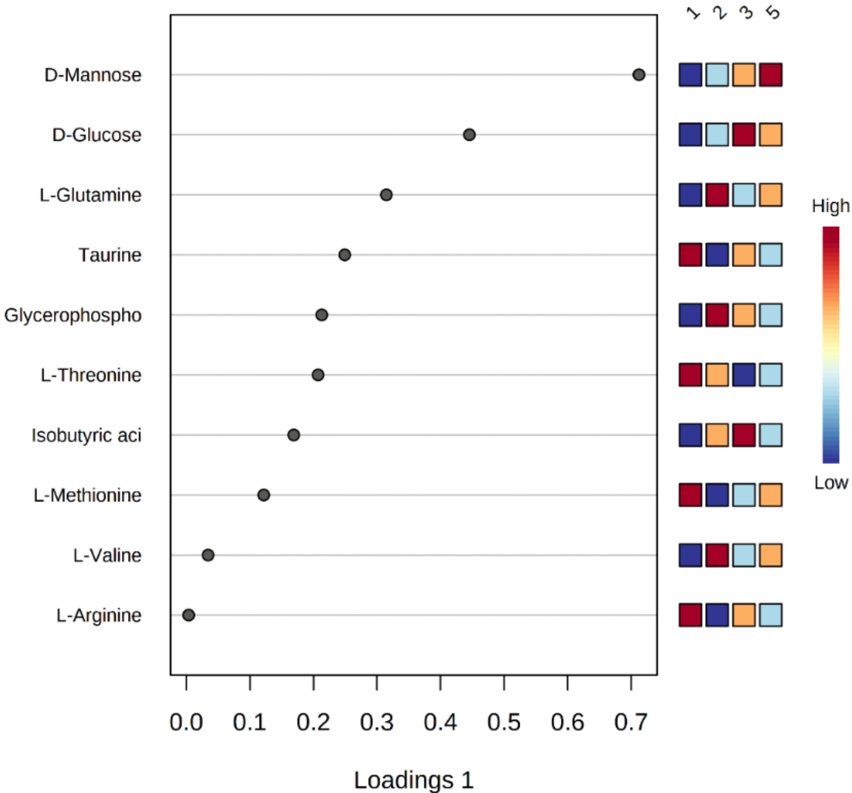


Figure 9 - The individual metabolites and the strength of their expression in the respective groups (features). 1= septic, 2= aseptic, 3= gout, 5= CPPD arthritis.

	1	2	3	5
1	Q^2 *p*	0.43 *< 0.01*	0.47 *< 0.01*	0.38 *< 0.01*
2			0.18 *< 0.01*	0.05 *0.01*
3				-0.01 *0.03*
5				

Figure 10 - Prediction value = Q^2 quantifying the algorithms discrimination power between different groups on an independent test set (sPLS-DA). 1= septic, 2= aseptic, 3= gout, 5= CPPD arthritis.

Figure 10 depicts the respective Q^2 values which are quantifying the sPLS-DA-algorithm discrimination power between different groups on an independent test set. Figure 11 shows the two single best performing metabolites in sepsis diagnosis (septic cases = SA and PJI; aseptic cases= aseptic arthritis, gout pseudogout) when analyzing the total study cohort, namely Mannose ($p<0.0001$) and Glucose ($p<0.0001$).

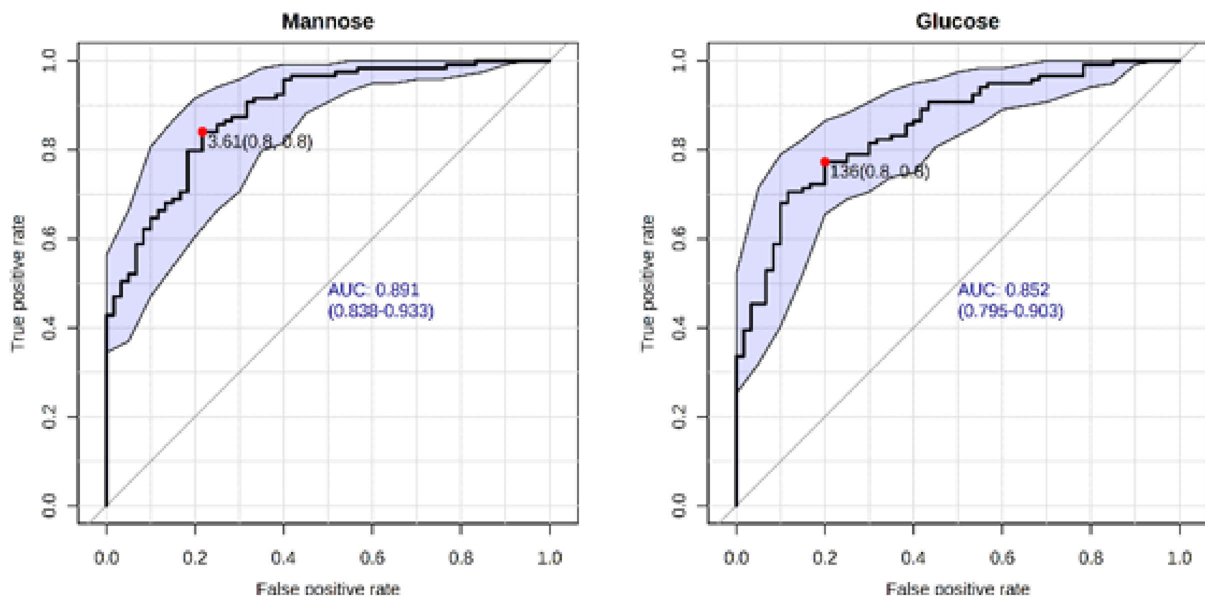


Figure 11 - Mannose ($p<0.0001$) and Glucose ($p<0.0001$) as the two best performing single metabolites in sepsis diagnosis (total study cohort).

Figure 12 reveals statistically significant differences among eight metabolites as calculated using one-way ANOVA. Parameters were selected based on a p value < 0.0014 (resulting from Bonferroni's correction with 37 parameters being investigated in targeted analysis). The y-axes represent concentrations in arbitrary units (a.u.) resulting from integration of peak intensities at the respective chemical shift intervals. The x-axes depict the categories septic/aseptic and prosthetic/native joint.

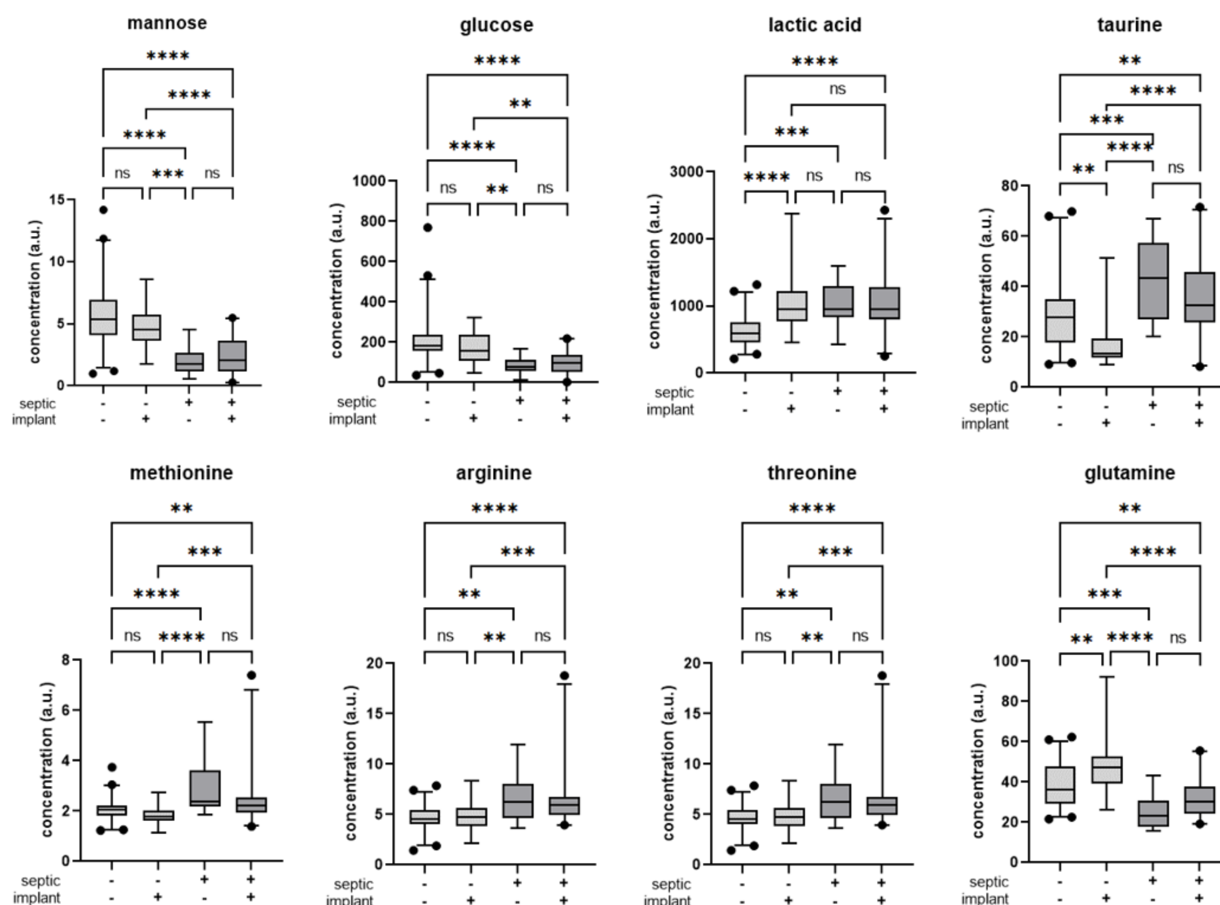


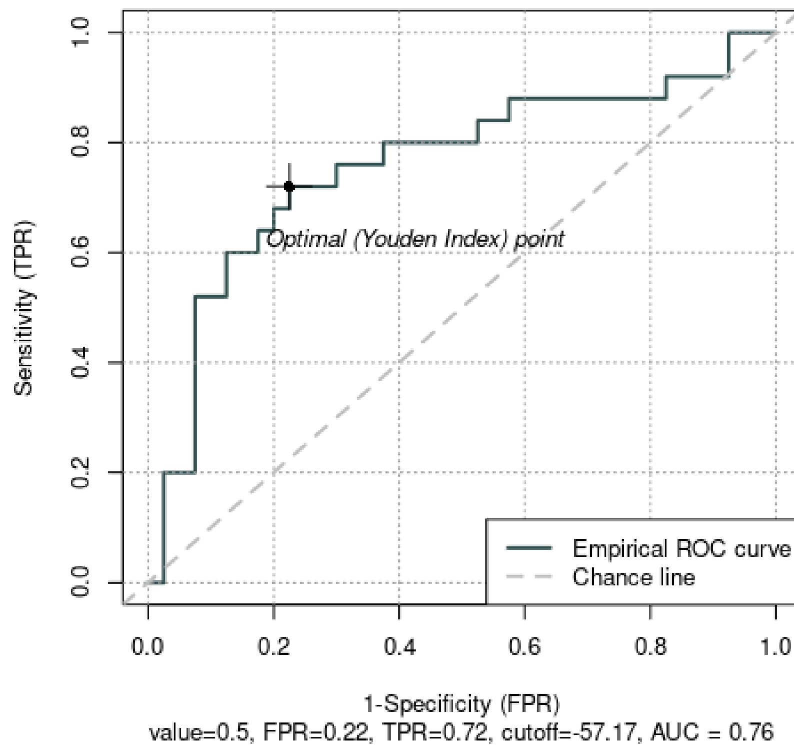
Figure 12 - one-way ANOVA calculation of differences among eight metabolites. The boxes extend from the 25th to 75th percentile, the bars represent 2.5-to-97.5 percentiles. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001

3.2 Soluble Urokinase Plasminogen Activator Receptor (suPAR)

3.2.1 Diagnostic Performance of suPAR in PJI

76 cases were included in this analysis, among those are 49 PJI cases and 27 aseptic cases. At the day of blood and synovial sample collection, the PJI group had a mean leukocyte count of 10.5 G/L (± 5.9), a mean CRP of 100.3 mg/dL (± 81.3), and a mean synovial WBC of 37964 cells/µL (± 41818). In the aseptic group, mean leukocyte count was 10.12 G/L (±5.7), mean CRP 35.3 mg/dL (± 40.6), and the mean synovial WBC 1408 cells/µL (± 4799). The most frequent microorganisms were *staphylococcus aureus* (n= 13; 27%) and *staphylococcus epidermidis* (n= 7; 14%). In 15 cases (30.6%), it was not possible to isolate a microorganism.

Figure 13 depicts the ROC curves including the AUC, sensitivity, and specificity of the plasma and synovial suPAR measurements. Figure 14 shows the established PJI biomarkers CRP and serum leucocytes (WBC) for performance comparison. In some cases, it was not possible to collect the whole data set according to the study protocol. Out of the included 76 cases, ten WBC, ten CRP, and eleven suPAR synovial samples were not available. Further, 38 missing suPAR plasma values are reported, this was due to the implementation of suPAR plasma measurement into the study in the form of an amendment after starting the patient recruitment.



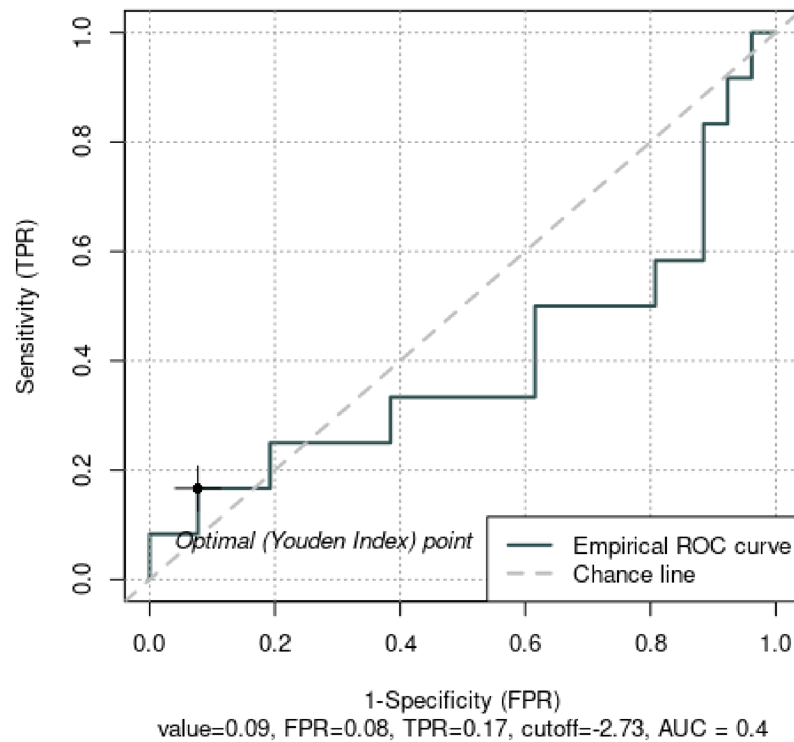
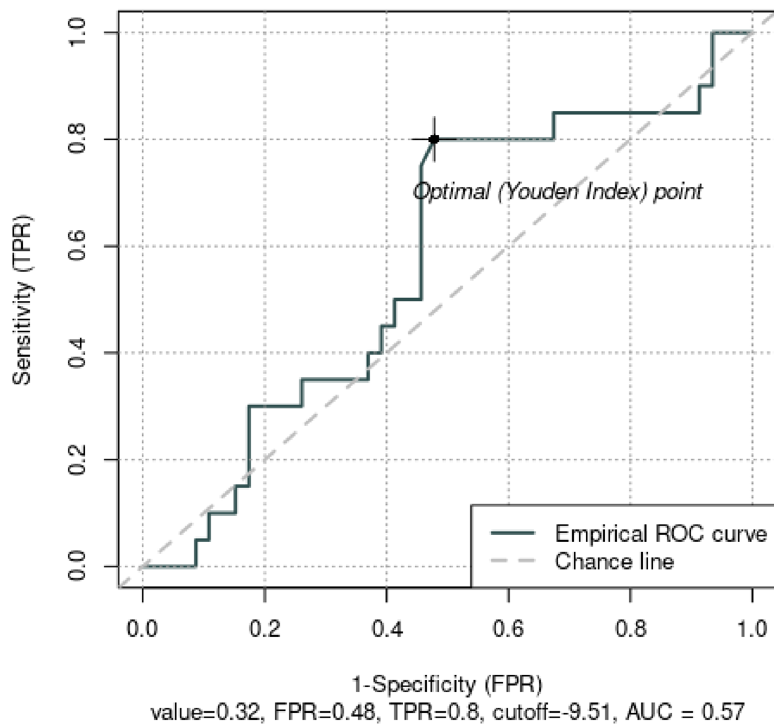


Figure 13 - ROC curves including the AUC, sensitivity, and specificity of the synovial (above) and plasma (below) suPAR measurements in the prosthesis group.



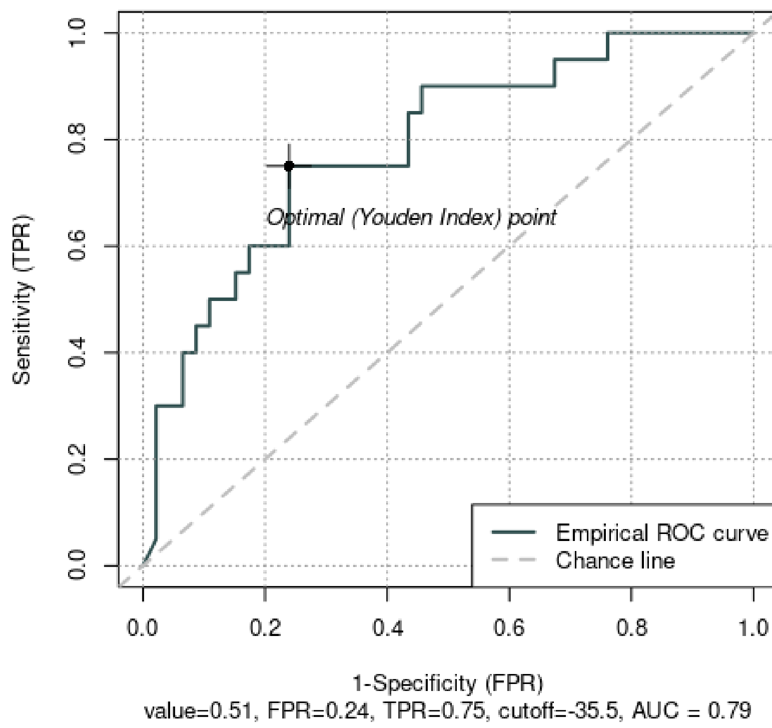
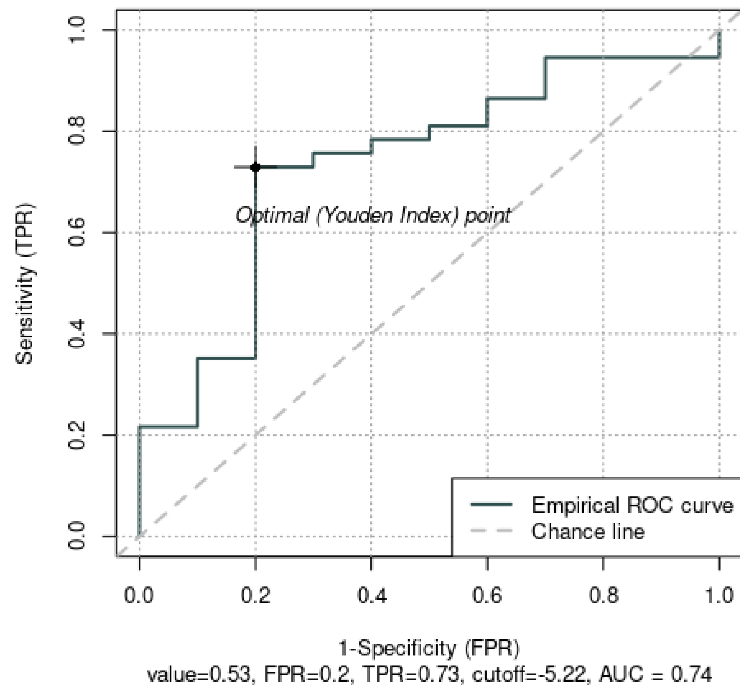


Figure 14 - ROC curves including the AUC, sensitivity, and specificity of the serum leucocytes (WBC, above) and C-reactive protein (CRP, below) measurements in the prosthesis group.

3.2.2 Diagnostic Performance of suPAR in SA

105 cases were included in this analysis, among those were 13 SA cases and 92 aseptic cases. The aseptic cases consisted of 40 cases of non-crystal aseptic arthritis, 27 cases of gout, and 25 cases of CPPD crystal arthropathy. At the day of blood and synovial sample collection, the SA group had a mean leukocyte count of 12.4 G/L (± 4.4), a mean CRP of 208.9 mg/dL (± 102), and a mean synovial WBC of 43617 cells/µL (± 47925). In the aseptic group, mean leukocyte count was 9.85 G/L (±3.0), mean CRP 90.5 mg/dL (± 83.7), and the mean synovial WBC 12977 cells/µL (± 13686).

Figure 15 depicts the ROC curves including the AUC, sensitivity, and specificity of the plasma and synovial suPAR measurements. Figure 16 shows the diagnostic performance of CRP and serum leucocytes (WBC). In some cases, it was not possible to collect the whole data set according to the study protocol. Out of the included 105 samples, 15 WBC, 13 CRP, and eight suPAR synovial values were not available. Further, 58 missing suPAR plasma values are reported due to the above-mentioned reasons.



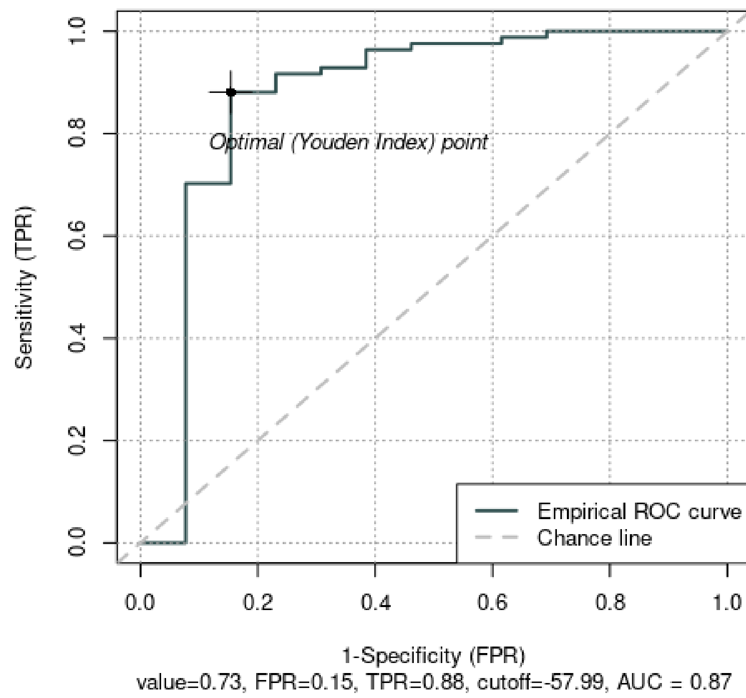
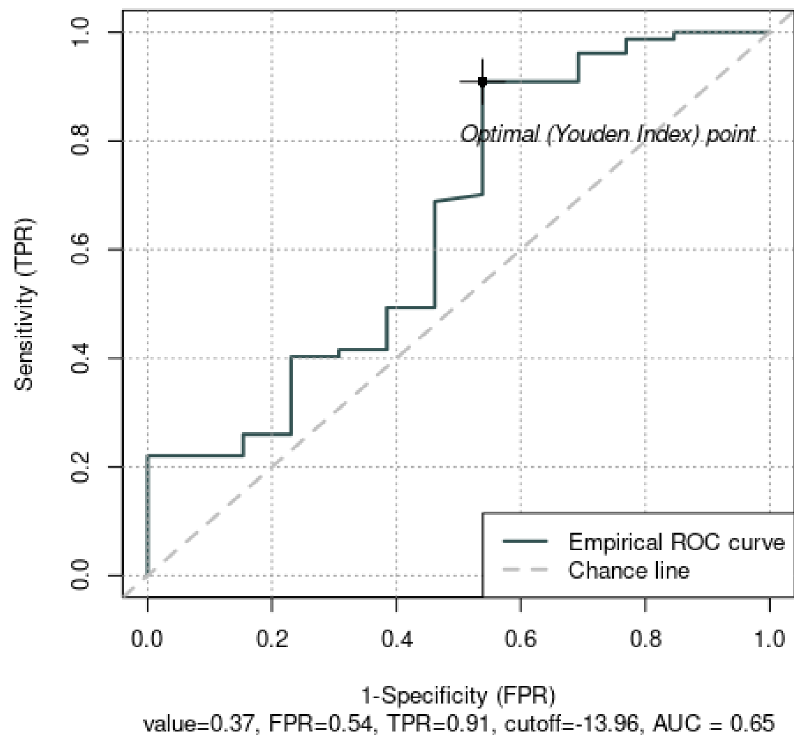


Figure 15 - ROC curves including the AUC, sensitivity, and specificity of the plasma (above) and synovial (below) suPAR measurements in the native joint group.



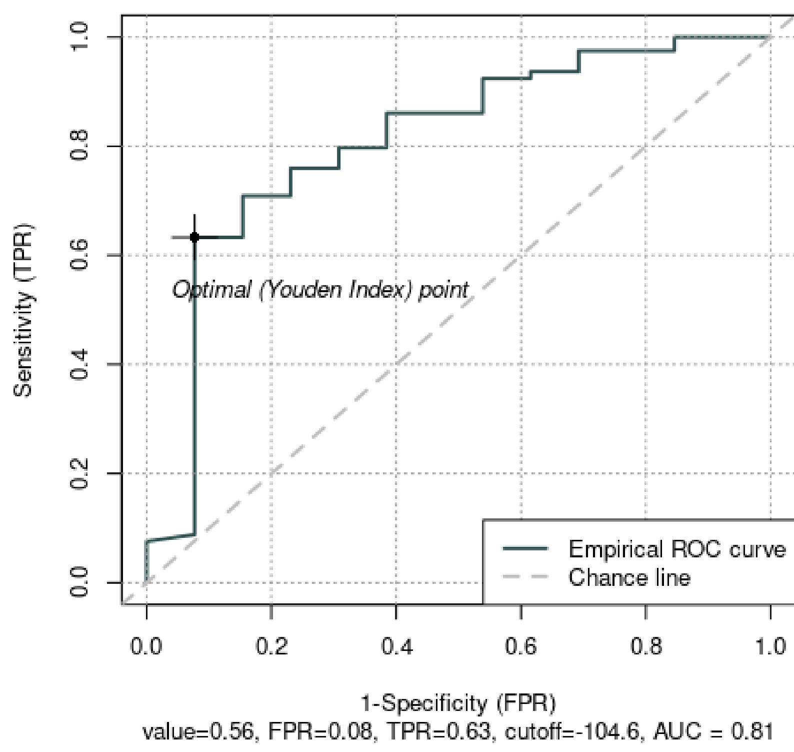


Figure 16 - ROC curves including the AUC, sensitivity, and specificity of the serum leucocytes (WBC, above) and C-reactive protein (CRP, below) measurements in the native joint group.

3.3 Multi Biomarker Model

In total, 124 surgical procedures were included in this analysis (78 with PJI and 46 PJI-negative cases). The final model integrated the ratio of fibrinogen to CRP (-0.00002), serum thrombocytes to CRP (-0.00632), CRP (0.00242), fibrinogen (0.00121), and an intercept (−0.24370), with the respective weights in brackets. Serum WBC, IL-6, interferon-alpha. and procalcitonin were excluded by the algorithm, as the inclusion resulted in a decreased diagnostic performance of the model. Figure 17 shows the ROC curves of the multi-biomarker model as well as the single biomarkers fibrinogen and CRP for comparison. While the model has shown a high diagnostic accuracy with an AUC of 0.95, a specificity of 91%. and a sensitivity of 72%, it did not show a significantly better performance than CRP (AUC 0.91, specificity 67%, sensitivity 90%) and fibrinogen (AUC 0.93, specificity 73%, sensitivity 94%) as previously reported by Klim et al (1).

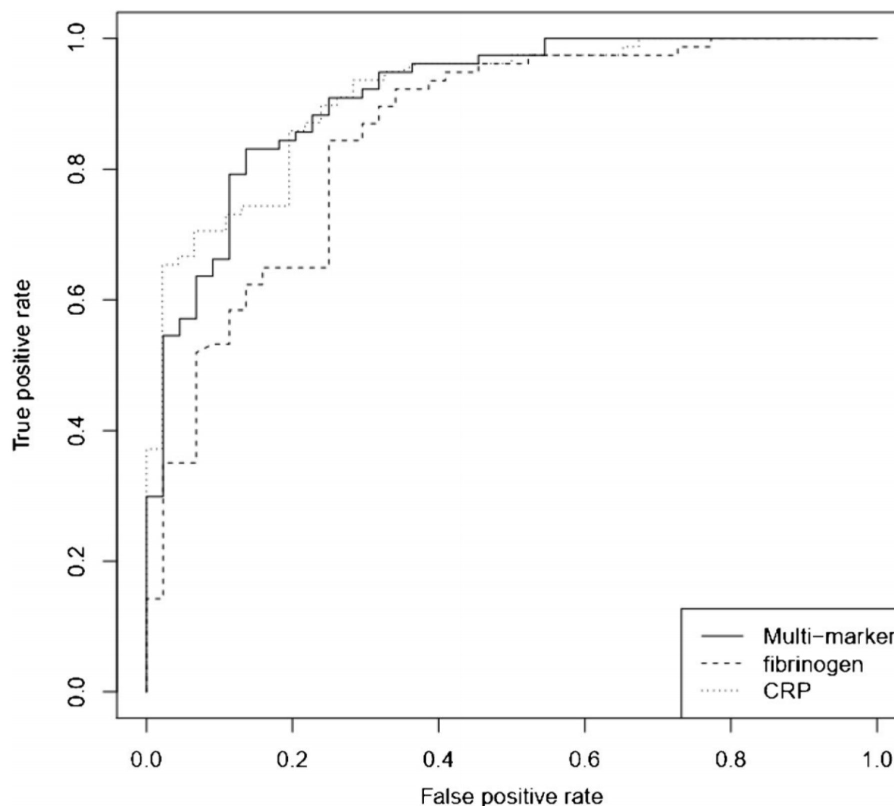


Figure 17 - ROC curves depicting the performance of the calculated multi-biomarker model (AUC 0.95, specificity 91%, sensitivity 72%; continuous line) in PJI diagnosis. CRP (AUC 0.91, specificity 67%, sensitivity 90%; small dotted line) and fibrinogen (AUC 0.93, specificity 73%, sensitivity 94%; big dotted line) for comparison. Reproduced from Klim et al. (Int Orthop. 2020 (1)) with permission of publisher "Springer".

4. Discussion

The aim of the present thesis was to investigate the methods of NMR and suPAR regarding their performance in SA and PJI diagnosis. Further, the aim was to evaluate whether a multi biomarker model can outperform established serum biomarkers in PJI diagnosis. In the following sections, the study results will be placed in the context of the current literature, compared with the published results and critically discussed. furthermore, the novelty value of the work will be analyzed and possible further research topics and focal points resulting from this dissertation will be considered. The strengths and weaknesses of each method will also be discussed. For a better overview, the discussion of the results is subdivided into the respective diagnostic methods.

4.1 Metabolomic Profiling via NMR

In the last 15 years, there has been a steady increase of papers investigating NMR-based metabolomics. With the other two major techniques in metabolom analysis being gas chromatography-based mass spectrometry and liquid chromatography-based single-stage mass spectrometry. Advantages of NMR-based metabolomics as used in the presented research are an relatively easy sample preparation, the quantifiability of metabolite levels, the high degree of experimental reproducibility, and the nondestructive nature making the method ideal for large-scale clinical research (173).

4.1.1 Context of the Current Literature

The metabolomic profiling and isolation of single metabolites as well as the ratios of two metabolites in synovial fluid showed very satisfactory results with high diagnostic significance in both native joints and prostheses. Thus, NMR seems to be an effective method to distinguish aseptic from septic arthritis and to diagnose PJI. The diagnostic properties of the method have already been demonstrated in other publications in different sample types and pathologies. In an earlier study from 2013, Young et al. assessed metabolic signatures in serum from patients with either an established rheumatoid arthritis or with early arthritis and found that NMR was able to reflect inflammatory disease activity in patients with synovitis (174). One year prior,

Hügle et al. first described different metabolomic patterns in human synovial fluid by distinguishing septic arthritis from osteoarthritis, crystal-associated arthritis, rheumatoid arthritis, and spondylarthritis, but found no differences between the aseptic pathologies. The study included 59 samples and concluded that NMR is a fast analytic method with a possible scope of application in synovial fluid diagnostics (139).

Currently, there are two further studies available investigating the metabolomic differences between septic and non-septic synovial fluid. Anderson et al. included 19 horses (seven septic vs. twelve aseptic) and found that synovial metabolites measured via NMR can distinguish between septic and aseptic equine arthritis, with glucose being the principal discriminator. Akhbari et al. investigated 16 samples (eight aseptic and eight septic) on differences in the metabolomic composition of the synovial fluid, and found three up-regulated metabolites and 13 down-regulated concentration in the septic group compared to the aseptic cohort, including lower glucose and mannose levels (141). The importance of glucose and mannose in the metabolism of bacterial arthritis was also a central finding of the present study and thus confirms these results. While healthy synovial fluid and serum have comparable glucose levels, the bacterial metabolism in septic arthritis leads to reduced glucose levels. However, this synovial glucose reduction is (although to a lesser extent) also possible in acute aseptic inflammation (175).

Attempts to use this diagnostically have been made using various methods. A recent study by Kinugasa et al. investigated the joint fluid glucose level in the diagnosis of septic arthritis. They described a cut-off of 40mg/dl (glucose in synovial fluid). Below this cut-off value, the authors were able to detect bacterial arthritis via culture in 11/11 (100%) patients, whereas only 6/19 (31.6%) of patients with glucose values above 40 mg/dl had a positive microbiologic culture (176). In a larger series including 102 patients, Omar et al. investigated the diagnostic performance of the synovial glucose levels using a glucometer. They reported a sensitivity of 100 % (95 % CI 78.2–100 %) and a specificity of 92.0 % (95 % CI 84.1–96.7 %) at a threshold of 1.4 mmol/l (177).

Grauslys et al. investigated whether NMR-based metabolomic profiling can distinguish between bacterial and viral infections and post-operative inflammation without infection in children following cardiac surgery. Furthermore, the significance of NMR based metabolomic profiling in blood plasma with regard to prognostic performance was investigated. Here, septic

organ dysfunction was used as a criterion (178). Similar to the present study, PCA and sPLSDA were used for multivariate analysis. Similar to the present study, a clear difference was shown between the group with bacterial infection (n= 25) and the control group (elective cardiac surgery without infection, n= 58) with an AUC of 0.94. The metabolomic profile of the viral infection group (n=30) was also significantly different from the control group (AUC 0.83). The distinction between the bacterial and the viral group (AUC 0.78) as well as between those children with sepsis and organ dysfunction and those without organ dysfunction (AUC 0.73) was somewhat more moderate. The authors thus concluded a good diagnostic and prognostic performance between the groups studied. The differentiation and further scientific investigation of the different phenotypes offers various possibilities for optimizing therapy in this severely ill patient population (178).

Another pilot study by French et al. has shown the potential of NMR metabolomics using cerebrospinal fluid samples in the differentiation of various causes of encephalomyelitis. They included patients with Lyme disease (n = 5), West Nile Virus meningoencephalitis (n = 5), Clinically Isolated Syndrome of multiple sclerosis (MS, n = 4) rabies (n = 10) and Histoplasma meningitis (n = 3) as well as 25 samples from discarded juvenile cerebrospinal fluid. Correct differentiation of the underlying cause of encephalomyelitis enables more effective and targeted treatment of this severe disease but is often clinically difficult or impossible. The authors could prove differences in the biochemical profiles of cerebrospinal fluid regarding the underlying pathology in encephalomyelitis cases. Especially Pyroglutamate had a high discriminatory value: Encephalomyelitis vs. controls (cut-off 35.44 µM; Sensitivity 96.3%, Specificity 96%); infection vs. aseptic (cut-off 35.44 µM; Sensitivity 100%, Specificity 93%); west nile virus, Lyme or histoplasmosis vs. controls (cut-off 35.67 µM; Sensitivity 100%, Specificity 100%). Other metabolites useful for diagnostic differentiation were glucose, 2-hydroxybutyrate, carnitine, acetamide and betaine (179). It must be considered that these results are data from a small cohort study - this is also pointed out by the authors. Due to the relatively small group size, the risk of bias must be considered. However, this study clearly shows the potential of NMR metabolome analysis and its possible clinical application in the diagnosis of encephalomyelitis.

4.1.2 Implications and Outlook

Compared to the available studies on NMR-based synovial metabolome analysis, one of the main strengths of our study is the significantly larger patient cohort in terms of both native and endoprosthetic joints. With this larger cohort, we were not only able to confirm the results of previous smaller studies and pilot studies, but also to prove the effectiveness of this new diagnostic method on a larger scale. However, there are still some steps to be taken on the way to the broad clinical application of NMR metabolomics as a diagnostic tool. Currently, we have been able to investigate this method in the context of university research due to the availability of a high-performance device at our University. Furthermore, the necessary know-how was available in the form of an internationally renowned expert on the field (Prof. Tobias Madl). One of the main problems at present is the organizational effort required to carry out a single measurement. In the context of this study, all samples were analyzed collectively after completion of patient recruitment. This is not feasible in everyday clinical practice, where a result should be available within a few hours. The results of this work can undoubtedly contribute to the expansion of the field of application of NMR analysis and thus to a faster development regarding the clinical applicability. While NMR has already taken major steps towards efficient clinical application in other countries, this is still necessary in Austria before it can be widely used in clinical diagnostics.

Compared to other methods of synovial analysis, the cost of metabolomic profiling via NMR are low amounting to about 15€ per measurement (consumable cost) (124). However, the purchase costs of the NMR spectrometer device, the maintenance and servicing as well as the special requirements for the laboratory infrastructure (non-vibrational floors and isolation from magnetic and radio frequency interference) are higher than for other metabolomic methods such as mass spectrometers. Further NMR operation requires highly skilled personal (173).

4.2 Soluble Urokinase Plasminogen Activator Receptor (suPAR)

4.2.1 Context of the Current Literature

We investigated suPAR in both plasma and synovial fluid regarding its diagnostic properties in SA and PJI. While plasma suPAR has shown inferior results in the PJI cohort (AUC 0.40),

synovial suPAR (AUC 0.76) was on the same level as serum CRP (AUC 0.79). In the native joint cohort, suPAR levels in plasma correlated considerably better with SA (AUC 0.74), however it was still outperformed by serum CRP (AUC 0.81) and the other tested biomarkers, including synovial suPAR (AUC 0.87). These findings showed that plasma suPAR has added no diagnostic value and should not be used in the standard diagnostic algorithm of SA and PJI. In contrast, synovial suPAR showed good diagnostic performance, especially in the native joint group. However, the difference in AUC to other biomarkers in the present study is too small to justify the use of joint puncture as the only factor in determining this biomarker. In this case, easier and periodically determined biomarkers such as serum CRP should be used. However, in cases where a joint puncture is to be performed anyway, suPAR can provide additional diagnostic value.

To our knowledge, there is currently one published study investigating the performance of serum suPAR (not plasma in contrast to the present study) in PJI diagnostics. Galliera et al. reported significantly higher values for PJI positive patients (6.76 ± 0.226 ng/mL $p < 0.0001$) compared to PJI negative patients, and thus the diagnostic performance was significantly better than in the present study (AUC 0.885). The study included 80 revision TKA or THA patients (45 PJI and 35 non infected cases) (154). Other studies focused on the potential of suPAR as a measurement tool for RA disease progression and joint destruction. In a recent paper, Enocsson et al. showed that baseline disease activity and joint damage at 36 months correlate significantly with suPAR serum levels. However, no predictive value of suPAR levels was observed. Closer monitoring of patients with elevated suPAR levels may be appropriate to detect joint destruction at an early stage (180). The diagnostic value of plasma/serum suPAR in joint infection diagnostics remains to be seen but has suffered a significant setback in the present study as described. However, SuPAR has already found its firm diagnostic and predictive value in other areas such as sepsis, cardiac disease, pneumonia, and autoimmune disorders (148) (149) (150) (151) (152).

In contrast to the studies cited above, a recent study from New Zealand did not investigate a specific pathology, but rather the relationship between suPAR as an inflammation marker and a faster ageing process regarding multiple organ systems and the central nervous system, as well as physical performance. In a population representative birth cohort of 843 patients, suPAR plasma measurements were performed at age 37 and 45 via the same ELISA-test protocol as described in the present study (suPARnostic® AUTO Flex ELISA, manufactured by ViroGates

A/S, Birkeroed, Denmark). Elevated suPAR levels were significantly associated with various negative effects on the investigated parameters: Faster ageing from 26 to 45 years, with an average of 6.4 years comparing the top to the bottom quintile and the central nervous system exhibited significantly more structural sings of older brain age (both $p < .0001$). Functional performance also showed a significant correlation with suPAR levels. Patients with higher suPAR levels showed poorer balance, reduced grip strength and more self-reported physical limitations (all $p>.0001$). These differences remained after correcting for sex, smoking, CRP, and current health conditions. The results of this study suggest a link between suPAR and a faster cognitive and physical ageing process. These insights are of great value for early detection of age-related pathologies and to maximize the effect of preventive measures (181).

SuPAR continues to be in the focus of clinical biomarker research. In June 2022, Holstein et al. published a study further investigating the elevated suPAR levels of elderly patients in the emergency department and its predictive performance for 30-day mortality. This could make suPAR a potential parameter to aid the decision whether a patient needs inpatient care or can be discharged. 1858 patients were included in the study with 1845 plasma samples available. Measurements were carried out using the same ELISA kit as in the present study. Elder patients (> 75 years) had significantly higher suPAR values (5.4 ng/mL vs. 3.7 ng/mL, $p < 0.001$) compared to the younger patients (< 75 years). SuPAR correctly predicted all-cause 30-day mortality in all investigated age groups. As a result, different cut-off values were determined to support the decision on whether an emergency department patient can safely be discharged: A safe option is a cut-off of 4 ng/mL for all patients. However, due to the generally higher suPAR levels in elderly patients, a cut-off value of 5 ng/mL should be considered for this subgroup (182). This was also used during the covid-19 pandemic when Stauning et al. were able to define a suPAR cut-off (< 2.0 ng/mL), which should help the attending physician in the emergency department to separate expected mild from severe courses of disease, and thus facilitate evidence-based triage (183).

Furthermore, suPAR is also used to evaluate new covid-19 therapies, including a large, double-blind, randomized controlled phase 3 trial of anakinra, a recombinant IL-1 receptor antagonist. The research group set a cut-off of plasma suPAR ≥6 ng ml-1 for patients at increased risk of progression to respiratory failure. The authors concluded that early treatment with anakinra guided by plasma suPAR levels significantly reduced the risk of a worse clinical outcome on day 28 in patients with moderate and severe covid-19 (184).

4.2.2 Implications and Outlook

The practical applicability of the suPAR ELISA kit used would enable rapid diagnostics in everyday clinical practice. SuPAR analysis is therefore the method that would be easiest to implement in clinical practice among the biomarkers investigated in this study. Further, the price per measurement is rather low with around 9€ per sample. However, the diagnostic performance of suPAR in synovial fluid is to be classified as acceptable at most. It is, at best, an addition to current diagnostic algorithms for the values of suPAR in synovial fluid. The plasma suPAR measurement in do not show any useful diagnostic value. While synovial suPAR shows essentially comparable performance to serum CRP in the diagnosis of PJI, synovial suPAR is superior to serum CRP in native joint infections. However, the different method of sample collection is decisive here. While serum CRP can be determined daily and thus represents a repeatedly measurable parameter, synovial suPAR can only be determined by joint puncture, which represents a decisive disadvantage in practical application. In conclusion, the suPAR analysis was, with a few exceptions, unable to confirm the expectations arising from the cited literature (154) (180). Therefore, it cannot be assumed that this method will be widely used in PJI or native joint infection diagnosis in the near future.

4.3 Multi Biomarker Model

4.3.1 Context of the Current Literature

The large category of serum PJI biomarkers was investigated in this dissertation using a separate cohort. The aim was to combine several biomarkers into a multi-biomarker model to improve diagnostic performance. The advantage of these serum markers is the ease with which the blood sample can be taken as well as the repeatability of the measurement. The serum biomarkers examined showed different performances. CRP (AUC 0.91) and fibrinogen (AUC 0.93) were the most accurate markers, followed by leukocytes (AUC 0.86), interleukin-6 (AUC 0.80), and procalcitonin (AUC 0.81), which are being used less frequently in clinical practice. Interferon alpha (AUC 0.36) did not provide significant added value in PJI diagnostics. Fibrinogen, a glycoprotein of the coagulation cascade, showed good diagnostic accuracy alongside the widely used PJI biomarker CRP, which is consistent with the results of other studies (103, 185, 186).

In addition to fibrinogen, d-dimer is increasingly being used as a biomarker in PJI diagnostic, as can be seen from the listing of d-dimer as a "minor criterion" for PJI in the ICM 2018 definition and various recent publications (3, 108, 187). It remains to be seen which of the two biomarkers will establish itself in the PJI diagnostic algorithm.

Several studies tried two or more biomarkers in a model with increased power. Wang et al. combined serum and synovial CRP, and reported two models, both of which had higher diagnostic accuracy compared to synovial CRP alone (sensitivity 84.62%, specificity 93.10% at a cut-off of 7.26 mg/l). The combined model I (Serum CRP > 10.2 mg/l OR SF CRP > 7.26 mg/l) showed a NPV of 96.67% and a sensitivity of 97.44%. Model II (Serum CRP > 10.2 mg/l AND Synovial CRP > 7.26 mg/l) showed a specificity of 1 and a PPV of 1 (188). Qin et al. combined D-dimer with the rate of CRP to ESR. They reported an increased sensitivity but decreased specificity compared to the performance of single serum biomarkers (187). This was in line with the reported results of Bottner et al., who found a high diagnostic accuracy for CRP (sensitivity 95%, specificity 96%) and IL-6 (sensitivity 95%, specificity 87%). Their combination led to a sensitivity of 100% and a specificity of 86% (87).

Hong et al. investigated 63 patients undergoing re-revision arthroplasty regarding the PJI diagnostic performance (32 PJI positive-cases) of CRP, ESR, fibrinogen and neutrophil–lymphocyte ratio (NLR). They report that fibrinogen had the highest AUC (0.885), followed by CRP (0.821), ESR (0.794), and NLR (0.702). Further they combined the tested biomarkers, showing a higher AUC (0.897) for CRP and Fibrinogen. The sensitivity (75%), specificity (93.5%) and the PPV (92.3%) as well as the NPV (78.4%) were also acceptable. The combination of all four markers led to a slightly improved AUC (0.903). The sensitivity (78.1%), specificity (90.3%), PPV (89.3%) and NPV (80%) were also reported. The authors concluded that combining fibrinogen with one (CRP) or multiple biomarkers does not significantly improve its already high diagnostic performance (189). These results confirm our published findings and the conclusion that fibrinogen and CRP alone are good PJI biomarkers and do not benefit significantly from combination with other biomarkers.

A recently published study by Hong et al. investigated a variation of different serum PJI biomarkers regarding their diagnostic performance as single biomarkers as well as their combination in 543 patients (245 PJI positive cases) (190). They further calculated classification trees with discriminatory cut-offs for an ideal biomarker combination (internally

cross-validated, 100 times repeat cycle). CRP was the best performing single biomarker with an AUC of 0.882), followed by IL-6 (0.845) and fibrinogen (0.834). The classification tree results have shown that the diagnostic performance basically decreased with each depth level added:

1. CRP (cut-off 5.91 mg/L, Sensitivity 86.1%, Specificity 79.5%);
2. CRP and ESR (cut-off 5.91 mg/L (CRP) and 32mm/h (ESR)), Sensitivity 71.8%, Specificity 89.9%);
3. CRP and ESR and platelet count/lymphocyte ratio (PLR; cut-off 5.91 mg/L (CRP), 32mm/h (ESR), 131.8 (PLR), Sensitivity 58%, Specificity 66.4%);

Both the study design and the results were similar to our work, showing no added value in a multi-biomarker model compared to the best performing single biomarker CRP (190).

4.3.2 Implications and Outlook

The application of the multi-biomarker model in clinical practice is limited by the complex calculations required. However, this could be simplified with an app and further prepared for practical use. Moreover, not all promising serum biomarkers were tested in this study or calculated into the multi-biomarker model. The increasing number of synovial biomarkers was also not investigated in this multi-biomarker analysis - this would be an important research question in future studies. Although the present work does not show any significant improvement in the diagnostic performance of the multi biomarker model compared to single biomarkers, some other published studies suggest an added value of such an approach (see citations above). Interestingly, no multi-biomarker model has yet been established as a standard in any of the major definitions (EBJIS, ICM, IDSA). This in turn confirms our findings that with the currently available biomarkers, there is no significant added value in a complex calculated multi-biomarker model. It is therefore very unlikely that a standard biomarker model will find its way into the clinical routine of PJI diagnostics in the near future, and different in-house algorithms will remain the rule in reference centers.

4.4 Strengths and Limitations

One of the main strengths of this study lies in the size of the cohort. With a few exceptions, the cohort sizes published so far have primarily been pilot studies and investigated whether the methods used have a fundamental diagnostic value. A study with a comparable number of participants on the topic under investigation has not yet been published. Especially in studies on the validity of new diagnostic methods, the superior group size leads to more robust and reliable data and represents a clear advantage. Another strength of the present study is the prospective design. Moreover, we were able to combine the resources available at our university center (renown experts in nuclear magnetic resonance analysis, infectious diseases, and musculoskeletal infections) with ideal interfaces between clinical and basic research.

The limitations of the present study include the implementation of blood plasma samples via a study amendment following the start of patient recruitment. This led to fewer samples in the suPAR plasma cohort compared to the synovial fluid cohort as listed in the corresponding results section. The implementation was deemed reasonable despite this, as a high diagnostic performance of plasma suPAR would have been the basis for a valuable diagnostic tool in SA and PJI detection. Further, due to the covid-19 pandemic and its impact on the daily working routine at the university clinic and the research institutions, patient inclusion had to be paused for four months in the year 2020, since adequate sample processing was not possible during this time. Inevitably, this led to a reduced study cohort size. Immediate sample storage at -80°C was not possible outside of the regular working time. In these cases, samples were stored at -15°C and transported to final storage at -80°C after 65 hours at the latest. According to the current state of knowledge, this should not have any negative effects on the measurements.

Furthermore, due to the type of patient selection and inclusion criteria (undergone a diagnostic joint puncture due to suspected arthritis of any cause (native or after arthroplasty treatment)), there is a risk of selection bias. In particular, the elevated SuPAR levels in older patients have to be mentioned here (182). However, in our opinion, this risk of bias is within an acceptable range and realistically reflects the patient population requiring SA or PJI assessment.

4.5 Conclusion

The investigation of new biomarkers in the diagnosis of joint infections within the framework of the present study yielded both positive and negative results.

On the one hand, we were able to show that NMR metabolome analysis is a promising method for diagnosing both septic arthritis and PJI. This analysis method consistently showed the best diagnostic performance of the new (NMR, suPAR, multi-biomarker model) as well as the established biomarkers (CRP, WBC) investigated. While NMR has already taken major steps towards efficient clinical application in other countries, this is still necessary in Austria before it can be widely used in clinical diagnostics.

On the other hand, the already commercially available synovial suPAR ELISA test did not provide any significant diagnostic value that would justify the more invasive sample collection of a joint puncture compared to venous blood sampling. In fact, slightly better AUC values were achieved with serum CRP. The poorest results in both joint categories were achieved with serum SPAR and serum WBC. In view of the current study results, no diagnostic added value is to be expected from these two methods.

The multi-biomarker model showed a good diagnostic performance. However, it must be considered that this model does not represent a significant improvement compared to the results of the individual biomarkers (CRP, fibrinogen) on the used dataset.

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Appendix

The following figure shows statistically significant differences between samples from septic and aseptic joints (with and without implants combined) as calculated by Student's t-test. The y-axes represent concentrations in arbitrary units (a.u.) resulting from integration of peak intensities at the respective chemical shift intervals. The boxes extend from the 25th to 75th percentile, the bars represent 2.5-to-97.5 percentiles. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001