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The Antimicrobial Properties of Exogenous Copper in Human Synovial Fluid Against Staphylococcus aureus: An In-Vitro Study

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Abstract

We sought to analyse the antimicrobial properties of exogenous copper in human synovial fluid against *Staphylococcus aureus*. We performed several in-vitro growth and viability assays to determine the capability of multiple *S. aureus* strains to survive in synovial fluid under different growth conditions. *S. aureus* UAMS-1 significantly died at 24 hours (p=0.017), and *S. aureus* USA300 WT survived at 24 hours. We confirmed a high sensitivity to killing with the addition of exogenous copper on both strains at 4 (p=0.011), 12 (p=0.011), and 24 hours (p=0.011). Both WT and CopAZB-deficient USA300 strains significantly died in synovial fluid, evidencing a minimum bactericidal concentration of copper of 50 µM against USA300 WT (p=0.011). Synovial fluid has antimicrobial properties against *S. aureus*, and the addition of 10µM of copper was highly bactericidal for both strains. Furthermore, we identified the CopAZB proteins as potential targets and the use of low exogenous copper concentrations as possible treatment alternatives against *S. aureus*.

Keywords: Antimicrobial properties; synovial fluid; copper; *Staphylococcus aureus*; periprosthetic joint infection; in-Vitro study

Summary for Lay Audience

Staphylococcus aureus is the most frequently isolated organism in periprosthetic joint infections. The mechanism by which the synovial fluid kills bacteria has not yet been elucidated. We sought to analyse the antimicrobial properties of exogenous copper in human synovial fluid against *S. aureus*. Synovial fluid samples were collected from patients undergoing total joint arthroplasty. Different *S. aureus* strains were used. We first performed in-vitro growth assays with the different *S. aureus* strains in human synovial fluid. Viability assays were then performed to determine the capability to survive in synovial fluid with the addition of exogenous cooper. After confirming the antimicrobial effect of copper against *S. aureus*, we compared the differences in sensitivity between a highly resistant and mutant deficient strain. The sensitive strain significantly died after 24 hours, whereas the resistant strain survived after 24 hours. Both strains significantly died after 4, 12, and 24 hours with the addition of exogenous copper, confirming its role as an antimicrobial agent against *S. aureus*. Finally, the protein deficient strain was susceptible to lower copper concentrations. In light of these findings, we confirmed the antimicrobial properties of synovial fluid and the bactericidal effect of exogenous copper against *S. aureus*. Although future and well-designed studies might be needed, we propose using exogenous copper and target bacterial proteins as possible treatment alternatives against *S. aureus* infections.

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Table of Contents

Abstract…….....i

Summary of lay audience……..…...ii

Acknowledgements……..…..iii

Table of contents…….....iv

List of tables……..viii

List of figures ……....ix

List of appendices…….....x

List of abbreviations…….....xi

Chapter 1 – Background…….....1

1.1 Periprosthetic joint infection in total hip and knee arthroplasty……..1

1.1.1 Epidemiology……..…….....2

1.1.2 Incidence..……2

1.1.3 Economic costs…..……...3

1.1.4 Risk factors……..…….....3

1.2 Clinical presentation…….....7

1.3 Classification schemes……...8

1.4 Pathogenesis……...9

1.4.1 Initiation…….....10

1.4.2 Biofilm importance……....11

1.4.3 Propagation……....12

1.5 The role of the immune system……..…...13

1.6 Synovial fluid, metals and their role in periprosthetic joint infection …….....14

1.7 Microbiology……....16

1.7.1 Frequently isolated microorganisms...…….....16

1.7.2 *Staphylococcus Aureus* as a pathogen…….....17

1.7.2.1 Epidemiology……..…….....18

1.7.2.2 Methicillin-resistant *S. aureus* periprosthetic joint infection…….....20

1.7.2.3 Mechanisms of antimicrobial resistance……..…21

1.8 Diagnosis…….....27

1.8.1 Diagnostic criteria……..30

1.9 Treatment…….....31

1.9.1 Treatment alternatives……....31

1.9.1.1 Debridement, antibiotics, and implant retention……...…..33

1.9.1.2 One-stage revision surgery…….....36

1.9.1.3 Two-stage revision surgery…….....37

1.10 Prevention……..39

1.10.1 Skin microbiota treatment…….....40

1.10.2 Perioperative antibiotic prophylaxis…….....41

1.10.3 Laminar airflow rooms and surgical body suits……..…42

1.10.4 Antibiotic-loaded PMMA at surgical implantation……...……43

1.10.5 Antibiotic prophylaxis before dental-urologic-gastrointestinal procedures…….....44

2 Chapter 2 - Project rationale…….....46

2.1 Research objectives…….....46

2.1.1 General objectives……..……..…..46

2.1.2 Specific objectives……..…….....47

3 Chapter 3 - Material and methods……48

3.1 Ethics statements……..48

3.2 Synovial fluid collection and preparation…….....48

3.3 *S. aureus* strains and routine culture…….……49

3.4 *S. aureus* survival in synovial fluid in-vitro…….....50

3.5 The effect of copper on *S. aureus* viability in synovial fluid……..……..….50

3.6 Determination of minimum bactericidal concentration of copper and differences in sensitivity to killing between WT and CopAZB-deficient USA300 strains in synovial fluid…….....52

3.7 Analysis of the acidic environment……..…….....53

3.8 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and protein identification in synovial fluid…….…….....54

3.9 Statistical analysis……....54

4 Chapter 4 – Results……...56

4.1 *S. aureus* survival in synovial fluid in-vitro……..56

4.2 The effect of copper on *S. aureus* viability in synovial fluid…….58

4.3 Determination of minimum bactericidal concentration of copper and differences in sensitivity to killing between WT and CopAZB-deficient USA300 strains in synovial fluid……..61

4.4 Analysis of the acidic environment……...65

4.5 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and protein

identification in synovial fluid……...…...67

5 Chapter 5 – Discussion and future directions…….....70

6 References……....76

7 Appendices…….123

7.1 Appendix 1: Synovial fluid study: Ethics approval…….123

7.2 Appendix 2: Synovial fluid study: Letter of Information and Consent Form..……125

8 Curriculum Vitae……....128

List of Tables

Table 1. *S. aureus* survival in saline solution in-vitro…….56

Table 2. *S. aureus* survival in synovial fluid in-vitro……..57

Table 3. The effect of copper on *S. aureus* viability in saline solution……....59

Table 4. The effect of copper on *S. aureus* viability in synovial fluid…….……61

Table 5. Minimum bactericidal concentration of copper on *S. aureus* USA300 WT……...….63

Table 6. Minimum bactericidal concentration of copper on *S. aureus* USA300 CopAZB……..64

Table 7. The effect of low-pH environment on *S. aureus* viability in saline solution……..66

Table 8. The effect of low-pH environment on *S. aureus* viability in synovial fluid……...……67

List of Figures

Figure 1. S. aureus biofilm formation in five stages……25

Figure 2. Mechanisms of copper toxicity……....51

Figure 3. *S. aureus* survival in saline solution in-vitro……57

Figure 4. *S. aureus* survival in synovial fluid in-vitro……58

Figure 5. The effect of copper on *S. aureus* viability in saline……60

Figure 6. The effect of copper on *S. aureus* viability in synovial fluid……60

Figure 7. Minimum bactericidal concentration of copper on *S. aureus* USA300 WT…….62

Figure 8. Minimum bactericidal concentration of copper on *S. aureus* USA300 CopAZB…….65

Figure 9. The effect of low-pH environment on *S. aureus* viability in saline solution…….66

Figure 10. The effect of low-pH environment on *S. aureus* viability in synovial fluid…..……..67

Figure 11. SDS-PAGE and protein identification in synovial fluid run for 90 min……...…..68

Figure 12. SDS-PAGE and protein identification in synovial fluid run for > 90 min…….69

List of Appendices

Appendix 1: Synovial fluid study: Ethics approval……..……..123

Appendix 2: Synovial fluid study: Consent to participate and form……..125

List of Abbreviations

Agr: Accessory gene regulator index

BMI: Body mass

CA-MRSA: Community-associated MRSA

CFUs: Colony forming units

CoNS: *Coagulase-negative staphylococci*

DAIR: Debridement, antibiotics, and implant retention

DMARDs: Disease-modifying antirheumatic drugs

DNA: Deoxyribonucleic acid

HA-MRSA: Healthcare-associated MRSA

IDSA: Infectious Diseases Society of America

MBC: Minimum bactericidal concentration

MRSA: Methicillin-resistant *Staphylococcus aureus*

MSSA: Methicillin-susceptible *Staphylococcus aureus*

MSIS: Musculoskeletal Infection Society

OSRS: One-stage revision surgery

PJI: Periprosthetic joint infection

PMMA: Polymethyl methacrylate

PCR: Polymerase chain reaction

PNAG: Poly beta-1,6-N-acetyl-D-glucosamine

RA: Rheumatoid arthritis

Rpm: Revolutions per minute

SD: Standard deviations

S. aureus: Staphylococcus aureus

SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SSI: Surgical site infection

SF: Synovial fluid

THA: Total hip arthroplasty

TJA: Total joint arthroplasty

TKA: Total knee arthroplasty

TSA: Tryptic soy agar

TSB: Tryptic soy broth

TSRS: Two-stage revision surgery

US: United States of America

WT: Wild type

Chapter 1

Background

1.1 Periprosthetic joint infection in total hip and knee arthroplasty

Osteoarthritis is the most frequent joint disease worldwide, with knee involvement representing over 80% of the total disease prevalence (1,2). Osteoarthritis is a degenerative disorder that affects joint cartilage and subchondral bone. Clinical presentation includes joint pain, inflammation, and decreased range of motion but can considerably progress, impacting the patient's quality of life.

Total joint arthroplasty (TJA) is a well-known and effective procedure that relieves pain, restores range of motion, and improves life quality in patients with end-stage osteoarthritis (3). While most TJAs restore pain-free function, a small number of cases will need revision surgery at some point during follow-up (4–7). The most typical reasons for revision surgery are infection, wear, loosening, instability, persistent pain, and fracture.

The total volume of primary TJA performed each year is increasing worldwide, and there is an expected annual volume of 4 million replacements in the United States (US) by 2030 (4,5). In this sense, the number of revision procedures is expected to increase with periprosthetic joint infections (PJI) as one of the most common and most challenging causes of revision surgery (4–6,8). Several studies reported an estimated infection rate of 1-2% after total hip arthroplasty (THA) and total knee arthroplasty (TKA) (7–10).

PJI affects the prosthesis and surrounding soft tissues, and there have not been any significant improvements in the diagnosis, treatments, or outcomes of PJIs. If anything, with increasing resistant organisms, our ability to eradicate infection is decreasing. Despite all efforts

to decrease the incidence of PJI (11,12), the infection burden is still increasing globally (7); hence PJI remains one of the most frequent reasons for TJA failure.

Indeed, PJI has been associated with increased morbidity, mortality and hospitalization, and significant physiological, psychological, and economic impact on the healthcare system (6,13,14). Even more, Zmistowski et al. (15) demonstrated a 5-year mortality rate of 87%, which is higher mortality than what is seen in frequent oncologic diseases, such as prostate, breast, and melanoma.

1.1.1 Epidemiology

1.1.2 Incidence

As the total number of TJA continue to increase, the incidence of PJI is expected to grow as well. Several studies have identified an increased incidence of PJI in hip and knee replacements. Kurtz et al. (14) reported an increase in the annual incidence from 1.99 to 2.18% and from 2.05 to 2.18% for hip and knee replacements, respectively. The Nordic Arthroplasty Register Association also demonstrated an increase in the cumulative 5-year revision rate for PJI after THA, from 0.46% to 0.71% during 1995-1999 and 2005-2009, respectively (16).

On the contrary, Tsaras et al. (17) performed a population-based study from 1969-2007 analysing 75 PJIs in 7,367 replacements and did not find evidence of an increase during their study. The cumulative incidence of PJI were 0.5, 0.8, and 1.4% at 1, 5, and 10 years, respectively. The most significant risk period was the first two years after the arthroplasty, accounting for 60 to 70% of infections diagnosed (10,17,18).

Although the increased incidence per joint per year is controversial, the overall number of PJIs will likely increase secondary to the increasing number of elective arthroplasties being performed and the total cumulative arthroplasties still functioning well in society.

1.1.3 Economic Costs

In addition to the patient morbidity and mortality, there is a significant economic impact associated with PJIs. The overall cost to treat PJI in the US was $566 million in 2009, which is expected to increase up to $1.62 billion in 2020 (14). Of course, individual costs in each patient are influenced by the elected treatment.

For example, a single-stage revision surgery due to PJI has higher costs than a revision for aseptic loosening due to an extended time of surgery, increased blood loss, and a higher complication rate (19).

Indeed, debridement, antibiotics, and implant retention (DAIR) triple the cost to treat a single PJI case compared to a primary TJA (20). Even more, the mean costs of one- and two-stage revision surgeries are approximately 3.4 and 6 times higher than the cost of a primary arthroplasty, respectively (21).

Additionally, Canadian data also confirms the tremendous economic impact of PJI in TJA. The authors reported a 5-fold increase in hospital expenditure in the management of PJI compared with primary THA and TKA, including a significant increase in mean length of hospital stay, mean number of clinic visits, number of readmissions, and average overall cost (22,23).

1.1.4 Risk Factors

The principal risk factors associated with PJI are obesity, diabetes mellitus, chronic inflammatory disorders such as rheumatoid arthritis (RA), smoking, length of primary surgery, prior interventions on the joint, and immunosuppression (18,24).

Obesity (body mass index (BMI>35) has been related to an increased risk of infection in several studies (18,25–33). The extended surgical time has been hypothesized as one of the causes of increased infection risk (34). Nonetheless, other studies have confirmed obesity as an independent risk factor after adjustment for confounders (26,28). Berbari et al. (35) could not demonstrate that correlation, and on the contrary, he also reported a higher risk of PJI with BMI < 25 (24).

Diabetes mellitus has also been related to an increased risk of PJI (26,36–38), being present in almost 30-40% of cases (125). Mraovic et al. (39) reported that before elective THA or TKA, perioperative hyperglycaemia was related to a higher risk of PJI, even in non-diabetic patients. This might be a consequence of an increased biofilm formation in the context of hyperglycaemia (40), inadequate leukocyte response, and microvascular pathology in diabetic patients, which may affect wound healing and lead to surgical site infections (SSI). Nevertheless, not all authors have found a strong correlation between diabetes and PJI (25,35), and some others have classified diabetes with other immunosuppressive diseases (29).

RA, immunosuppressive therapy, and malignancy have been related to a higher risk of PJI in several studies (18,35,41–44). Frequently, it is challenging to isolate the essential role of each comorbidity in highly comorbid patients. Bongartz et al. (43) reported an infection rate of 2.3% during the first postoperative year for patients with RA. Berbari et al. (29) showed a 2.2-fold increase in the risk of PJI when multiple comorbidities such as RA, systemic immunosuppression, diabetes mellitus, chronic kidney disease, and malignancy were considered.

Biologic disease-modifying antirheumatic drugs (DMARDs) have also been associated with an increased risk of SSI after TJA; however, the small number of included patients did not allow the authors to reach categoric conclusions (45,46). Current RA guidelines strongly recommend withholding tumour necrosis factor-alpha inhibitors (DMARDs) close to the date of the surgical procedure (47,48). In clinical practice, the management of DMARDs is diverse and must be individualized for each patient. Akkara Veetil et al. (49) suggest withholding one cycle of biologic DMARDs before TJA and resume treatment one or two weeks after the procedure. Although scarce evidence recommends continuing nonbiologic DMARDs during TJA, methotrexate can be withheld if wound healing concerns are expected (50,51). When revision surgery due to PJI is indicated, weekly methotrexate and biologic DMARDs should be withheld for one or two cycles before the procedure. Once the surgery is performed, nonbiologic DMARDs may be reinitiated when the wound is completely healed. The reinitiation of biologic DMARDs depends on the surgical treatment employed. If a two-stage revision surgery (TSRS) is indicated, biologic DMARDs might be restarted once the incision is healed after the reimplantation. When one-stage revision surgery (OSRS) or DAIR is performed, biologic DMARDs reinitiation can be indicated once suppressive antibiotic treatment is started. It is essential to highlight that the perioperative management of DMARDs should be individualized for each patient and monitored by the treating rheumatologist.

The PJI rate after a revision surgery is higher than that after elective TJA (29,35,52,53). Some related risk factors are extended surgical time, a possible PJI undiagnosed during revision surgery, and soft tissue deficiency.

Other variables have been reported to increase the risk of infection after THA or TKA, such as male gender (8,10,16,27,54), smoking (12), previous history of bacteraemia during the

previous year (55), history of septic arthritis (35), and intra-articular corticosteroid injections three months before TJA (56,57). Additionally, a high American Society of Anesthesiologists preoperative score has also been related to a higher risk of PJI (18,25,26,29).

Surgical factors may also influence the risk of infection. Although several studies reported no difference between cemented and cementless TJA (35,58), cemented fixation has the theoretical benefit of antibiotic addition and the consequent local release to prevent an infection. Postoperative wound-related complications have been related to a high risk of infection, such as dehiscence, wound drainage, SSI, and hematoma (18,29,35,41,55). An extended surgical time is related to a higher risk of infection (8,10,35,54), with a 9% increase risk every 15-min increment of surgery (26) due to more prolonged exposure to bacterial contamination.

Pulido et al. (18) also identified postoperative myocardial infarction and atrial fibrillation as additional risk factors for an increased risk of PJI. The authors stated that the necessary anticoagulation therapy would predispose to the development of a subclinical hematoma.

Blood transfusion has been reported as an additional risk factor in several studies. It is essential to differentiate the allogenic transfusion from the autologous, as the first one has been related to a higher risk than the autologous procedure (18,35,59,60). Several hypotheses have been developed, and most of them highlight the role of the immune response to transfusion.

Perioperative urinary or respiratory tract infections are related to a higher risk of PJI (12,18,29), probably because of transient bacteraemia from the distant infection sites during the immediate postoperative period (61). In this sense, patients must be thoroughly assessed for possible signs and symptoms suggesting urinary or respiratory infections at the pre-admission visits and treated consequently to decrease the risk of infections.

1.2 Clinical presentation

Clinical findings can be challenging unless the responsible microbe is aggressive enough to activate the immune system. In this sense, the presentation may vary based on the microbe's virulence, the type of infection, the host immune system, the surrounding soft tissue, and the affected joint.

Acute compromise usually manifests as deep wound infections. The presentation might include different signs and symptoms, including pain, inflammation, erythema, increased temperature, fever, wound drainage, or a sinus tract communicating with the joint. While several studies recognized pain as the most commonly identified symptom (17,62–64), Peel et al. (65) reported wound drainage as the most common manifestation, with pain being diagnosed in only 42% of patients. Indeed, wound-related complications, such as abscess, sinus tract (17,65,66), or dehiscence, were more frequent in perioperatively PJI than in hematogenous PJI (63,66). Clinical confirmation of wound infection is the strongest risk factor for acute infection (Odds Ratio: 52, 95% Confidence Interval: 21 to 130) (67).

Some chronic PJIs can also be oligo or asymptomatic (6,17). Typically, patients do not have overt signs of infection but instead complain about chronic pain, poor function, and history of discomfort. Most patients with PJI or aseptic failure will probably complain about pain; thus, pain does not seem helpful for the differential diagnosis between them. Fever has been reported to be present in only 4.5% of all cases (68), and a systemic compromise is considerably more frequent in hematogenous infections. The only exception would be the evidence of a sinus tract communicating with the joint, which is considered a major criterion in most international consensus (3,69,70).

The varied presentation can make the diagnosis of PJI challenging for the clinician. It is crucial to assimilate all the clinical findings and risk factors and use them to guide investigations to confirm the diagnosis and establish a treatment plan.

1.3 Classification schemes

Different classification schemes have been published, but the most accepted is based on the time to presentation and dissemination mechanisms (71).

An acute or early PJI results from microorganism contamination during the surgical procedure or the initial postoperative period. These relatively virulent microorganisms are usually part of the normal cutaneous flora and compromise wound healing with prolonged post-operative wound drainage. These infections are usually symptomatic and diagnosed within 90 days of implantation.

Late chronic PJIs can occur between 3 to 12-24 months after the surgery. They may also result from intraoperative contamination but are caused by less virulent microorganisms. The difference between acute and late chronic can be affected by the organism's virulence and the needed inoculum to develop the infection, resulting in a more indolent presentation. The time between inoculation and initial symptoms is the time needed for microorganisms to grow and proliferate.

Late hematogenous PJIs result from a spread from a distant focus and may appear any time after TJA with an acute presentation in a previously healthy joint. Additionally, as distinguished from chronic types where general symptoms are usually absent, hematogenous infections cause malaise, chills, fever, generally of the septic type (13).

Most early and hematogenous PJIs are caused by aggressive microorganisms, especially *Staphylococcus aureus* (*S. aureus*), beta-haemolytic *streptococci*, Gram-negative bacteria, and polymicrobial infections. On the contrary, chronic PJIs are usually caused by indolent microbes, including *Coagulase-negative staphylococci* (CoNS), *Enterococcus* and *Cutibacterium species*. Nevertheless, there is a universal consensus to recognize *S. aureus* as the most frequent causative microorganism, responsible for 18 to 73% of all PJIs (72).

Tsukayama et al. (73,74) developed another type of classification in the 1990s. They divided four categories according to the time of the surgery and the mechanism of infection. The first category includes cases with positive intraoperative cultures in patients with presumed aseptic failures. The second category includes early postoperative PJI occurring during the first postoperative month. The third category includes late chronic infections one month after the replacement with an indolent presentation. Finally, the fourth category includes acute hematogenous PJIs.

To conclude, McPherson et al. (75,76) described a classification analysing the type of PJI and the host immune system. This classification includes early, hematogenous, and late chronic infections, categorized as type I, II, or III, respectively. The host immune system can be categorized as A (not compromised), B (immunocompromised), or C (significant immunocompromised), based on the evidence of neutropenia, low CD4 T-cell count, or patients older than 80 years. The joint can be classified as 1 (not compromised), 2 (compromised), or 3 (significantly compromised), based on the evidence of chronic active infection, including soft tissue deficiency, sinus tract, dehiscence, or abscess.

1.4 Pathogenesis

1.4.1 Initiation

Most PJIs diagnosed during the first postoperative year result from direct contact or aerosolized contamination of the implants or surrounding soft tissues during the surgical procedure. After contamination and consequent colonization, a low inoculum of bacteria is necessary to produce the infection. Southwood et al. (61) demonstrated that less than 10^2 CFU of *S. aureus* are needed to establish an infection after inoculation during a hip hemiarthroplasty in an animal model study, compared with 10^4 CFU if no device is implanted.

Another mechanism of initiation is contiguous dissemination from a nearby focus. During the early postoperative period, superficial SSIs can progress and affect the prosthesis and deep layers. On the other hand, a late PJI can also occur if old wounds are disrupted after a traumatic episode or a new adjacent surgery.

Lastly, hematogenous dissemination from a distant site is infrequent but still possible during the entire follow-up of the patient. Uckay et al. (77) reported only seven hematogenous PJIs in 551 distant infections after 6,101 TJAs. PJIs were found in 5/81 (6%) patients with confirmed bacteraemia, with *S. aureus* as the most frequently identified microbes.

Multiple authors reported almost 30 to 40% risk of hematogenous PJI after confirmed *S. aureus* bacteraemia (78,79), compared to a 3 to 10% risk in native joints (80–82). Although *S. aureus* was the most commonly isolated microorganism, CoNS, Streptococcus and Enterococcus species, and aerobic Gram-negative bacilli have also been reported as causative pathogens of bacteraemia and later hematogenous PJI (18,83,84). Usually, bacteraemia and PJI manifestations occur at the same time. Nevertheless, some microbes may need more time to establish the infection between bacteraemia and PJI presentation (85).

1.4.2 The Importance of Biofilm

Biofilms are any syntrophic consortium of microorganisms embedded in an extracellular matrix in which cells stick to each other and grow on different surfaces. Usually, microorganisms that form biofilms include bacteria, fungi, and protists.

They can be mono or polymicrobial with different characteristics and growth behaviour. In this sense, mixed bacteria biofilms might be differentially affected by antibiotics or the immune response, making them challenging to diagnose.

Biofilm formation has different "stages," including attachment, initial growth, maturation, and detachment. Mature biofilms are formed by multicellular structures in which their components communicate with each other and have different functions (86).

Biofilms can grow on different surfaces, including medical devices and implants. This characteristic allows several microorganisms to cause medical device infections, including PJIs. Indeed, microorganisms that are part of the normal flora hardly cause infections but can become pathogens in the presence of medical devices due to biofilm formation (86).

The extracellular matrix is made of varying concentrations of polysaccharides and proteins. The biofilm is protective against antibiotics and the immune response (87), making treatment challenging and may require surgical procedures, including implant removal. The acquired antibiotic resistance is associated with the "persisters" cells and the protection provided by the biofilm structure (88,89).

Biofilms have been associated with PJI, and there is strong evidence of their importance in the pathogenesis of infections. Stoodley et al. (90) demonstrated viable *S. aureus* biofilm in an infected prosthesis after removal.

On the other hand, different theories have been described regarding gene expression for biofilm formation. Some authors believe that key gene loci involved may differ between pathogens and contaminants. Galdbart et al. (91) demonstrated that the ica genes in *Staphylococcus epidermidis* are implicated in biofilm formation during PJI. Despite these findings, several studies have demonstrated that the ica genes are not needed to establish an infection (92,93). Definitive evidence regarding gene expression and its association with biofilm formation is needed to elucidate this controversy.

Besides, it is necessary to mention the importance of biofilms in the diagnosis of PJI. Usually, microorganisms are settled around the implants and influence the sensitivity of culture samples. A diagnostic alternative to solve this challenge is to obtain samples applying sonication techniques (86).

1.4.3 Propagation

At first, the infection is typically only present in the intraarticular space, and histology may reveal large granulomas with neutrophils and abscesses. The next step involves disseminating to the proximal bone, with the upper 1/3 of the metaphysis being compromised during the third week. If the infection progress, it finally compromises the remainder of the metaphysis and affects the immediate area of the diaphysis (86).

On the other hand, hematogenous infections are believed to have some differences regarding the initiation process. Cremieux et al. (94) reported that hematogenous long bone osteomyelitis might begin at the metaphysis, and consequently, it is possible that it progresses to the joint space and affect the prosthesis. More studies are needed to prove whether this difference would alter the diagnosis and treatment of infections.

1.5 The role of the immune system

Physical barriers are the most basic form of nonspecific defence. They aim to prevent microbes from reaching tissues and provide mechanical defences that physically remove microbes and debris from areas of the body susceptible to infection.

At the cellular level, barriers are formed by tightly joined cells to prevent invasions from deeper tissues. Cell junctions are formed by cell membrane proteins related to the extracellular matrix or complementary proteins from other cells. Different cell junctions are available in different tissues, including tight junctions, desmosomes, and gap junctions. Pathogens may try to break down these barriers chemically, using enzymatic proteases, causing structural damage, and creating an entry point (95–97)

Additionally, the normal microbiota competes with pathogens for nutrients. Microorganisms that are part of the normal flora of the cutaneous tissue infrequently cause infection unless the host is immunocompromised. The human cutaneous tissue is rich in fatty acids and sodium, helping prevent bacterial growth (95,96).

When pathogens break down these physical and chemical defences, they may invade human tissues. If they effectively defeat initial barriers, they may face the primary humoral immune response, including the complement system (98). The complement system is formed by small proteins synthesized by the liver, circulating in the bloodstream as inactive precursors. When stimulated, proteases initiate an amplifying cascade of further events. This complement activation stimulates phagocytes, inflammation, and final activation of the cell-killing membrane attack complex. The membrane attack complex is a protein complex formed on the pathogen's cell

membrane, and its assembly leads to cell membrane disruption of target cells, leading to cell lysis and death (99).

1.6 Synovial fluid, metals and their role in periprosthetic joint infection

Osteoarthritis is a frequent pathology that affects joint cartilage and synovial fluid (SF), resulting in increased wear, inflammation, pain, and decreased mobility (100,101). SF is the neutral pH, viscous fluid that lubricates and cushions synovial joints during movement (102). Its proteins and hyaluronic acid concentrations, thermostability, and osmolality are essential in osteoarthritis and are therefore compromised (101).

The host's immune system includes an innate response against pathogens in different body fluids, including SF. Current literature suggests that SF has defence factors to prevent infections apart from the cell-mediated immune response and might participate in the joint's natural defence. Gruber et al. (103) showed bactericidal activity of synovial fluid against different *Staphylococcus* species, including *S. aureus*.

Iron is a necessary micronutrient for microorganisms due to its wide range of redox potentials. The iron participates in catalytic enzymatic processes in DNA synthesis and electron transport in any of its presentations. Although iron is abundant, the amount of free iron in living organisms is deficient due to its trend to form insoluble oxyhydroxides under aerobic and neutral pH conditions. Moreover, iron availability is further reduced by sequestration into proteins such as transferrin and lactoferrin (104,105).

It has been reported that iron is scarce in SF from patients with osteoarthritis due to transferrin activity as an iron-binding protein. Ahmadzadeh et al. (106) demonstrated higher transferrin concentration in SF from osteoarthritic patients than patients with RA, which showed

higher free iron levels. In this sense, transferrin would reduce bacterial growth in SF, as they need iron to survive (107). Additionally, Watson et al. (108) demonstrated that SF from patients with osteoarthritis has minimum free iron concentration and bactericidal effect against *S. aureus*. As a result, the iron restriction would contribute to SF's bactericidal activity, and the complement system would kill bacteria.

In addition to iron, microbes require zinc, manganese, and copper as essential micronutrients to survive. Copper is the third most abundant essential transition metal in humans, and it is required by several cellular enzymes that are involved in redox reactions (109). As a redox-active metal, copper is an ideal cofactor for enzymes involved in different physiological processes such as photosynthesis, respiration and detoxification and iron metabolism (110). Nevertheless, an excess of copper can be harmful to cells, potentially producing toxic reactive oxygen species (111,112). Consequently, transport and cellular copper homeostasis are strictly regulated.

The entire human body contains between 75–100 mg of copper, and the highest amounts are in the liver, kidney, and brain (113). Usually, more than 90% of the copper in the blood is bounded to ceruloplasmin, which is responsible for transporting it to different tissues. It is estimated that the concentration of protein-free copper is less than 5%, leaving the remaining amount bound to transcuperin, albumin, and amino acids. The intracellular cooper is usually bound to chaperones because free cooper is potentially harmful to cells (114).

The excess copper, especially the free proportion, may be toxic due to its redox potential. In this sense, the measurement of unbound copper in circulation would theoretically be helpful to detect potentially toxic copper overload. The normal range for total copper in blood is 85-180 µg/dL (13.3-28.3 µmol/l = 13.3-28.3 µM), and serum-free copper reference values have been

routinely identified between 0-10 µg/dL (0-1.6 µmol/l = 0-1.6 µM). However, the mechanism by which SF kills bacteria is yet to be defined. A better understanding of its antimicrobial properties and the role of different metals, especially copper due to its bactericidal activity, might help develop new treatment alternatives.

1.7 Microbiology

1.7.1 Frequently isolated microorganisms

Identification of the infecting microorganism is a crucial aspect of successful treatment. Several studies have analysed the frequency of the isolated microbes causing PJI (29,35,65,115–119).

The results show that Gram-positive cocci are the causative microorganism in most infections. *S. aureus* and CoNS are the most common, accounting for 50 to 60% of all PJIs, whereas streptococci and enterococci represent approximately 10% of infections. Aerobic Gram-negative bacilli are responsible for less than 10% of hip and knee PJIs, and culture-negative infections may vary between 5 to 34% (65,119,120). These results might be influenced by several variables, such as the administration of preoperative antibiotics, the interpretation of positive culture results, the possibility of a positive result as contamination, and the amount and type of samples obtained.

Isolation of the microorganism at the time of presentation with a PJI is relevant because causative microbes may vary and require tailored treatments. In acute settings (<1 to 3 months), *S. aureus* and aerobic Gram-negative bacilli account for 60% of infections (73,121–126). The high virulence of these microbes contributes to the clinical presentation during the early postoperative period. Nevertheless, CoNS and polymicrobial infections remain essential due to the low virulence

inoculation with multiple microorganisms during the surgical procedure or local dissemination. Late chronic PJIs (3 to 12-24 months) are usually a consequence of low virulence microorganisms during the replacement, including *CoNS* and *enterococci* as the most frequent, and aerobic Gram-negative *bacilli* as less common (127). Late hematogenous PJIs (>12 to 24 months) result from spread from a distant site, with *S. aureus* as the most frequent isolated microorganism (78,79,128).

1.7.2 *Staphylococcus aureus* as a pathogen

Sir Alexander Ogston first discovered *S. aureus* in 1881, when *Staphylococcus* was found as a possible cause of wound infections (72,129,130). The name was a consequence of its clustered appearance, evidenced under microscope. In 1884, Friedrich Rosenbach identified *Staphylococcus Aureus* (Aurum, Latin word for "gold"), differencing it from *Staphylococcus Albus* (Albus, Latin word for "white"), a related bacterium (131).

S. aureus is a Gram-positive, round-shaped commensal bacterium that forms pairs, tetrads, irregular "grape-like" structures and is part of the body's normal microbiota (129,132). It is usually found in the upper respiratory tract and on the cutaneous tissue and is positive for catalase and nitrate reduction. Additionally, it has unique characteristics to survive in adverse environmental conditions, being a facultative anaerobe that can survive without oxygen supply (133).

Although *S. aureus* is an endogenous microorganism of the human microbiota, it can also behave as an opportunistic pathogen, being one of the most frequently isolated bacteria that causes a wide range of clinical infections. Pathogenic strain infections result from the expression of virulence factors such as potent protein toxins and cell-surface proteins that bind and inactivate human antibodies (72).

Approximately 20% to 30% of all healthy patients have their anterior nares colonized and are *S. aureus* carriers, which has been shown to be related to a higher risk of PJI (72,134–136). *S. aureus* can also be isolated from the normal cutaneous flora and women's lower reproductive tract (137). Wertheim et al. (138) reported that almost 80% of nosocomial *S. aureus* infections were related to previous nasal colonization.

A wide range of symptomatic affections has been reported due to acute and chronic *S. aureus* infections, from minor skin infections, osteoarticular, implant-related, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, bacteraemia, and sepsis (72,129). Acute bacteraemia and cutaneous infections are usually a result of toxins and enzyme activity (139). On the other hand, chronic infections are related to biofilm production, where *S. aureus* attaches and survives, affecting bone, heart valves, and medical implants (140).

1.7.2.1 Epidemiology

S. aureus is the most isolated microorganism in all three major osteoarticular infections, including osteomyelitis (141), native joint septic arthritis (142), and PJI (83).

In almost all series and for all types of PJI, *S. aureus* is the most common causative organism, accounting for 18 to 73% of cases (72,86). In patients with TJA who suffer *S. aureus* bacteraemia, a PJI occurs in 29 to 39% (79). Additionally, it is one of the most frequent causes of invasive nosocomial and healthcare-associated bloodstream infections (143,144). Several studies (145,146) reported an increased risk of invasive infection when risk factors such as medical devices, intravenous drug use, haemodialysis, RA, diabetes, and *S. aureus* nasal colonization are present.

One of *S. aureus*'s principal characteristics is its capacity to develop antibiotic resistance, making treatment even more difficult. The first episode of penicillin resistance was reported in 1942, although its mechanism was first identified in the mid-1940s based on an inducible beta-lactamase.

On the other hand, Beecham developed methicillin in 1959, initially used to treat penicillin-resistant *S. aureus* strains. The first methicillin-resistant *S. aureus* (MRSA) strain was evidenced in 1960 (129,147,148), and it is defined as an oxacillin minimum inhibitory concentration ≥ of 4 micrograms/mL. Its resistance mechanism is not related to beta-lactamase production, like with penicillin. Still, it is a consequence of the Penicillin-Binding Protein 2a expression, which has a low affinity for beta-lactams (129,132). Penicillin-Binding Protein 2a is expressed by the gene *mecA*, localized in the *Staphylococcal* cassette chromosome mobile genomic element. The *mecA* gene is present in almost all S*taphylococcal species*; consequently, the methicillin resistance determinant (*mec* determinant) might be easily transmissible between different strains (129,149).

In the 1970s, MRSA spread worldwide, acquiring resistance to different antibiotics. The additional resistance to gentamicin was initially discovered in Europe and the US, reaching Australia and Latin America. In 2003, several authors reported MRSA prevalence of 60% in US intensive care units (ICU) (147,150). It has been identified in several cutaneous and soft tissue outbreaks in the healthy population in Europe, the US, and Canada (151). According to Kavanagh et al. (152), MRSA is endemic in the US, with 2% carriers in the general population and 43% of *S. aureus* cultures' oxacillin-resistant.

MRSA was first identified in Canada in 1964, but the first outbreak was reported in 1978 in Montreal. Until the 1980s, MRSA was primarily described as a hospital-acquired infection but has become community and livestock-acquired, being highly resistant to most oral antibiotics

(153). Healthcare-associated MRSA (HA-MRSA) infections affect patients with predisposing risk factors, and community-associated MRSA (CA-MRSA) infections usually occur in the healthy population. CA-MRSA strains are widely spread in some areas, being more virulent and transmissible than HA-MRSA (154).

1.7.2.2 Methicillin-resistant *Staphylococcus aureus* periprosthetic joint infection

The treatment of PJI after TJA has been complicated with the increasingly frequent appearance of antibiotic resistance strains (155). The actual evidence suggests an increasing number of PJI caused by MRSA. Parvizi et al. (156,157) reported a 50% prevalence of MRSA in the US.

Usually, PJIs are related to an increased readmission rate, length of stay, follow-up visits, and lower functional results (22,23,158). Several studies have found an estimated cost of more than $100,000 per case, generating an economic burden for the patient and the healthcare system (22,158).

The economic costs of MRSA PJI are certainly higher than that of methicillin-susceptible *Staphylococcus aureus* (MSSA) infections. Essentially, they are a consequence of the increased length of stay and ICU admissions (156). Parvizi et al. (156) reported a significant increase in expenses associated with MRSA PJI over the past decade, with estimated annual costs near $450 million.

MRSA has been identified as a frequent cause of acute PJI, secondary to its aggressive virulence. Due to the growing number of TJA performed worldwide, PJI caused by MRSA would have negative consequences on implant survival and are related to higher morbidity and mortality rates than MSSA (155).

Management is generally prolonged, and most implants are compromised (155). Poor DAIR outcomes have been reported when MRSA caused PJI. As expected, the most effective treatment for this type of infection is TSRS, with an extended antibiotic treatment before reimplantation. Regarding antibiotic treatment, different studies reported a minimum duration of 5 weeks for TKA and 8-12 weeks for THA, including i.v. vancomycin combined with oral rifampin (159).

Although different pre and postoperative measures, such as skin decolonization, routine antimicrobial prophylaxis, increased hand hygiene compliance, and patient isolation, contributed to reducing MRSA infections, it still is a significant cause of morbidity and mortality (147); therefore, high surveillance and novel treatment options are needed (129).

1.7.2.3 Mechanisms of antimicrobial resistance

Several glycopolymers have been described on the surface of *S. aureus*, including wall teichoic acid, peptidoglycan, lipoteichoic acid, and capsular polysaccharides. These cell-surface proteins are then recognized by the host's immune system, resulting in the complement system activation (160). The essential characteristic of *S. aureus* is adapting and surviving under unfavourable conditions. It is well-known for evolving and generating resistance to almost all available antibiotics (147,161).

Resistance is defined as an increase in an antibiotic's minimum inhibitory concentration value due to bacteria mutation. On the other hand, tolerance is the capability to resist the antibiotic effect due to reversible phenotypic changes (162). Antibiotic resistance is a natural event since bacterial evolution included resistance to antimicrobials even before human existence (163). Antibiotic tolerance has been identified in several microorganisms for a wide range of antibiotics.

The main characteristics implicated in antibiotic tolerance/resistance are bacterial dormancy (164), biofilm thickness (165), persister cells (164), and sub-minimum inhibitory antibiotic concentration (165).

Biofilm formation is a common characteristic of most bacteria; hence antibiotic tolerance is a frequent consequence. In biofilms, antibiotic tolerance is associated with the type of biofilm growth (166). Most of the usual microorganisms isolated in PJI have been proven to form biofilms, including pathogens such as *Cutibacterium acnes, Staphylococcus epidermidis, Enterococcus faecium, Pseudomonas aeruginosa,* and *S. aureus*, among others (167). Interestingly, biofilms can be formed even before PJI diagnosis. Saeed et al. (168) reported that progressive biofilm formation results from several microbial, host, and environmental characteristics without a direct correlation with symptoms appearance. In this sense, there are limited chances to act before biofilm formation.

Survival of *S. aureus* relies on biofilm formation (169,170). A biofilm is a sessile microorganism community where microbes attach to surfaces protected by an extracellular polymeric matrix (140). This polymeric matrix structure acts as an environmental reservoir, enables S. aureus to adhere to implanted devices, and protects against antibiotics and the immune system. Additionally, microorganisms usually begin a stationary phase where they stop growing due to biofilm formation, and as a result, they develop a higher resistance to antibiotic killing, thereby increasing their virulence (72,171).

Any type of implant is vulnerable to biofilm attachment and, therefore, at risk of developing an infection. *S. aureus* biofilms have been found in chronic infections, affecting different orthopaedic implants, including wires, pins, screws, plates, and prostheses (172). The infection can occur during the surgery, due to a traumatic event, or after hematogenous dissemination, after microorganism attachment to surface proteins (172). Biofilm formation

around implants and surrounding soft tissues have been proven to be difficult to eradicate, and the optimal management, including antibiotic therapy, remains unclear (168,173,174). A clear understanding of biofilm formation is crucial to developing new alternatives for treating these aggressive pathogens.

Biofilm formation was traditionally described as a three-stage process consisting of (1) attachment, (2) accumulation/maturation, and (3) detachment/dispersal (175). The development of new technologies using time-lapse microscopy, such as the BioFlux1000 system, allowed scientists to analyse a more detailed process, describing five stages instead of three: attachment, multiplication, exodus, maturation, and dispersal (Figure 1) (170).

(1) Attachment: *S. aureus* cells use several specific proteins to attach to different host matrix surfaces. These surface-attached proteins are known as the microbial surface components recognizing adhesive matrix molecules. Several of them have similar cell wall structures (176) but have different binding characteristics for host matrix components (177).

(2) Multiplication: after attachment and with enough nutrient supplies, *S. aureus* cells will start division and accumulation. Before the self-production of an extracellular matrix, the new cells are at risk of detachment, mainly associated with the fluid flow forces. To maintain this immature biofilm's stability, *S. aureus* cells produce various proteins to stabilize cell-to-cell bindings (170).

During this phase, microorganisms begin to biosynthesize the cell wall. It is a complex structure covering mature *S. aureus* cells, and it is formed by host factors, secreted and lysis-derived proteins, polysaccharides, and eDNA. This polymer is the main structure of the bacteria and is mainly made of peptidoglycans. It is made of sugars and amino acids that forms a mesh-like surface outside the plasma membrane. It provides structural support, regulates the cytoplasm's

osmotic pressure, and is involved during bacterial cell division. The peptidoglycan cell wall is formed by a polysaccharide intercellular adhesin, known as poly beta-1,6-N-acetyl-D-glucosamine (PNAG) (178). The polysaccharide intercellular adhesin polymer is made of β-1,6-linked N-acetylglucosamine and is an essential component in *S. aureus* biofilm structure (179). Due to the DNA polymer's negative charge, eDNA acts as an electrostatic polymer that binds *S. aureus* cells to a surface, host factors, and each other. The enzyme DD-transpeptidase is responsible for the cross-linking process, resulting in a solid and rigid 3-dimensional structure (169). Immature biofilms are most susceptible to DNAase treatment, suggesting that eDNA may be implicated during attachment (180). The peptidoglycan layer is significantly thicker in Gram-positive bacteria (20 to 80 nanometres) compared to Gram-negative bacteria (7 to 8 nanometres) (181). It forms 40 to 90% of the cell wall's dry weight of Gram-positive microorganisms but only 10% of Gram-negative. Therefore, these high amounts of peptidoglycan are the primary differentiation of bacteria as Gram-positive, participating in attachment and serotyping purposes (181).

(3) Exodus: this phase is one of the new biofilm formation concepts discovered using time-lapse microscopy. It consists of a unique and coordinated release of cells approximately 6 hours after the beginning of the multiplication phase. Basically, it is an early dispersal with microcolony formation resulting in biofilm restructuration. It is regulated by nuclease-dependent degradation of eDNA and is independent of the accessory gene regulatory (Agr) quorum-sensing system (170)

(4) Maturation: this stage consists of microcolony formation that provides an increased surface for nutrient and waste exchange. It also encourages biofilm cell dissemination to distal sites (182). In this sense, rapidly growing microcolonies are formed from different groups of slower-growing cells that persist in the basal layer immediately after the exodus' initiation (183).

(5) Dispersal is the final phase where microorganisms can spread to distant sites and disseminate the infection. This phase is under the Agr quorum-sensing system's control, responding to bacterial cell density and allowing dispersion and bacteraemia (170,184).

Virulence regulation includes complex global regulatory circuits that sense environmental conditions and determine the activation of master regulators to modulate gene expression. *S. aureus* responds to cell density through an auto induced, quorum-sensing signal.

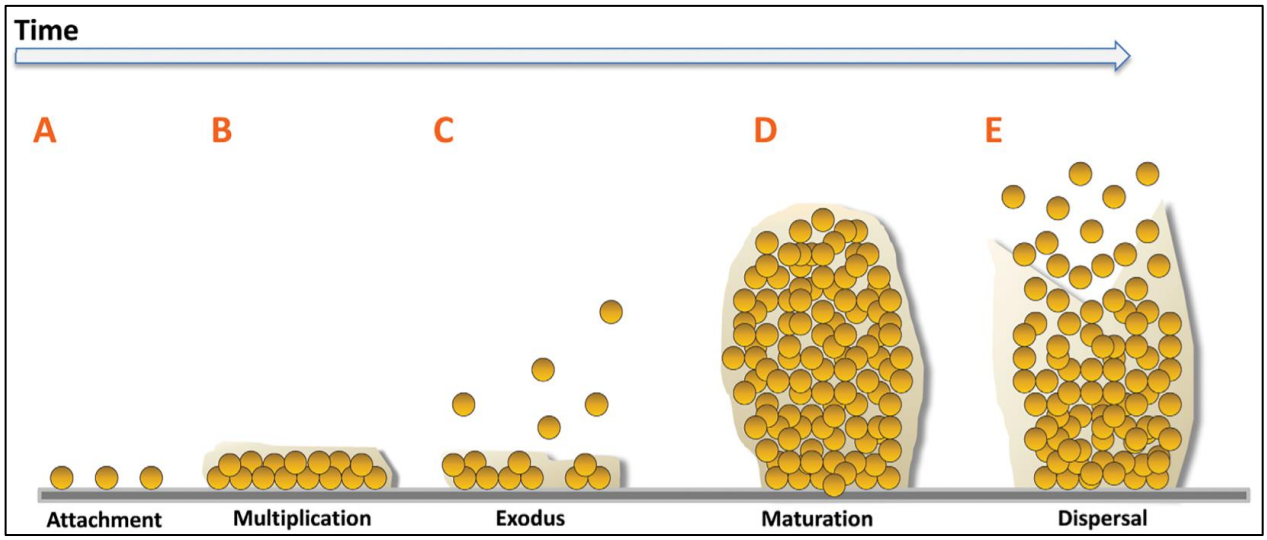

Figure 1. *S. aureus* biofilm formation in five stages: A) Attachment: *S. aureus* cells attach to a surface, B) Multiplication: after attachment, the biofilm develops into a confluent group of cells made of eDNA and proteinaceous matrix, C) Exodus: after confluency, a massive exodus occurs, and a minor subgroup of cells is released from the biofilm to allow three-dimensional microcolonies development, D) Maturation: this stage is defined by rapid cell division forming big collections of cells. These microcolonies are formed from different groups of cells that have remained attached during the exodus stage, E) Dispersal: activated Agr-mediated quorum sensing initiates biofilm matrix modulation and dispersal of cells.

In 1986, Recsei et al. (185) identified this Agr quorum-sensing system as the master virulence regulator in *S. aureus*. The Agr quorum-sensing system depends on cell density and the accumulation of signal molecules called autoinducers. In *S. aureus*, an auto-inducing peptide (186) accumulates in the culture medium, and after reaching a specific threshold concentration, binds to

and activates the histidine kinase, AgrC. Once activated, AgrC phosphorylates the response regulator, AgrA, which then initiates transcription from the P3 promoter of the Agr operon, producing a regulatory RNA molecule (RNAIII) that regulates the expression of several virulence factors and biofilm-associated genes (187,188). BioFlux1000 system demonstrated an increased P3 promoter expression within microcolonies due to auto-inducing peptide accumulation (189). There is a direct correlation between P3 activation and biofilm dispersal, which may be due to increased protease activity and subsequent degradation of the protein-based extracellular matrix (190).

Regarding copper, there is evidence in the literature of an increased systemic and local copper availability during infections (191). This suggests that the host environment uses copper's toxic properties to fight microbes. Free copper is toxic for most bacteria, and they go to great lengths to avoid its accumulation in the intracellular/cytoplasmic compartment. Most identified bacterial cuproproteins are within the cytoplasmic membrane or in the periplasmic space. Usually, microbes strictly regulate copper, and they use it in complex physiologic processes. Indeed, several studies showed less than 10^4 free copper atoms in each bacterial cell (192,193).

Usually, bacteria can avoid copper toxicity by three principal mechanisms, 1) copper efflux across the plasma membrane into the periplasmic space or the extracellular compartment, 2) copper sequestration within the cytoplasm or periplasm by copper-binding proteins, or 3) Cu(I) oxidation to generate the less toxic Cu(II) ion (194,195). Different types of copper export proteins have been identified in bacteria, and almost all Gram-negative or Gram-positive microorganisms appear to possess at least one copper exporting P1B-type ATPase that prevents the cytoplasmic accumulation of copper (196–198). Examples of these proteins include CopA of *E. coli*, CopA1 and CopA2 of *Pseudomonas aeruginosa* and CopA, GolT of *Salmonella typhimurium* and CopZ

of *Enterococcus hirae*. CopA is a copper exporting P1B-type ATPase that is usually activated under high extracellular copper concentrations (199–202). Solioz et al. (196) identified the presence of P1B-type ATPases in Gram-positive bacteria, such as *S. aureus*, that export Cu(I) out of the cytoplasm across the cell membrane.

1.8 Diagnosis

The complete assessment of a painful TJA must include a detailed history, complete physical examination, and radiographic views to evaluate the corresponding implants. The diagnosis of PJI is usually based on clinical manifestations, laboratory results, including blood work and synovial fluid tests, microbiological data, histological analysis of tissue samples, intraoperative evaluation, and radiographic assessment (13).

Multiple diagnostic guidelines within the joint arthroplasty literature recommend physicians suspect a PJI in any patient with a TJA and persistent wound drainage or acute pain without previous trauma or chronic pain at any moment since the initial replacement (69).

High-risk patients include cases with the previous compromise of the same joint, multiple procedures, previous PJI or history of SSI, immunocompromised patients (i.e., diabetes mellitus, inflammatory arthropathy, human immunodeficiency virus, chronic kidney disease, etc.), and finally, patients with a high risk of cutaneous lacerations (i.e., intravenous drug abuse, skin ulcers, chronic venous insufficiency). During physical assessment, suggestive findings of PJI include joint erythema, effusion, and increased temperature (71). The radiographic analysis is generally performed to rule out other pain reasons such as implant loosening, wear, and periprosthetic fractures (13). No changes might be seen in the acute onset of symptoms. However, some

nonspecific characteristics like bone resorption, periosteal reaction, and osteolysis might suggest chronic infection.

Different serological, synovial, microbiological, and histological investigations are performed, being some of them expensive, invasive, and inaccurate. Usually performed studies for PJI diagnosis includes direct or indirect methods.

Direct methods consist of the identification of the pathogen microorganism. A confirmed PJI due to *S. aureus* requires its isolation from intraoperative fluid and/or tissue samples. Positive results from three intraoperative samples for the same microorganism represent a 95% probability. In contrast, two positive results represent a 20% probability, and one represents only 13% (203). Nevertheless, as *S. aureus* is never considered a contaminant, a single positive culture with a compatible clinical presentation can be considered diagnostic. Biofilm microorganisms can be difficult to identify due to poor growth and isolation characteristics (168). In order to improve isolation accuracy, novel techniques have been developed, including sonication of removed implants, the use of disclosing agents, and polymerase chain reaction (PCR) (86,168).

On the other hand, indirect methods include several tests associated with the host's immune response to infection without microorganisms' isolation. Some of them are the erythrocyte sedimentation rate (3), C-reactive protein (3), synovial cell count and neutrophil percentage (204), leukocyte esterase (205), alpha-defensin (206), d-dimer (207), and histology (Polymorphonuclear neutrophils per high-powered field). Despite significant progress in the past years, laboratory analysis for the diagnosis of PJI remains a challenge, and no gold standard test exists. In this sense, in low-grade infections, some of these studies may not be routinely used (86,168). Recently, novel biomarkers and molecular methods have shown promising results in the current literature. Still, the most used inflammatory parameters among surgeons include the erythrocyte sedimentation

rate and serum C-reactive protein. An increased value of both markers after more than 90 days from the replacement suggests PJI (13,71,208).

When suspecting PJI, one of the most critical diagnostic exams is the culture of SF samples, obtained through closed-needle joint aspiration (13). The SF evaluation must analyse the total cell and differential leukocyte counts and include culture analysis for aerobic and anaerobic microorganisms (3). However, contradictory results have been reported. Some studies (209) highlight closed joint aspiration as a highly specific procedure for infection, and others (210) confirm its lack of sensitivity. A variable false positive and false negative rate of Gram strains have been reported. Stirling et al. (211) reported a false-negative rate of 78% for 143 positive SF cultures. In this sense, special considerations must be followed when indicating joint aspiration. According to the American Academy of Orthopaedic Surgeons guidelines (212), avoiding any antibiotic therapy 14 days before aspiration or biopsy is necessary. If PJI can not be confirmed, the next step would be open surgery collecting at least five tissue samples for routine culture and histology (203).

If components are extracted, they should be analysed. Routine sampling and cultures of removed implants lack sensitivity due to bacteria's biofilm, antibiotic treatment, or antibiotic-impregnated cement. Sensitivity from prostheses can be increased by applying sonication techniques and culturing the sonicated fluid. This method has 75% sensitivity for culturing microorganisms, compared to 34 to 45% sensitivity for culturing multiple tissue samples. It is especially relevant if there has been recent antibiotic therapy (213,214). Nevertheless, false-positive results due to contaminants are still possible if the correct threshold is not applied (215).

Finally, the precise role of PCR is unknown, as it seems to add limited evidence to routine cultures and would also have false-positive and -negative results (216). Although *S. aureus* can be

easily cultured, in PJI, it is usually related to biofilm formation and more virulent and resistant infections (217). Consequently, they result in challenging isolation using standard microbiological methods.

1.8.1 Diagnostic criteria

The diagnosis of PJI continues to be challenging. While many diagnostic criteria have been developed over the past decades, including the one from the Infectious Diseases Society of America (IDSA) and the Musculoskeletal Infection Society (MSIS) (3,69,70), a universal consensus for its diagnosis is still lacking. Furthermore, different organizations, like the MSIS and the European Bone and Joint Infection Society, developed an international consensus for the definition and diagnosis of PJI (218).

The MSIS definition has major and minor criteria for PJI diagnosis (69). A major criterion of PJI is the evidence of a sinus tract in communication with the joint or the same isolated microorganism in two different culture samples. The evidence of at least 4/6 minor criteria can also confirm the diagnosis.

Similarly, the IDSA definition requires evidence of a sinus tract and at least two cultures with the same microorganism to confirm the diagnosis (3). On the contrary, the evidence of purulence itself, without confirmed aetiology, is considered a definitive criterion of PJI. Being said, if using the IDSA definition, excluding other causes of purulence is mandatory (219). The evidence of purulence has been removed from the International Consensus Meeting definition (218) to unify criteria. Additionally, the IDSA definition does not consider serum inflammatory markers or SF cell counts and does not follow major and minor criteria as the MSIS definition.

Furthermore, it considers different variables, such as the growth of an aggressive microorganism from a single culture or histopathology confirmation of acute inflammatory response.

1.9 Treatment

1.9.1 Treatment alternatives

Despite new surgical techniques, modern designs, and routine antibiotic prophylaxis, PJI still is a major concern after TJA. Although PJI prevention should be the primary objective, the treatment aims to eradicate the infection and preserve a functional joint (220).

In order to define the most accurate treatment option, several characteristics must be considered, including the host immune status and comorbidities, previous PJI history, duration of symptoms, characteristics of the wound, microorganism virulence, and patient expectations (13).

Different treatment alternatives have been reported based on PJI classification (71), which should be individualized in each patient to enhance success. One of the most critical aspects of effective treatment is a collaborative and multidisciplinary approach, including surgeons, microbiologists, and infectious disease specialists (3,221).

Current alternatives include non-operative treatment with long term antibiotic suppression, DAIR (222,223), one- (220,224,225) or two-stage revision surgery (226), partial implant retaining surgery (227), arthroplasty resection without reimplantation, arthrodesis and amputation.

The use of antibiotic-loaded cement/calcium sulphate beads has been another alternative. They deliver high doses of antibiotics at the infection site (228). Calcium sulphate beads are biodegradable and, therefore, do not need to be removed. The selected antimicrobial agent should be effective against the isolated microorganism and available as a powder without loosening its

properties and effect after polymerization (229). The release process is performed in a biphasic curve. The first peak is identified between the first hours and days after the surgery. A slow and constant release characterizes the second phase during weeks, months, or even years (230,231). Antibiotic elution characteristics depend on the polymer used, room temperature, and pH conditions. Palacos cement, for example, is known for allowing a complete elution of antibiotics, including most aminoglycosides.

On the other hand, the oldest registered medical use of copper is documented in the Smith Papyrus (232). This Egyptian medical manuscript describes the use of copper to sterilize chest wounds and drinking water (232). Several civilizations, including Greeks, Romans and Aztecs, also used copper to treat headaches, burns, intestinal worms, and ear infections. In the 19th century, a new interest in copper's medical utility was generated by the evidence that copper workers appeared to be immune to cholera (232). Finally, the use of copper in medicine became universal in the 19th and 20th centuries, being used to treat chronic adenitis, eczema, impetigo, scrofulosis, tubercular infections, lupus, syphilis, anaemia, chorea, and facial neuralgia (232).

The use of copper as an antimicrobial agent continued until antibiotics became available in 1932. Unfortunately, due to antibiotic resistance, new alternatives to keep pathogenic microorganisms at bay are needed. In this sense, the use of copper surfaces in hygiene-sensitive areas has been developed (233). At this point, additional studies are needed to determine copper's potential role in treating PJIs and the most cost-effective presentation to deal with this devastating complication.

Unfortunately, current treatment options involve aggressive procedures with high comorbidity for patients, and therefore, are not recommended in high-risk cases. Moreover, the high prevalence of antibiotic resistance raises concerns and limits antibiotic options (234,235). In

this sense, new and less invasive treatment alternatives must be developed to avoid aggressive and long revision surgeries, hoping to improve the effectiveness of current strategies.

1.9.1.1 Debridement, antibiotics, and implant retention

As we previously mentioned, PJI is a devastating complication after TJA with significant morbidity and surgical challenges. While TSRS is known as the gold standard for the treatment of PJI, it is related to high economic costs, morbidity and mortality. The DAIR procedure is a well-known therapeutic alternative for acute PJIs. However, the success rate varies widely in the literature, and its efficacy and indication are still controversial.

Several authors (236,237) identified different risk factors affecting DAIR outcomes, such as type of microorganisms, duration of symptoms, and previous antibiotic treatment. Even though patient comorbidities may affect DAIR outcomes (236,237), no clear association with DAIR failure was found (238).

Obesity is a well-known risk factor that has been related to an increased PJI risk, but no direct association has been evidenced between DAIR failure and increased BMI (239). Systemic diseases affecting immune response, such as diabetes mellitus and inflammatory arthritis, may increase the risk of PJI. However, similarly to obesity, no strong association was reported with DAIR failure. In this sense, specific comorbidities may not contraindicate the surgery itself (239,240).

It has been proven that the time between initial symptoms and intervention affects the success rate. Kunutsor et al. (240) reported poorer outcomes when DAIR was performed >21 days from clinical presentation. On the contrary, better outcomes were found when the surgery was performed <21 days from the onset of symptoms.

Acute PJI has been traditionally treated with TSRS, with more than 90% satisfactory outcomes (241,242). However, DAIR has been increasingly performed during the past two decades, reporting 70 to 82% satisfactory results in highly selected patients (243–246), being a less aggressive procedure.

On the contrary, some other authors report poorer outcomes. Cobo et al. (123) analysed 117 acute PJIs, and reported a 57% success rate. A systematic review including 710 cases of acute PJI treated with DAIR showed a 46% success rate for those undergoing a single procedure and 52% when multiple surgeries were performed (247). Additionally, outcomes can be influenced by the responsible microorganism. Kunutsor et al. (240) reported a higher success rate in *streptococcal* PJI (89,5%) when compared to *staphylococcal* species (75%). Moreover, Triantafyllopoulos et al. (248) found higher failure rates when DAIR was performed due to *S. aureus* or MRSA infections.

The outcomes of DAIR for acute hematogenous PJI are less accurate. Several studies reported poorer success rates than acute PJI, ranging from 50 to 70% (63,73,246,249). In patients where DAIR is not a possible option, the principal treatment alternative would be single or TSRS.

On the other hand, chronic PJI is considered an absolute contraindication of DAIR. The mature biofilm formation around the implant requires removal of the prosthesis to achieve infectious control (250). In this sense, when the risk of failure is high enough, DAIR should not be recommended, and implant removal should be considered.

DAIR seems to be an attractive alternative as it is cheaper and would avoid multiple procedures and prolonged postoperative periods. Zimmerli et al. (173) suggested an algorithm for the precise indication of DAIR for the treatment of PJI. In this sense, it should only be indicated if

the patient is less than three weeks since symptomatic presentation, has a stable TJA, no soft tissue deficiency, and susceptible microorganism to rifampin and/or quinolones. In all other cases, the intervention selected should be either a single or TSRS, or even long-term antibiotic suppression therapy if the patient is unfit for any surgical procedure.

Two primary antibiotic therapies have been described for staphylococcal PJIs after adequate debridement in appropriately selected patients. The first alternative consists of 6 weeks of i.v. vancomycin (for MRSA or CoNS) or anti-staphylococcal penicillin (for MSSA). Different studies report success rates of 70% in acute PJI of the hip (73,125). The second alternative includes 2 to 6 weeks of i.v. vancomycin or anti-staphylococcal penicillin, with 3 to 6 months of oral rifampin combined with a second oral antibiotic, frequently ciprofloxacin or fusidic acid.

Zimmerli et al. (251) analysed rifampin's role in treating orthopaedic implant-related staphylococcal infections. Patients were randomized to receive either two weeks of i.v. flucloxacillin or vancomycin with rifampin or placebo, followed by either ciprofloxacin-rifampin or ciprofloxacin-placebo therapy for 3 to 6 months (251). Although the study has significant limitations and conclusions must be cautiously interpreted, the authors found successful results at 24 months of follow-up for 12/12 patients in the rifampin group, compared with 7/12 in the placebo group. Other non-controlled studies of this second alternative were also controversial. Several authors demonstrated success rates varying from 57% (123) to 85% (243,244). Notwithstanding all limitations of these studies, the 2013 IDSA recommendation guidelines (3) suggest combined rifampin antibiotic treatment 3 to 6 months after DAIR.

It is necessary to highlight the importance of an aggressive debridement to achieve successful results, even more critical than selecting antibiotic therapy. In this sense, Lora-Tamayo

et al. (63) reported that polyethylene liner exchange independently predicted satisfactory outcomes (adjusted Odds Ratio: 0.65; 95% Confidence Interval: 0.44 to 0.95).

1.9.1.2 One-stage revision surgery

OSRS was initially described by Buchholz et al. (252). Nowadays, it is performed worldwide, but it is more frequent in Europe. Several advantages have been associated with OSRS, such as only one intervention, anaesthesia, shorter length of stay, earlier postoperative rehabilitation, and fewer costs (253,254).

Not every patient is a candidate for OSRS; on the contrary, specific characteristics should be considered for its indication. This includes a healthy patient, correct microorganism isolation before the surgery, aggressive intraoperative debridement, complete implant extraction with cement mantle if was used, and a precise postoperative antibiotic treatment. Additionally, it is necessary to have a collaborative and multidisciplinary approach, including experienced surgeons, infectious disease specialists, and microbiologists (254).

On the other hand, several factors are contraindications for OSRS (220,224,255), such as more than two previous OSRS failures, vascular or neurovascular involvement, unidentified microorganisms before the surgery, microorganisms with high virulence or resistance, and unavailability of specific antimicrobial therapy.

OSRS would include an aggressive debridement and all implant removal with cement mantle if it was previously used. Before antibiotic treatment initiation, a minimum of 5 fluid or tissue samples should be sent for further microbiology analysis. Irrigation is usually performed with at least 9 litres of saline solution and pulsatile lavage with an antiseptic product. The incision is then irrigated, and temporary wound closure is performed with interrupted sutures. The next

step includes the second draping in a sterile fashion with new surgical instruments. The new prosthesis implantation is generally fixed with antibiotic-loaded cement, considering the isolated microorganism and its sensitivity. Postoperative intravenous antibiotic treatment is indicated for a minimum of 6 weeks, adjusted to culture results. Additional oral antibiotic administration depends on the antibiogram and the microorganism sensitivity (255,256).

Although TSRS is the most frequent type of treatment, OSRS seems to have comparable outcomes when performed by skilled surgeons. Haddad et al. (256) reported a 67% to 95% success rate in highly selected patients. In a meta-analysis, including 2,500 patients comparing OSRS vs TSRS for PJI, similar success rates were evidenced at 24 months of follow-up (91% vs 90%) (242). Different complications have been related to OSRS, including 10% to 15% risk of re-infection and reoperation (220).

1.9.1.3 Two-stage revision surgery

TSRS was originally described by Insall et al. (257). It has better reported outcomes than DAIR and is considered the current gold standard for chronic PJI. Mahmud et al. (119) reviewed 253 TSRS for infected TKA and reported an infection-free survival rate of 85% at five years and 78% at ten years.

The treatment involves at least two procedures to treat the infection. The initial surgery includes removing the implants and all infected bone, tissue, and cement, followed by 2 to 8 weeks of i.v. antibiotics. A temporary antibiotic-loaded cement spacer is implanted to treat the infection and preserve a functional joint. Finally, a second procedure is indicated, where a new replacement is implanted once the infection is eradicated and inflammatory markers are normalized. Antibiotics are selected according to isolated microorganisms, the culture samples' results, antibiogram, and

sensitivity (258,259). Evidence related to the duration of antibiotic treatment between both surgeries and the selection of antibiotics is scarce.

No universal consensus exists regarding the ideal type of spacer. Spacers are meant to preserve the joint space, reduce soft tissue tension, and deliver high doses of antibiotics while preserving a functional joint. Static spacers are specifically indicated in patients with massive bone loss, collateral ligament insufficiency, extensor mechanism injury, and soft tissue deficiency. On the other hand, articulating spacers can be indicated in almost all cases, except for the aforementioned characteristics. The principal difference between them is the supposed better functional outcome when articulating spacers are used.

Similar results have been reported regarding infection eradication (258). Still, Park et al. (260) reported better functional outcomes with an improved range of motion and a more straightforward second-stage procedure using articulating spacers. However, evidence concerning spacer-related complications is scarce. Struelens et al. (261) reported a 57% complication rate, with spacer tilting and mediolateral translation as the most frequent. On the contrary, Lanting et al. (262) reported only an 8.4% spacer complication rate. In this study, posterior and lateral subluxation cases were not included, as the authors believed these complications were prevalent due to spacer mispositioning and incorrect soft tissue tension.

Another controversial topic is the antibiotic treatment duration and the correct time of reimplantation. Insall et al. (257) initially described a prolonged intravenous therapy of 6 weeks before the second stage. Kuzyk et al. (263) questioned the standard six-week protocol and stated that the infection and the aggressive debridement might compromise the bone blood supply and surrounding tissues. In this sense, the antibiotic effect and its presence in the infection site would be attenuated.

Antibiotic-loaded cement spacers deliver high doses of antibiotics and can maintain an effective concentration for almost four months after implant removal (264). On the other hand, a shorter intravenous antibiotic protocol would decrease the risk of systemic toxicity and minimize the possible emergence of resistant microorganisms (159). Several authors reported similar success rates using short antibiotic protocols, with lower patient morbidity than standard therapies (159,264).

Most recommendation guidelines suggest vancomycin for MRSA PJI and anti-staphylococcal penicillin for MSSA, with or without additional rifampin (3). Due to vancomycin's poor bone penetration and low success rates, there is an increasing concern for the indication of different antibiotics, such as linezolid (265,266), daptomycin (267) and rifampin combined with quinolones or fusidic acid (243,268).

Byren et al. (267) compared daptomycin versus standard-of-care therapy (vancomycin, teicoplanin, or nafcillin) to treat *staphylococcal* PJI undergoing TSRS. Although the study has several limitations, as the analysis was performed only 1 to 2 weeks after the second surgery, the authors reported higher creatinine kinase (CK) levels more frequently in the daptomycin group than in the standard-of-care group (CK level >500 in 19% of patients vs. 8%), and they also found a higher success rate in the daptomycin group (60% vs. 38%).

1.10 Prevention

Management of modifiable risk factors before surgery is crucial to prevent PJIs. Regarding diabetes, blood sugar levels should be improved. Strong recommendations (269) were developed suggesting strict blood glucose controls in diabetic patients, patients with hyperglycaemia, and all cases treated with therapies that can increase blood sugar levels resulting in hyperglycaemia.

Smoking cessation should be strongly suggested. Concomitant infections in other joints or sites must be treated before joint replacement. When considering RA, perioperative management of DMARDs should be decided with a rheumatologist. Finally, it must be remembered that TJA must be considered as elective surgery and should be carefully scheduled to decrease the impact of the patient's underlying risk factors.

1.10.1 Skin microbiota treatment

Due to the high frequency of *S. aureus* PJIs, preoperative detection and decolonization of patients colonized with *S. aureus* have been strongly recommended to decrease the infection rate after TJA. Current prevention guidelines suggest mupirocin nasal unguent when nasal colonization is detected (270).

Nevertheless, different studies regarding preoperative decolonization have produced mixed results. Bode et al. (271) found an 80% decrease in deep infection rate among different surgeries after PCR *S. aureus* nasal detection followed by a 5-day protocol of twice-daily nasal mupirocin and daily chlorhexidine bathing. Additionally, Chen et al. (272) demonstrated an apparent reduction of nasal *S. aureus* colonization on the day of primary TJA using a similar treatment protocol. However, these data together cannot confirm whether this treatment is effective in decreasing SSI or PJI. A systematic review showed reductions in SSI varying between 13 and 200% (273). It is essential to mention that this review included 19 heterogeneous studies using universal or selective decolonization protocols in orthopaedics, including joint arthroplasty. Webster et al. (274) performed a meta-analysis showing no difference in SSI rates between chlorhexidine bathing and placebo. Again, this study included heterogeneous surgical procedures, and different types of protocols were used.

On the other hand, Johnson et al. (275) demonstrated a reduced SSI rate after using chlorhexidine wipes the night before knee arthroplasty. However, Farber et al. (276) could not find a difference when chlorhexidine wipes were used 1 hour before TJA. Of course, none of these studies would have the same skin decolonization rate compared to the conventional 5-day treatment. The absence of a clear and consistent benefit, the risk of secondary bacterial resistance to mupirocin or chlorhexidine, and the likelihood of side effects should be considered before considering this strategy.

1.10.2 Perioperative antibiotic prophylaxis

SSI is a well-known risk factor for later PJI (18,29,35,41,55), and perioperative antibiotic prophylaxis decreases the risk of infection by >80% (277,278). The general principles described in SSI guidelines can be easily applied to TJA (270).

Importantly, cefazolin is the most used due to its antistaphylococcal activity, availability, and low cost (277). The concomitant use of cefazolin and vancomycin might have a theoretical benefit for patients with MRSA colonization. Still, Sewick et al. (279) evidenced no significant reduction in SSI rate with dual antibiotic prophylaxis.

Antibiotic allergies and previous adverse reactions must be identified and recorded at the preadmission assessment. Most β-lactam allergic patients may safely receive cefazolin as antibiotic prophylaxis, avoiding using other antimicrobials (280). On the other hand, vancomycin or clindamycin should only be considered in patients with a positive penicillin skin test or a type I hypersensitivity reaction to cefazolin.

The first cefazolin dose should be administrated within 60 minutes before skin incision to reach proper tissue concentrations, whereas vancomycin should be administrated 60 to 120 min

before (270). A single preoperative dose is enough to maintain appropriate tissue concentrations during the surgical procedure. However, a second intraoperative dose might be necessary if the surgery is longer than 4 hours or excessive blood loss (281). A second dose of vancomycin is only necessary when the length of the surgery lasts more than twice the expected half-life of the antimicrobial. Moreover, Nelson et al. (282) evidenced no benefit in extending the antibiotic prophylaxis after 24 hours of the surgery.

Finally, it is relevant to highlight the importance of weight-based dosing. Of course, different indications, physiological characteristics, and types of antibiotics may influence the applicability of this strategy. In some scenarios, like vancomycin or linezolid therapy, weight-based dosing is superior to currently established fixed-dose treatment in adult patients (283–286). The usual daily dose of intravenous vancomycin is 2 g (500 mg/6 hours or 1 g/12 hour) for adults without renal failure. However, several authors reported better results with weight-based dosing (283,284,287). Catanzano et al. (284) retrospectively reviewed MRSA-positive patients before TJA or spine surgeries. The antibiotic prophylaxis was the same for all patients, including 1 g of vancomycin within an hour before skin incision. They were classified as either underdosed or overdosed based on the weight-based dosing protocol (15 mg/kg) for vancomycin. 69% of patients were underdosed, and 10% of patients were overdosed. Moreover, 60% of patients had a vancomycin level < 15 mg/L at the end of the surgery with a fixed-dose regimen compared to 12% with a weight-based dose ($p=0.0005$). These data suggest that weight-based dosing of vancomycin may be better than fixed dosing to prevent MRSA SSIs.

1.10.3 Laminar airflow rooms and surgical bodysuits

Theoretically, an “ultraclean” flow in the operating room would reduce intraoperative contamination and subsequent PJI. Laminar airflow rooms, in which a positive-pressure ventilation

system moves air at a uniform velocity in either a horizontal or vertical flow pattern, significantly reduce the number of contaminated particles in the air (288).

Initially, Lidwell et al. (288) reported a decreased PJI rate with the additional use of surgical bodysuits at one year of follow-up. Nevertheless, only 25% of the included patients had received perioperative antibiotics. On the contrary, later studies could not show clear benefits when current infection guidelines were also applied (25,26,35,289). Indeed, Hooper et al. (289) reported a strong correlation between laminar airflow rooms, surgical bodysuits, and the later revision surgery due to infection after six months of implantation. Overall, additional studies are still required before a definitive conclusion can be reached to support using these technologies when other well-known strategies are being used.

1.10.4 Antibiotic-loaded PMMA at surgical implantation

The purpose of adding antibiotics in the PMMA is to prevent a possible infection after primary arthroplasty or aseptic revision surgery or also increase the local antibiotic effect to continue treating an established infection during staged surgeries for PJI. Usually, 0.5 to 1 g of antibiotics per 40 g of PMMA are used for primary arthroplasty or aseptic revision, and 1 to 2 g during reimplantation after arthroplasty exchange due to PJI (290).

Lautenschlager et al. (291) reported reduced bone cement mechanical properties when large doses of antibiotic powders are added. Parvizi et al. (292) demonstrated an almost 50% decrease in deep infection rate when reviewing more than 20,000 primary or aseptic hip revision surgeries. However, some patients included in these six studies did not receive systemic perioperative antibiotic prophylaxis (293). Two other authors showed a decreased infection rate in diabetics (294) and nondiabetics patients (295) after TKA with 2 g of cefuroxime in 40 g of

PMMA. Nevertheless, the infection rate in the control groups was high in both studies, 13.5% in the diabetics and 3.1% in the nondiabetics despite the use of systemic antibiotic prophylaxis. Hinarejos et al. (54) found no difference in the incidence of infection at one year when erythromycin- and colistin-loaded cement or plain cement were used during TKA.

Additional randomized trials using more common antibiotics should be considered to generalize these results. Although Dale et al. (16) reported more than 90% of antimicrobial-loaded PMMA primary arthroplasties in some countries, no substantial evidence recommends this indication. As a consequence, the use of antibiotic-loaded PMMA for primary TJA remains controversial (296). Antibiotic-loaded PMMA is generally used during one-stage (297–301) or two-stage (302–304) revision surgeries for PJI. Additionally, Engesaeter et al. (305) found a similar or higher risk of reinfection when uncemented implants are used for PJI.

1.10.5 Antibiotic prophylaxis before dental, urologic or gastrointestinal procedures

For several years, both orthopaedic and dental professionals recommended antibiotic prophylaxis before dental procedures for patients with TJAs (306). Initially, this indication was based on older evidence and small cohort studies (307–311).

Current studies reported no increased risk of PJI after low- or high-risk dental procedures (29,312). Moreover, antibiotic prophylaxis before dental procedures does not decrease the risk of later PJI (29,313).

It is important to note that a lower risk of PJI was found among patients with good oral hygiene. Consequently, the American Dental Association and the American Academy of Orthopedic Surgeons recommend “changing routine antibiotic prophylaxis for patients with orthopaedic implants who undergo dental procedures” (314). Up to date evidence does not

recommend antibiotic prophylaxis before dental procedures. Patients should aim at optimal oral hygiene and routine dental visits to avoid possible infections.

Antibiotic prophylaxis is also not recommended in patients undergoing urologic or gastrointestinal procedures. However, Coelho-Prabhu et al. (315) reported a 4-fold increased risk of PJI related to oesophagus-gastro-duodenoscopy with biopsy. Other endoscopic procedures were not related to an increased risk, highlighting the different available procedures, and encouraging individual prophylaxis in each case.

Infections are a complex problem with a significant burden on patients and the healthcare system. Although much work has been done, we have not improved our diagnosis or treatment of infections, and with the increasing resistance, we have the danger of poorer outcomes in the future unless improvements are made.

Chapter 2

Project rationale

Osteoarthritis is the most prevalent joint disease worldwide. Fortunately, TJA is an effective procedure that restores quality of life. The worldwide number of TJA increases yearly and is projected to reach at least 4 million annual cases by 2030. PJI is the main reason for implant failure after TJA, affecting almost 2% of all replacements. *S. aureus* is a commensal bacterium, human pathogen, and is the leading cause of PJIs. Due to increasing multi-resistant microorganisms and MRSA prevalence, the eradication rate of chronic PJI continues to decrease, severely affecting patients, surgeons, and the healthcare system. In this sense, treatment options are limited, and new therapeutics are needed. Several studies have indicated that SF may participate in innate joint defence. However, the exact mechanism by which SF kills bacteria is yet to be defined, and an improved understanding of its antimicrobial properties would facilitate new diagnosis and treatment strategies for *S. aureus* infections. Thus, adding exogenous copper to human synovial fluid is expected to produce a high killing of the different *S. aureus* strains in our in vitro study. This multidisciplinary study will provide a better understanding of the behaviour of *S. aureus* in human SF and try to identify specific synovial proteins that might influence bacterial survival. Additionally, it is anticipated that this work will help develop novel strategies such as new antimicrobials, and therapies which could, in turn, lead to significant cost savings and reduce patient morbidity and mortality associated with this challenging complication.

2.1 Research objectives

2.1.1 General objectives

1. Analyse the specific antimicrobial properties present in human SF from osteoarthritic patients and evaluate how *S. aureus* can survive in human SF to cause infections
2. Investigate the pathogenesis of *S. aureus* and evaluate future alternatives to treat PJI after TJA

2.1.2 Specific objectives

1. Assess *S. aureus* survival in human SF in-vitro
2. Analyse the effect of exogenous copper on *S. aureus* viability in human SF
3. Determine the minimum bactericidal concentration of copper (MBC-Cu) and differences in sensitivity to killing between WT and CopAZB-deficient USA300 strains in human SF
4. Protein identification in human SF by running a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Chapter 3

Material and methods

3.1 Ethics statement

This research was approved by the Western University Research Ethics Board and Lawson Research Institute. Informed consent was signed before patient enrolment. See Appendix 1 and 2 for Research Ethics Board and informed consent, respectively.

3.2 Synovial fluid collection and preparation

SF samples were collected from patients undergoing elective primary TKA or THA to treat symptomatic osteoarthritis. All patients with inflammatory arthropathy (i.e., rheumatoid arthritis, gout) or fibromyalgia were excluded, as well as cases with multiple severe comorbidities.

The same surgical team performed all procedures under sterile conditions in the operating room at Concordia Hospital, Winnipeg, Manitoba, Canada. When TKA was performed, to minimize blood contamination, a tourniquet was applied around the thigh before starting the intervention. After the skin incision and deep dissection, the joint capsule was exposed, and SF was aspirated using a sterile 18-gauge needle and 20 cc syringe.

After collection, all samples were centrifuged for 10 minutes at 4700g within 12 hours of the procedure to remove any possible red blood cell and lymphocyte contamination. The cell-free SF supernatant was then aspirated and transferred to cryotubes under sterile conditions. The samples that remained with evident blood contamination after centrifugation were excluded from the analysis. All eligible samples were then frozen and stored in a -80°C freezer until further

analysis. All SF samples were labelled and separated by donor with a unique identification number without recording any patient's identifiable information.

After sterile collection at Concordia Hospital (Winnipeg), the human SF samples were shipped on dry ice to ensure they remained frozen during shipping to the Siebens Drake Research Institute at Western University (London). At the time of experimentation, randomly selected samples were taken out from the -80°C freezer and put in the incubator at 37°C for 5 to 10 minutes to thaw. After each experiment was completed and analysis was finished, the SF samples used were destroyed.

All patients were followed according to the surgeon's criteria with routine appointments to depict any unexpected complications. No follow-up assessment was necessary for the purpose of the present analysis.

3.3 *Staphylococcus aureus* strains and routine culture

Different *S. aureus* strains previously found to be sensitive and resistant, UAMS-1 and USA300 Wild Type (WT), respectively, were used throughout this study. Bacteria were routinely cultured at 37°C in liquid tryptic soy broth (TSB) (Difco) with shaking overnight at 200 Rpm or on solid tryptic soy agar (1.5% w/v) (TSA) plates. When necessary, *S. aureus* strains were cultured in the presence of 3µg/mL erythromycin to allow for selection of resistance markers.

It is essential to mention that all analysis and comparisons between strains were ideally made with the same human SF sample to avoid a possible bias in the results. When it was not possible, a different SF sample was used following the same methodology. A total of 55 different human SF samples were used in this study.

At the same time, a minimum of 8 different tubes (n=8) containing each *S. aureus* strain (UAMS-1 and USA300 WT) were used in every assay to reach appropriate statistical results. The only exception was the low-pH environment viability assay, where only four tubes for each strain were included in the analysis.

3.4 *Staphylococcus aureus* survival in synovial fluid in-vitro

Eleven human SF samples from different living donors were removed from the freezer, thawed, and diluted in sterile saline solution immediately before in-vitro growth assays. To analyse the effect of SF against *S. aureus*, in-vitro growth assays were performed with different *S. aureus* strains. We decided to use two of the most prevalent strains associated with human infections, including one sensitive and one resistant strain, such as the osteomyelitis isolate UAMS-1 and USA300 WT, respectively.

Overnight cultures of each *S. aureus* strain were grown in 5mL of TSB and then normalized to an optical density (OD_{600}) of 1.0 in sterile saline solution. Tubes containing either 20% SF (UAMS-1) or 50% SF (USA300 WT) in sterile saline solution were inoculated with 10µL of the *S. aureus* strain suspension at OD_{600}=1.0 to start each tube at an initial OD_{600} 0.01, equivalent to ~2-4x10^6 CFU/mL. The samples were incubated at 37°C for 2, 4, 12, and 24 hours, after which the samples were serially diluted, and 10µL were droplet plated on TSA plates. After overnight incubation at 37°C, the colonies were counted to determine the final number of colony-forming units per ml (CFU/mL) for each sample.

3.5 The effect of copper on *Staphylococcus aureus* viability in synovial fluid

Due to the well-known toxicity of copper (Figure 2) and the presence of ceruloplasmin in SF, viability assays were performed to determine the capability of different *S. aureus* strains to

survive in SF with the addition of exogenous copper. As done previously, 12 random SF samples were thawed immediately before dilution in sterile saline solution to determine *S. aureus* survival or death. SF samples were diluted in saline to varying concentrations from 20% to 50% along with no synovial fluid controls, and 1µL of 10mM copper sulphate ($CuS0_4$) was added to achieve a working concentration of 10µM copper in each sample. Similarly, tubes containing paired samples of SF or saline alone were included as controls without adding copper. All samples were plated on TSA at 0 hours after inoculation to assess the starting CFU/mL. Samples were again plated on TSA after 4, 12, and 24-hours incubation at 37°C to determine the resultant number of CFU/mL.

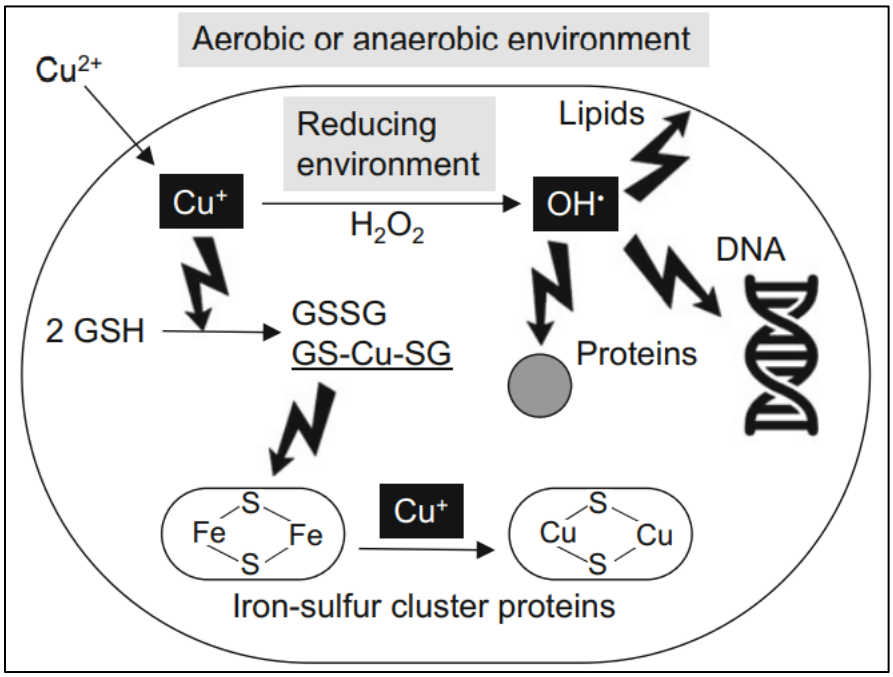

Figure 2. Mechanisms of copper toxicity. Copper violates the bacterial wall. The reducing environment of the cytoplasm reduces copper (Cu++ to Cu+), which can participate in Fenton type reactions, produce highly reactive hydroxyl radicals, and affect lipids, proteins and DNA. Cu+ can also lead to thiol depletion in the GSH pool, proteins and free amino acids. Under anaerobic conditions, glutathione-copper complexes (GS–Cu–SG) can act as copper-donors for metalloenzymes. The most common mechanism of copper toxicity is the displacement of iron from iron-sulfur cluster proteins.

3.6 Determination of minimum bactericidal concentration of copper and differences in sensitivity to killing between WT and CopAZB-deficient USA300 strains in synovial fluid

The MBC is the lowest amount of an antimicrobial agent required to cause a 3-logarithmic microbial death (99.9% killing) in the size of the standard inoculum. To identify the MBC-Cu, we performed viability assays using a two-fold dilution series of copper in synovial fluid and saline. On the other hand, different copper export proteins have been identified in bacteria (196–198), and Solioz et al. (196) identified P1B-type ATPases in *S. aureus*. All *S. aureus* strains have a conserved operon encoding a P1B-1-type ATPase copper efflux transporter (copA) and a copper chaperone protein (copZ) (316) encoded as part of the core genome. Some HA-MRSA strains also have an additional copper exporting P1B-3 type ATPase, designated copB, encoded on a plasmid that is either free or integrated into the genome (317).

Different mutant microorganisms with CopA deficiency have been demonstrated to have inadequate copper efflux and increased copper sensitivity (318–320). To try elucidating the mechanism of bacterial killing, we used the USA300 WT strain due to its inherent high resistance and the availability of mutants deficient for CopAZB protein that collaborate with bacterial survival by pouring copper out of its cytoplasm. In this sense, we tried to analyse the effects of deficient CopAZB protein on sensitivity to SF killing and determine the MBC-Cu.

Both the WT and CopAZB-deficient USA300 strains were streaked onto TSA plates and grown overnight at 37°C. Isolated colonies were inoculated into individual glass test tubes containing 5 ml TSB and grown overnight with shaking. The next day, bacteria were diluted in sterile saline solution to obtain an OD_{600} of 1.0. Next, these bacterial suspensions were used to inoculate SF and sterile saline solution with 1 in 100 dilutions (final OD_{600} of 0.01) with and without the addition of copper at a range of concentrations. Exogeneous copper sulphate was added

to the SF and saline in a two-fold dilution series ranging from 100 µM down to 1.5 µM along with a no copper control. A total of 12 different human SF samples were used for this analysis.

All samples were plated on TSA at 0 hours after inoculation to assess the starting CFU/mL. Samples were again plated on TSA after a 24-hours incubation at 37°C to count the resultant number of CFU/mL. The MBC-Cu was defined as the lowest concentration of copper, resulting in significant bacterial killing following overnight incubation at 37◦C.

3.7 Analysis of the acidic environment

Low-pH environments (<6.5) can reduce the survival of gram-positive pathogens (321). Indeed, weak acids would have antimicrobial activity because the undissociated form of weak acids passes easily through the cell membrane (321). Because of the acidic characteristic of local environmental ph. with the addition of exogenous copper, we then sought to analyse if the low-pH environment in SF would influence the bacterial killing.

As done previously, four random SF samples were thawed immediately before dilution in sterile saline solution to determine *S. aureus* survival or death. SF samples were diluted in saline to varying concentrations from 20% to 50% along with no synovial fluid controls, and 1µL of 10mM copper sulphate (CuS04) was added to achieve a working concentration of 10 µM copper in each sample. Similarly, tubes containing paired samples of SF or saline alone were included as controls without adding copper. Finally, additional samples of SF or saline were included as an experimental analysis with the addition of hydrochloric acid (HCl) solution to determine if the acidic environment influence the killing of the bacteria. Overnight cultures of each *S. aureus* strain were grown in 5mL of TSB and then normalized to an optical density (OD_{600}) of 1.0 in sterile saline solution. All samples were plated on TSA at 0 hours after inoculation to assess the starting

CFU/mL. Samples were again plated on TSA after 4, 12, and 24-hours incubation at 37°C to determine the resultant number of CFU/mL.

3.8 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and protein identification in synovial fluid

The amount of copper available in the host environment depends on binding proteins such as ceruloplasmin. Ceruloplasmin is a serum ferroxidase responsible for more than 90% of copper transportation (322), and its weight ranges between 120-132 kDa (108,323). In this sense, ceruloplasmin may be present in SF and may be responsible for the binding of copper.

To determine whether ceruloplasmin is responsible for the availability of copper in SF, we tried to identify the presence of ceruloplasmin in SF. Eighteen random SF samples were thawed immediately before dilution in sterile saline solution to an initial concentration of 10% (vol/vol) in saline. Then, 10 µL of the previous diluted SF samples from different donors were mixed 1:1 with Laemmli protein buffer and boiled for 10 min.

After cooling all samples, 7 µL of the ladder control solution was loaded. Then, 10 µL of 9 of the SF samples were loaded and run a 12% SDS-PAGE in Tris-glycine buffer at 150 V and 500 mA for 90 min. The remaining nine samples were loaded and run a second 12% SDS-PAGE in Tris-glycine buffer at 150 V and 500 mA but for more than 90 min. Upon completion, the gel was removed from the dock and stained overnight with Instant Blue® Coomassie Protein Stain. After staining the gel, we used an automated spot picker to identify the individual protein bands from the gel.

3.9 Statistical analysis

Continuous variables were expressed as means and standard deviations (SD) or medians and interquartile ranges depending on whether they had a normal distribution. Data were compared using the independent-samples t-test, where data were normally distributed, and the Mann-Whitney U test otherwise. Variables were considered statistically significant at $P < 0.05$ (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). All analyses were performed using IBM SPSS Statistics (IBM Corp., Armonk, NY, USA).

Chapter 4

Results

4.1 *Staphylococcus aureus* survival in synovial fluid in-vitro

Human SF has a heterogenous bactericidal response against different *S. aureus* strains. Both control strains, in sterile saline solution and without SF, clearly died at the final endpoint. We only found a higher sensitivity to killing for UAMS-1 after 12 hours (***p=0.001) when compared to USA300 WT (Table 1).

Table 1. *Staphylococcus aureus* survival in saline solution in-vitro

Time (hours)	UAMS-1 (n=8) (CFU/mL)	USA300 WT (n=8) (CFU/mL)	P value
0	7.66 x 10e5 (SD, 9.03 x 10e5)	1.36 x 10e6 (SD, 1.01 x 10e6)	P=0.14
2	1.01 x 10e6 (SD, 1.74 x 10e6)	1.32 x 10e6 (SD, 1.55 x 10e6)	P=0.92
4	6.1 x 10e5 (SD, 6.4 x 10e5)	1.37 x 10e6 (SD, 1.21 x 10e6)	P=0.09
12	2.12 x 10e5 (SD, 1.16 x 10e5)	1.45 x 10e6 (SD, 4.7 x 10e5)	***P=0.001
24	4.12 x 10e5 (SD, 1.11 x 10e6)	5.6 x 10e5 (SD, 4.5 x 10e5)	P=0.29

CFU/mL: Colony-forming units per ml; SD: standard deviation

Despite an initial growth after the first 2 hours (p=0.33), UAMS-1 died after 4 (p=0.06), 12 (p=0.33), and 24 hours (p=0.09), without being statistically significant. On the contrary, USA300 WT survived for the first 12 hours but showed significant bacterial death after 24 hours (*p=0.04) (Figure 3).

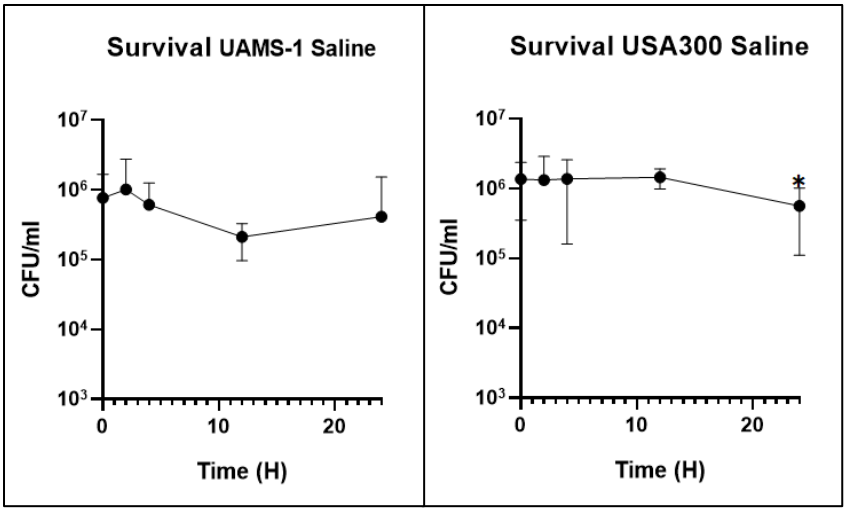

Figure 3. S. aureus survival in saline solution in-vitro. Variables were considered statistically significant at $p < 0.05$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

In contrast, we observed a different bactericidal effect of SF against each strain. UAMS-1 was highly sensitive and significantly died after 12 (***p=0.001) and 24 hours (*p=0.03) when compared to USA300 WT (Table 2).

Table 2. *Staphylococcus aureus* survival in synovial fluid in-vitro

Time (hours)	UAMS-1 (n=8) (CFU/mL)	USA300 WT (n=8) (CFU/mL)	P value
0	9.35 x 10e5 (SD, 5.72 x 10e5)	1.66 x 10e6 (SD, 9.09 x 10e5)	P=0.082
2	1.74 x 10e6 (SD, 1.1 x 10e6)	9.38 x 10e5 (SD, 5.33 x 10e5)	P=0.114
4	1.1 x 10e6 (SD, 7.8 x 10e5)	5.74 x 10e5 (SD, 7.23 x 10e5)	P=0.206
12	5.9 x 10e5 (SD, 1.66 x 10e5)	2.03 x 10e6 (SD, 8.98 x 10e5)	***P=0.001
24	1.29 x 10e5 (SD, 2.05 x 10e5)	3.67 x 10e6 (SD, 3.96 x 10e6)	*P=0.03

CFU/mL: Colony-forming units per ml; SD: standard deviation

Despite an initial growth after the first 4 hours (p=0.67), UAMS-1 died after 12 hours (p=0.09), achieving statistical significance at the 24 hours endpoint (*p=0.02). On the other hand,

even though USA300 WT showed a significant decrease of CFU/ml after 2 (*p=0.04) and 4 (*p=0.04) hours, it could finally survive and even grew after 12 (p=0.53) and 24 hours (p=0.67), suggesting an increased resistance to SF activity (Figure 4).

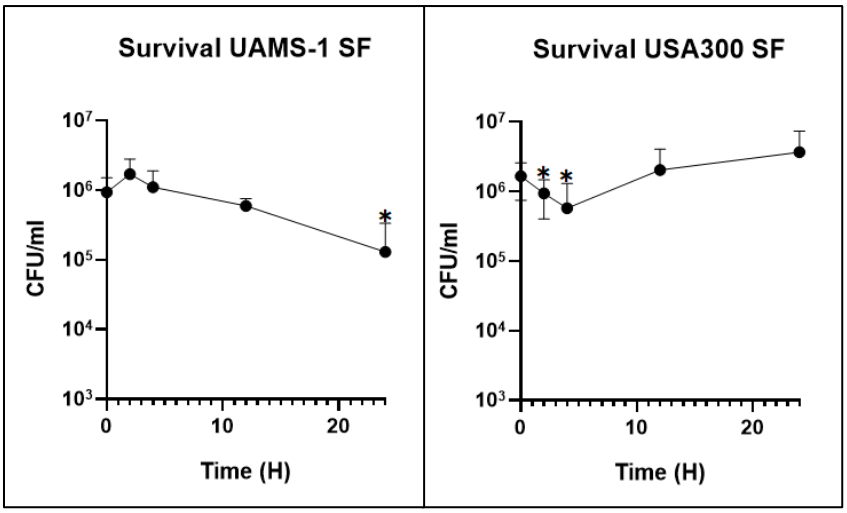

Figure 4. S. aureus survival in synovial fluid in-vitro. Variables were considered statistically significant at p <0.05 (*p < 0.05, **p < 0.01, ***p < 0.001).

Although we confirmed the bactericidal activity of SF, the sensitivity varied significantly between both strains, with UAMS-1 being more sensitive than USA300 WT affecting the overall survival of each strain.

4.2 The effect of copper on *Staphylococcus aureus* viability in synovial fluid

The addition of exogenous copper contributes to and increases the antimicrobial activity of SF against *S. aureus*. Due to the presence of ceruloplasmin as a copper-binding protein in SF and after demonstrating the bactericidal behaviour of SF against *S. aureus*, we then sought to analyse whether the addition of exogenous copper would contribute to bacterial killing.

Tables 3 and 4 show the results of bacterial viability assays separated by *S. aureus* strains, while Figures 5 and 6 show the same data but as survival curves for each strain. Both control

strains in saline solution and, with exogenous copper, significantly died after 4 hours. We observed a higher sensitivity to killing for UAMS-1 after 12 (**p=0.003) and 24 hours (***p=0.0008) when compared to USA300 WT (Table 3).

Table 3. The effect of copper on *Staphylococcus aureus* viability in saline solution

Time (hours)	UAMS-1 (n=8) (CFU/mL)	USA300 WT (n=8) (CFU/mL)	P value
0	6.83 x 10e6 (SD, 5.50 x 10e5)	6.4 x 10e6 (SD, 2.7 x 10e6)	P=0.92
0 + copper	6.1 x 10e6 (SD, 7.31 x 10e5)	6.34 x 10e6 (SD, 2.24 x 10e6)	P=0.67
4	3 x 10e6 (SD, 1.26 x 10e6)	5 x 10e6 (SD, 1.84 x 10e6)	P=0.06
4 + copper	1.83 x 10e6 (SD, 1.66 x 10e6)	3.25 x 10e6 (SD, 2.14 x 10e6)	P=0.14
12	3.6 x 10e5 (SD, 1.8 x 10e5)	4.28 x 10e6 (SD, 2.1 x 10e6)	***P=0.0008
12 + copper	6 x 10e4 (SD, 6.4 x 10e4)	3.52 x 10e5 (SD, 3 x 10e5)	**P=0.003
24	3.8 x 10e4 (SD, 1.5 x 10e4)	1.25 x 10e6 (SD, 9.9 x 10e5)	***P=0.0007
24 + copper	1.8 x 10e31.800 (SD, 1.6 x 10e3)	1.6 x 10e4 (SD, 2.2 x 10e4)	***P=0.0008

CFU/mL: Colony-forming units per ml; SD: standard deviation

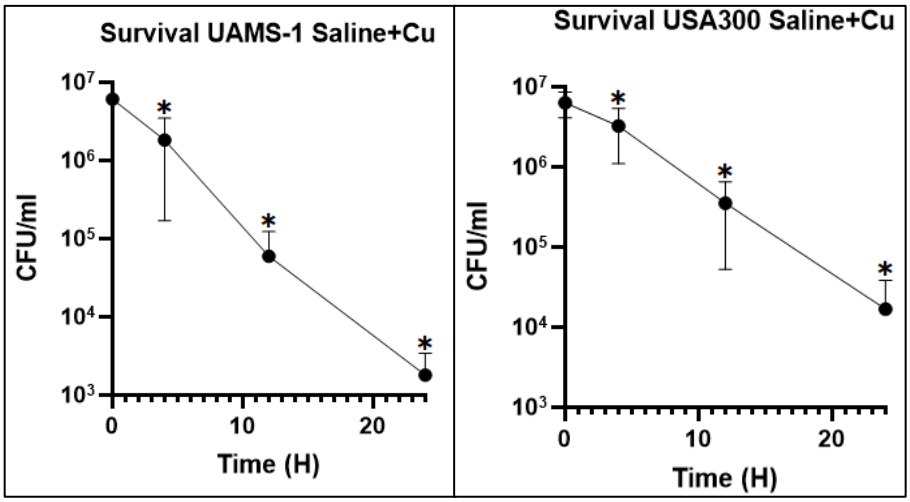

Figure 5. The effect of copper on S. aureus viability in saline. The addition of copper resulted in significant killing of both strains after 4, 12 and 24 hours. Variables were considered statistically significant at $p < 0.05$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Similarly, the addition of exogenous copper in SF resulted in a significant killing of both strains after 4, 12 and 24 hours (Figure 6).

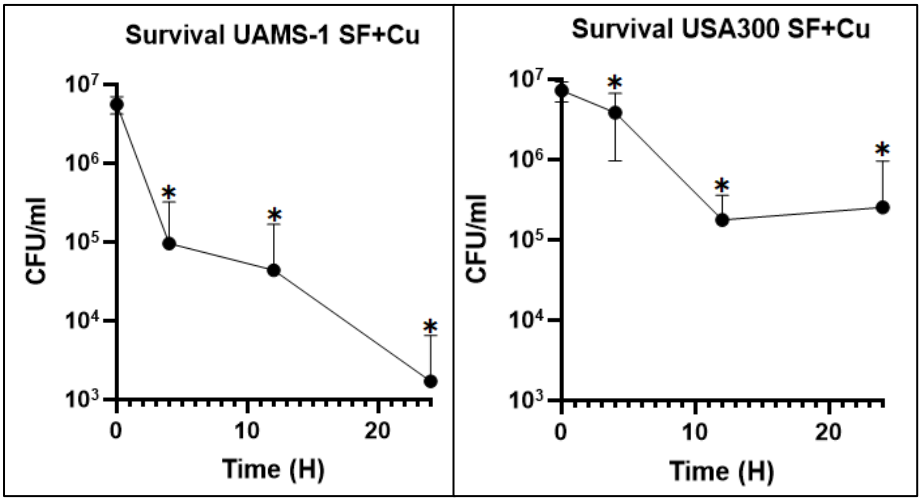

Figure 6. The effect of copper on S. aureus viability in synovial fluid. The addition of copper resulted in significant killing of both strains after 4, 12 and 24 hours. Variables were considered statistically significant at $p < 0.05$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

As expected, UAMS-1 was more sensitive and significantly died after 4 (***p=0.001), 12 (**p=0.005) and 24 (**p=0.006) hours when compared to USA300 WT (Table 4).

Table 4. The effect of copper on *Staphylococcus aureus* viability in synovial fluid

Time (hours)	UAMS-1 (n=8) (CFU/mL)	USA300 WT (n=8) (CFU/mL)	P value
0	6.5 x 10e6 (SD, 1.6 x 10e6)	7 x 10e6 (SD, 2.7 x 10e6)	P=0.67
0 + copper	5.6 x 10e6 (SD, 1.4 x 10e6)	7.25 x 10e6 (SD, 2 x 10e6)	P=0.24
4	5.1 x 10e6 (SD, 2.2 x 10e6)	5.1 x 10e6 (SD, 2.1 x 10e6)	P=0.87
4 + copper	9.6 x 10e4 (SD, 2.2 x 10e5)	3.84 x 10e6 (SD, 2.9 x 10e6)	***P=0.001
12	4.7 x 10e6 (SD, 1.9 x 10e6)	5.8 x 10e6 (SD, 1.7 x 10e6)	P=0.17
12 + copper	4.3 x 10e4 (SD, 1.2 x 10e5)	1.76 x 10e5 (SD, 1.8 x 10e5)	**P=0.005
24	4.4 x 10e6 (SD, 1.7 x 10e6)	6.9 x 10e6 (SD, 1.2 x 10e6)	*P=0.015
24 + copper	1.7 x 10e3 (SD, 4.8 x 10e3)	2.5 x 10e5 (SD, 7.1 x 10e5)	**P=0.006

CFU/mL: Colony-forming units per ml; SD: standard deviation

These data again demonstrate that SF from 12 different donors has a higher bactericidal effect against UAMS-1 compared to USA300 WT, and the addition of exogenous copper significantly increased bacterial killing of both strains.

4.3 Determination of minimum bactericidal concentration of copper and differences in sensitivity to killing between WT and CopAZB-deficient USA300 strains in synovial fluid

The deficiency of CopAZB protein enhances SF bacterial killing with lower copper concentrations. We found that the USA300 WT was significantly sensitive to killing with higher copper concentrations in SF after 24 hours. On the other hand, the control strain in saline solution

was significantly sensitive to killing with any copper concentrations after 24 hours (Table 5). Consequently, a 50µM copper concentration was identified as the MBC-Cu (Figure 7).

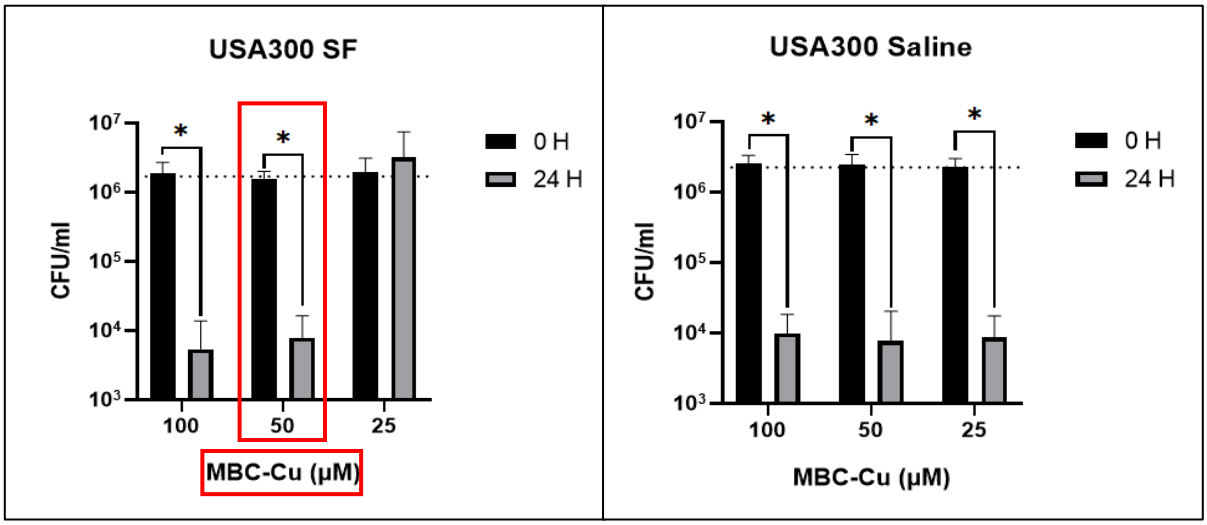

Figure 7. Minimum bactericidal concentration of copper on S. aureus USA300 WT. Variables were considered statistically significant at $p < 0.05$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Table 5. Minimum bactericidal concentration of copper on *Staphylococcus aureus* USA300 WT			
Time (hours)	SF (n=8) (CFU/mL)	SL (n=8) (CFU/mL)	P value
0 + 100 µM copper	1.9 x 10e6 (SD, 7.9 x 10e5)	2.5 x 10e6 (SD, 7.8 x 10e5)	*P=0.04
0 + 50 µM copper	1.6 x 10e6 (SD, 4.2 x 10e5)	2.4 x 10e6 (SD, 9.7 x 10e5)	*P=0.01
0 + 25 µM copper	1.9 x 10e6 (SD, 1.1 x 10e6)	2.3 x 10e6 (SD, 7.2 x 10e5)	P=0.53
0 + 12,5 µM copper	1.5 x 10e6 (SD, 1.4 x 10e6)	2.1 x 10e6 (SD, 8.5 x 10e5)	*P=0.03
0 + 6,25 µM copper	1.2 x 10e6 (SD, 7.1 x 10e5)	2.5 x 10e6 (SD, 1.5 x 10e6)	*P=0.03
0 + 3,125 µM copper	1.1 x 10e6 (SD, 5.5 x 10e5)	1.9 x 10e6 (SD, 6.6 x 10e5)	*P=0.02
0 + 1,5 µM copper	2.1 x 10e6 (SD, 7.9 x 10e5)	2.1 x 10e6 (SD, 6.5 x 10e5)	P=0.87
0 + 0 µM copper	2.1 x 10e6 (SD, 9.1 x 10e5)	2 x 10e6 (SD, 1.1 x 10e6)	P=0.92
24 + 100 µM copper	5.3 x 10e3 (SD, 8.4 x 10e3)	9.9 x 10e3 (SD, 8.3 x 10e3)	P=0.29
24 + 50 µM copper	7.9 x 10e3 (SD, 8.4 x 10e3)	7.7 x 10e3 (SD, 1.2 x 10e4)	P=0.92
24 + 25 µM copper	3.2 x 10e6 (SD, 4.3 x 10e6)	8.5 x 10e3 (SD, 8.9 x 10e3)	P=0.06
24 + 12,5 µM copper	3.7 x 10e6 (SD, 4.3 x 10e6)	1.2 x 10e4 (SD, 1.6 x 10e4)	**P=0.008
24 + 6,25 µM copper	6.7 x 10e6 (SD, 4.3 x 10e6)	1.8 x 10e4 (SD, 2.1 x 10e4)	**P=0.008
24 + 3,125 µM copper	1.2 x 10e6 (SD, 2.3 x 10e6)	8.6 x 10e3 (SD, 9.4 x 10e3)	*P=0.01
24 + 1,5 µM copper	6.5 x 10e4 (SD, 1.2 x 10e5)	2 x 10e4 (SD, 2.5 x 10e4)	P=0.46
24 + 0 µM copper	2 x 10e6 (SD, 3.6 x 10e6)	7.3 x 10e5 (SD, 6.1 x 10e5)	P=0.40
SF: synovial fluid; SL: saline solution; SD: standard deviation; M: molar; CFU/mL: Colony-forming units per ml			

Interestingly, we demonstrated that the USA300 CopAZB mutant strain was significantly sensitive to killing in SF with as little as 1,5 µM copper concentration or without adding it (0 µM)

after 24 hours (Table 6). Moreover, the mutant strain significantly died even without copper (0 µM) at 0 hours compared with the control group in saline solution (*p=0.03) (Table 6).

Table 6. Minimum bactericidal concentration of copper on *S. aureus* USA300 CopAZB

Time (hours)	SF (n=8) (CFU/mL)	SL (n=8) (CFU/mL)	P value
0 + 100 µM copper	1.9 x 10e6 (SD, 5.1 x 10e5)	2.1 x 10e6 (SD, 8.8 x 10e5)	P=0.15
0 + 50 µM copper	1.4 x 10e6 (SD, 6.9 x 10e5)	2.5 x 10e6 (SD, 8.7 x 10e5)	*P=0.03
0 + 25 µM copper	2.1 x 10e6 (SD, 8.4 x 10e5)	2 x 10e6 (SD, 8.1 x 10e5)	P=0.96
0 + 12,5 µM copper	2 x 10e6 (SD, 6.5 x 10e5)	1.6 x 10e6 (SD, 5.5 x 10e5)	P=0.09
0 + 6,25 µM copper	1.3 x 10e6 (SD, 6 x 10e5)	1.7 x 10e6 (SD, 7.6 x 10e5)	P=0.60
0 + 3,125 µM copper	1.6 x 10e6 (SD, 7.4 x 10e5)	2.1 x 10e6 (SD, 9 x 10e5)	P=0.21
0 + 1,5 µM copper	1.8 x 10e6 (SD, 9.1 x 10e5)	2.1 x 10e6 (SD, 7 x 10e5)	P=0.56
0 + 0 µM copper	9.9 x 10e5 (SD, 8.5 x 10e5)	1.9 x 10e6 (SD, 6 x 10e5)	*P=0.03
24 + 100 µM copper	6.7 x 10e4 (SD, 1.6 x 10e5)	0 (SD, 0)	***P=0.0003
24 + 50 µM copper	2.9 x 10e4 (SD, 3.1 x 10e4)	275 (SD, 778)	**P=0.002
24 + 25 µM copper	1.1 x 10e5 (SD, 1.9 x 10e5)	13 (SD, 35)	***P=0.0005
24 + 12,5 µM copper	3.8 x 10e5 (SD, 6.6 x 10e5)	0 (SD, 0)	***P=0.001
24 + 6,25 µM copper	3.8 x 10e5 (SD, 6.6 x 10e5)	313 (SD, 579)	**P=0.005
24 + 3,125 µM copper	1.1 x 10e4 (SD, 1.6 x 10e4)	75 (SD, 139)	**P=0.006
24 + 1,5 µM copper	4.6 x 10e4 (SD, 6.5 x 10e4)	8.8 x 10e3 (SD, 1.6 x 10e4)	P=0.05
24 + 0 µM copper	8.3 x 10e4 (SD, 1.8 x 10e5)	1.7 x 10e5 (SD, 2.7 x 10e5)	P=0.43

SF: synovial fluid; SL: saline solution; SD: standard deviation; M: molar;

CFU/mL: Colony-forming units per ml

These results highlight the importance of the CopAZB protein for bacterial survival, showing that the mutant strain cannot survive with small amounts of copper exposure. When analysing the results in saline solution, we found a higher sensitivity to killing with any copper concentrations after 24 hours, being statistically significant (Figure 8 and Table 6). These data suggest that *S. aureus* requires CopAZB protein for resistance to killing when free or exogenous copper is available in SF; however, other proteins or defence mechanisms might play a role in resistance to the killing by SF.

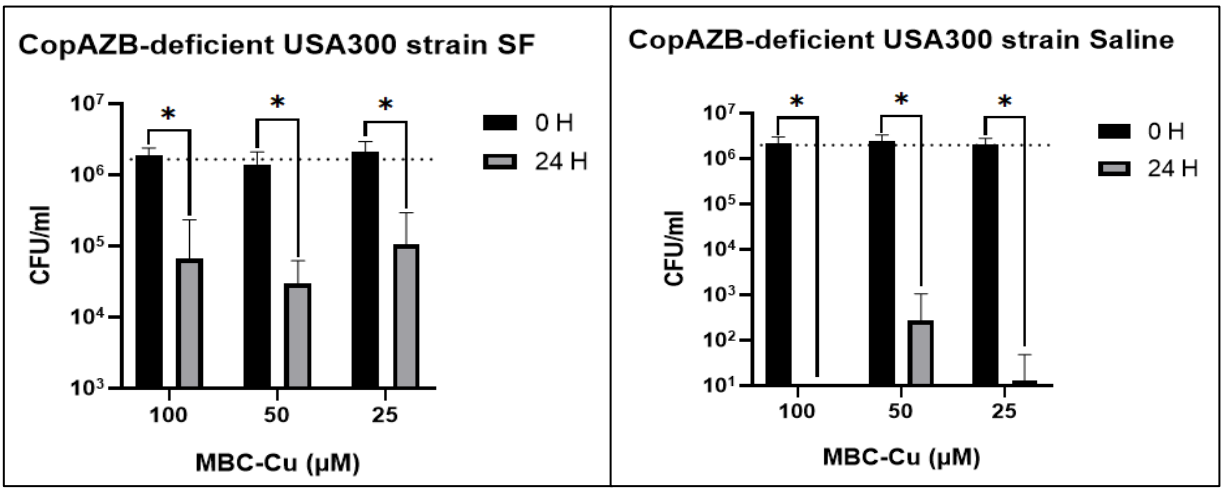

Figure 8. Minimum bactericidal concentration of copper on S. aureus USA300 CopAZB. Variables were considered statistically significant at $p < 0.05$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

4.4 Analysis of the acidic environment

The addition of exogenous copper is responsible for the increased antimicrobial activity of SF against *S. aureus*. As low-pH environments can reduce the survival of gram-positive pathogens, we then sought to analyse if the acidic environment could contribute to bacterial killing because of the addition of exogenous copper. For this purpose, additional samples of SF or saline were included with hydrochloric acid (HCl) to determine whether the acidic environment influences the killing of the bacteria.

As shown in Tables 7,8, and Figures 9,10, the addition of copper was the critical factor for the increased bacterial killing in SF after 24 hours (*p=0.02). Similar to the previous analysis, UAMS-1 showed a higher sensitivity to killing when compared to USA300 WT.

Table 7. The effect of low-pH environment on *S. aureus* viability in saline solution			
Time (hours)	UAMS-1 (n=4) (CFU/mL)	USA300 WT (n=4) (CFU/mL)	P value
0 + copper	1.6 x 10e6 (SD, 1.4 x 10e6)	1.2 x 10e6 (SD, 1.6 x 10e6)	P=0.56
0 + HCl	1.5 x 10e6 (SD, 7.1 x 10e5)	2.1 x 10e6 (SD, 9.9 x 10e5)	P=0.24
0 -	1.8 x 10e6 (SD, 7.5 x 10e5)	2.3 x 10e6 (SD, 5.9 x 10e5)	P=0.38
24 + copper	1.3 x 10e3 (SD, 1.1 x 10e3)	1.4 x 10e4 (SD, 1.7 x 10e4)	P=1.00
24 + HCl	2 x 10e4 (SD, 1.1 x 10e4)	4.4 x 10e4 (SD, 1.8 x 10e4)	P=0.10
24 -	1.2 x 10e5 (SD, 1.7 x 10e5)	2.8 x 10e5 (SD, 3.3 x 10e5)	P=0.56
CFU/mL: Colony-forming units per ml; SD: standard deviation			

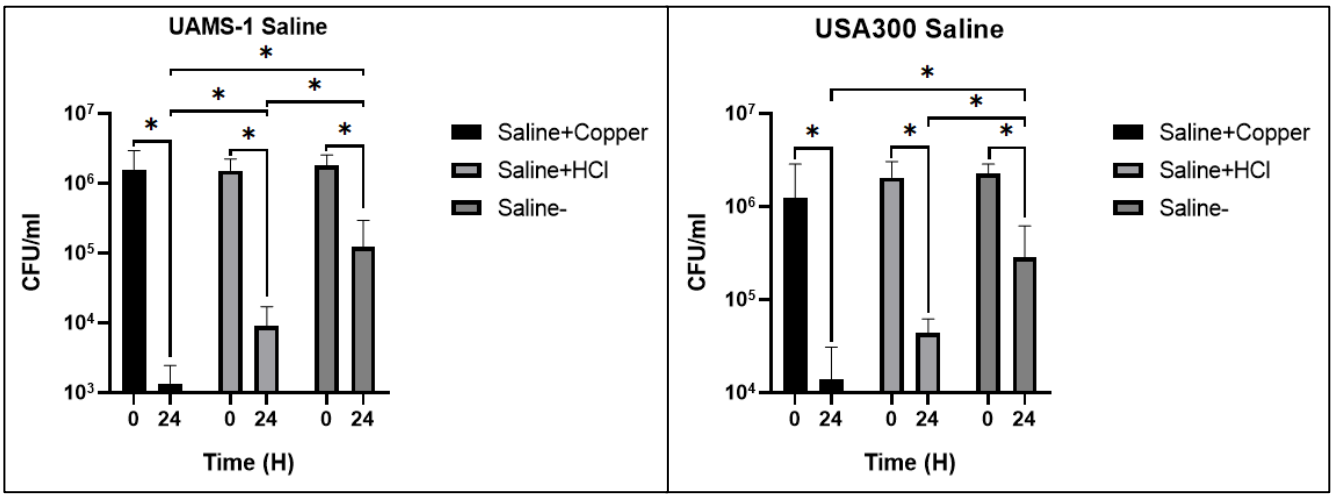

Figure 9. The effect of low-pH environment on S. aureus viability in saline solution. Variables were considered statistically significant at p <0.05 (*p < 0.05, **p < 0.01, ***p < 0.001).

Table 8. The effect of low-pH environment on *S. aureus* viability in synovial fluid			
Time (hours)	UAMS-1 (n=4) (CFU/mL)	USA300 WT (n=4) (CFU/mL)	P value
0 + copper	2.8 x 10e6 (SD, 1.1 x 10e6)	1.65 x 10e6 (SD, 1.8 x 10e6)	P=0.38
0 + HCl	2.6 x 10e6 (SD, 1.6 x 10e6)	3.3 x 10e6 (SD, 7.9 x 10e5)	P=0.55
0 -	3.1 x 10e6 (SD, 7.1 x 10e5)	2.9 x 10e6 (SD, 1.8 x 10e5)	P=0.24
24 + copper	25 (SD, 29)	3.7 x 10e4 (SD, 2.3 x 10e4)	*P=0.02
24 + HCl	1.4 x 10e6 (SD, 2.6 x 10e6)	5.3 x 10e6 (SD, 3.6 x 10e6)	P=0.24
24 -	1.1 x 10e6 (SD, 1.9 x 10e6)	5.8 x 10e6 (SD, 3.9 x 10e6)	P=0.24
CFU/mL: Colony-forming units per ml; SD: standard deviation			

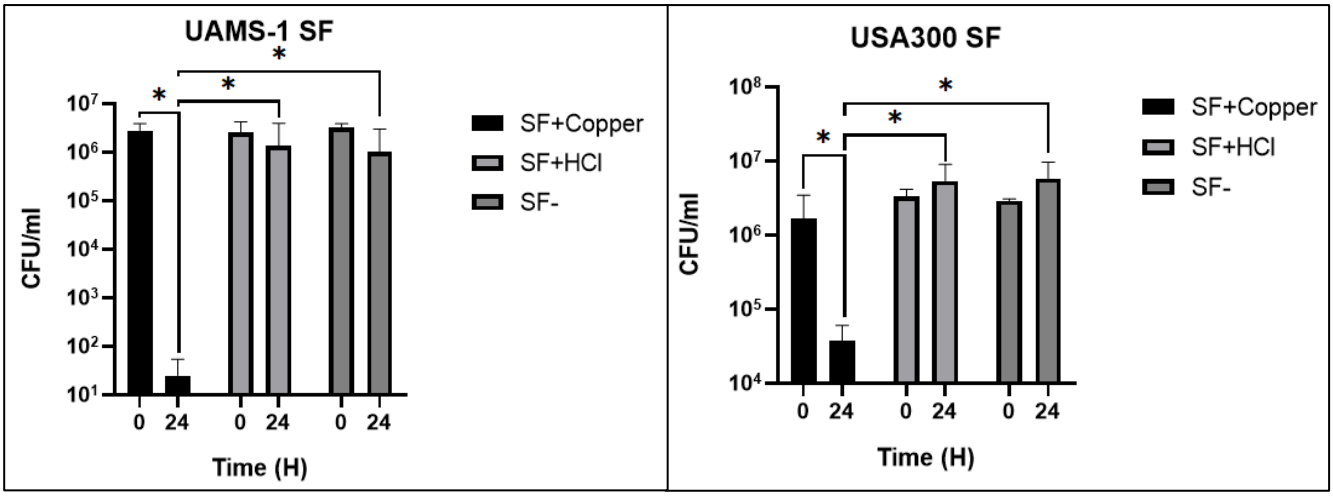

Figure 10. The effect of low-pH environment on S. aureus viability in synovial fluid. Variables were considered statistically significant at p <0.05 (*p < 0.05, **p < 0.01, ***p < 0.001).

4.5 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and protein identification in synovial fluid

Two 12% SDS-PAGE were run using nine randomly diluted SF samples from different donors in each gel. The first SDS-PAGE was run for 90 min, and the 9 SF samples ended the

analysis successfully. The second SDS-PAGE was run for more than 90 to achieve a better identification of proteins. Still, unfortunately, one of the SF samples was deficient, and only eight samples ended the run correctly.

The gel demonstrated a heterogeneous amount of protein, around 122 kDa, compatible with the estimated size of ceruloplasmin in SF as Watson et al. (108) previously demonstrated (Figure 11 and 12). In this sense, ceruloplasmin would be responsible for the presence of copper in SF.

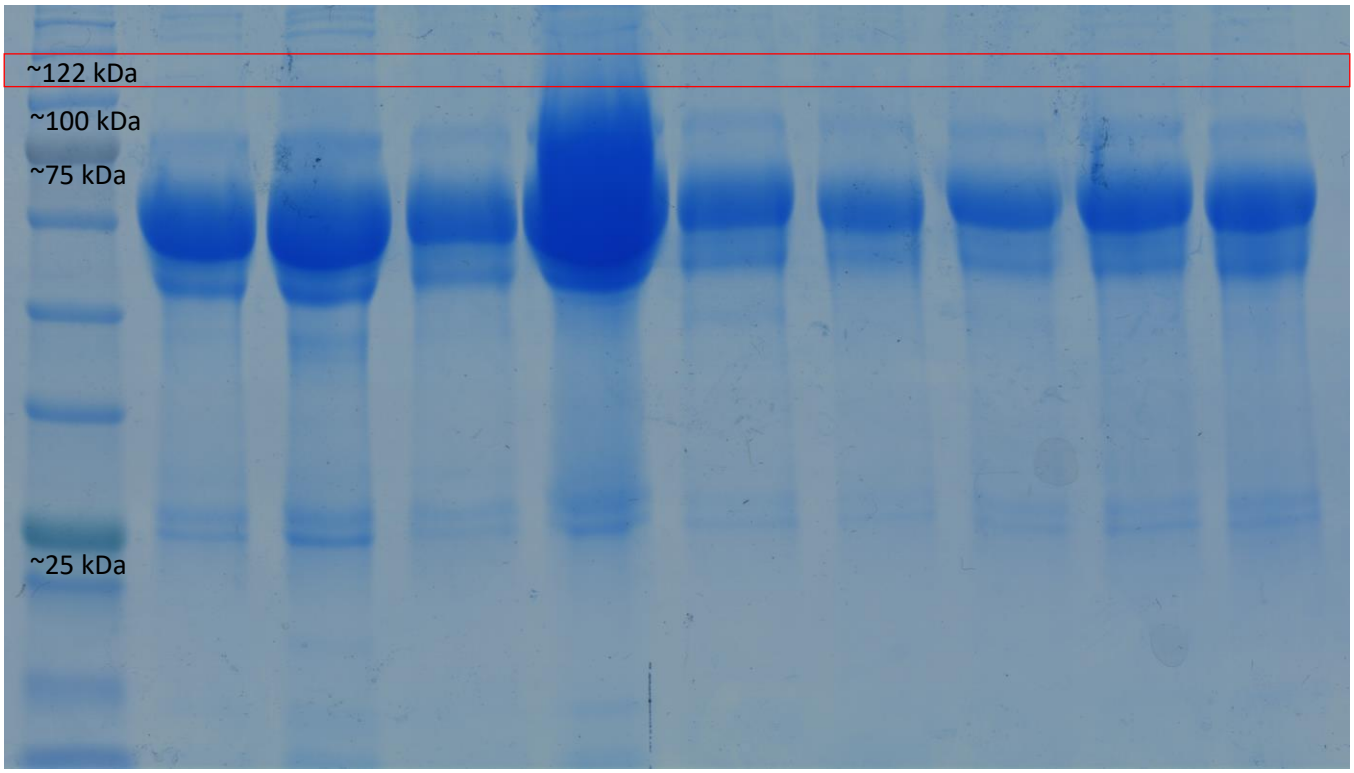

Figure 11. SDS-PAGE and protein identification in synovial fluid run for 90 min. Upon completion, the gel was removed from the dock and stained overnight with Instant Blue® Coomassie Protein Stain. After staining the gel, we used an automated spot picker to identify the individual protein bands from the gel. The red box identifies the heterogeneous band in the different synovial fluid samples compatible with the estimated size of ceruloplasmin (122 kDa).

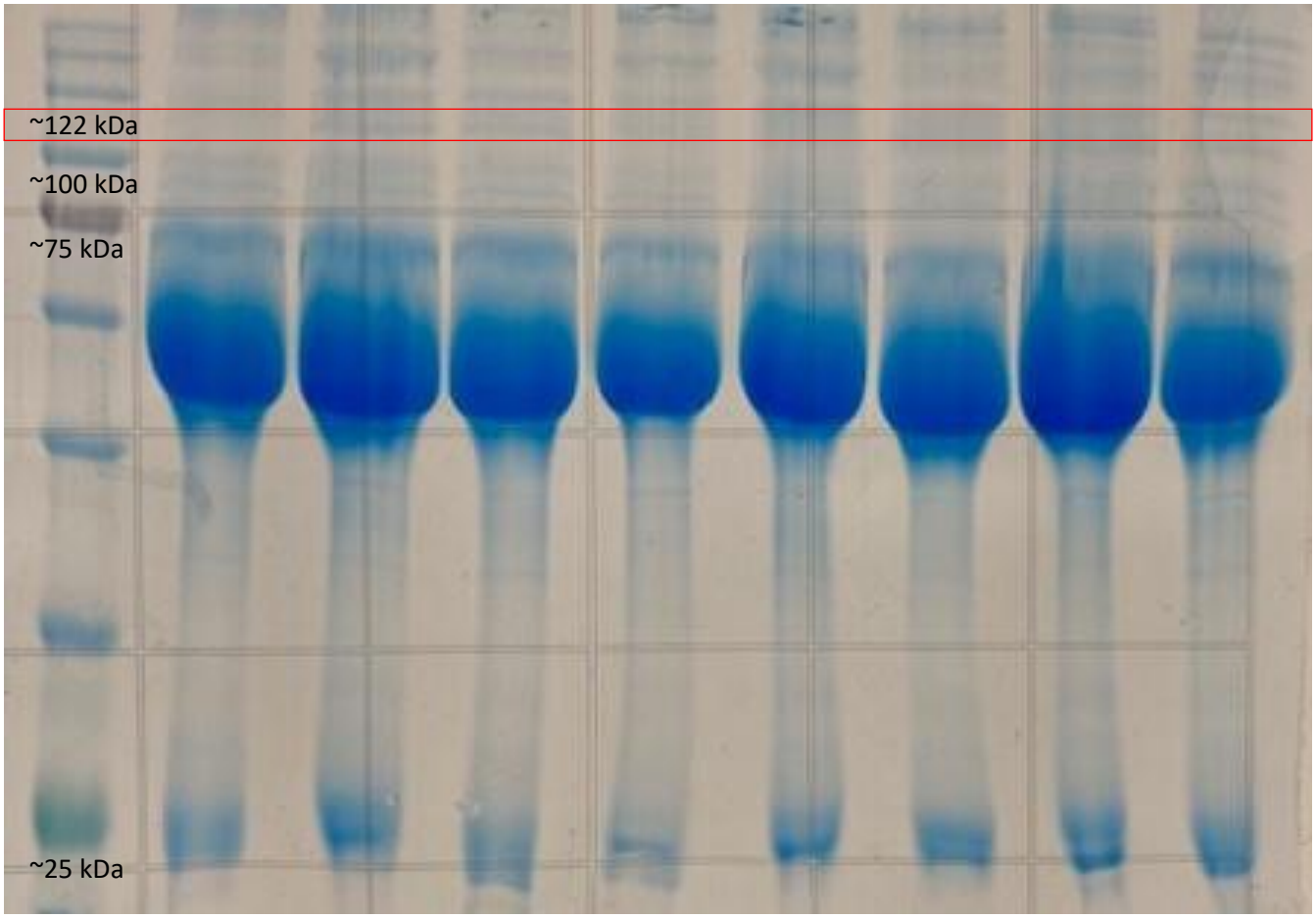

Figure 12. SDS-PAGE and protein identification in synovial fluid run for > 90 min to better identify the protein band. Again, upon completion, the gel was removed from the dock and stained overnight with Instant Blue® Coomassie Protein Stain. After staining the gel, we used an automated spot picker to identify the individual protein bands from the gel. Similarly, the red box identifies the heterogeneous band in the different synovial fluid samples compatible with the estimated size of ceruloplasmin (122 kDa).

Chapter 5

Discussion and future directions

The number of TJA performed increases every year, and consequently, the number of revision surgeries is expected to grow as well (4,5). PJI is known as the most challenging complication after TJA and has been reported as the leading cause of revision surgery (8), with a prevalence between 1 to 2 % among all replacements. As previously mentioned, *S. aureus* is part of the human microbiota and is the most frequently isolated bacterial pathogen responsible for PJI after THA or TKA. It has a well-known ability to cause infections, plasticity to mutate and develop high resistant strains, which has resulted in it being a real threat for surgeons and the health care system. Although there is evidence of the bactericidal role of SF, the exact mechanism and the specific antimicrobial properties that might be implicated in killing *S. aureus* still need to be defined (101). This study provides evidence that SF from hips and knees of osteoarthritic patients is bactericidal against *S. aureus*. Moreover, we have demonstrated that the addition of low copper concentrations exacerbates the bactericidal effect of SF and that S. aureus CopAZB protein-deficient strain without the capacity to efflux copper ions from the cytoplasm have increased sensitivity to synovial fluid and the lowest copper concentrations.

Regarding *S. aureus* survival analysis in SF, Watson et al. (108) showed that SF from osteoarthritic patients has antimicrobial factors that restrict and kill *S. aureus*, but sensitivity variations exist, with the CA-MRSA strain LAC showing a high level of resistance. Similarly, we confirmed the heterogenous bactericidal activity of human SF against different *S. aureus* strains. UAMS-1 was highly sensitive and significantly died, whereas USA300 WT survived and even grew at the final endpoint, suggesting an increased resistance to SF activity.

It is challenging to confirm how SF can kill bacteria, partly because the precise SF composition of osteoarthritic joints is not widely known (100). For example, as we already know, iron is an essential micronutrient required by most bacteria for crucial intracellular processes and their growth (105,324–326). Nevertheless, free iron is almost non-existent in most host environments, as different iron-binding proteins like ferritin, transferrin, lactoferrin, and haemoglobin actively bind to iron and sequester most of it from the extracellular space competing with bacteria (106,107,324). On the other hand, bacteria, specifically *S. aureus*, can steal iron from the host iron-binding proteins by producing siderophores (327,328). In this sense, Watson et al. (108) identified the presence of transferrin in SF of osteoarthritic knees and confirmed the capacity of SF to decrease iron availability for bacteria, resulting in a higher siderophore synthesis by *S. aureus*. They also demonstrated increased survival of *S. aureus* when adding free iron in SF, consistent with the concept that iron restriction decreases bacterial growth.

Many publications have already dealt with the kinetics of contact killing upon exposure of bacteria. It seems that dry metallic copper surfaces are even more antimicrobial than moist ones, resulting in bacteria inactivation within a few minutes of exposure (329,330). Touch surfaces frequently found in hospitals can be highly contaminated, and *S. aureus* can persist on such surfaces for months (331). Systematic and efficient cleaning, combined with proper hand hygiene, decreases infections, but complete elimination seems to be impossible (332). With the high prevalence of MRSA, nosocomial infections have become a primary concern for hospitals, and copper seems to be a promising alternative (330). Thus, the use of metallic copper surfaces would protect from microorganisms by reducing surface contamination (233,333,334). Indeed, the antimicrobial properties of copper surfaces have been shown to reduce bacterial counts, indicating

that copper surfaces are a reliable alternative to decrease the number and severity of hospital-acquired infections (335–337).

Microbes might have difficulty facing the bioavailability of copper and other essential and deleterious metals during infections. In this sense, host environments take advantage of this challenging situation by responding with different strategies to starve pathogens of essential metals (338,339). There is reduced availability of iron (338,340,341), as well, manganese and zinc are also withheld at local sites of infection by high-affinity metal-binding proteins (342,343). On the contrary, it seems to happen exactly the opposite with copper concentrations. Instead of starvation, the host environment increases copper during infection to kill pathogens, with copper toxicity behaving as an antimicrobial agent (344–347). In our study, we demonstrated that the addition of low copper concentrations increases the antimicrobial activity of SF against *S. aureus*. Although we demonstrated a higher sensitivity to killing for UAMS-1 than for USA300 WT, interestingly, the addition of a working concentration of 10µM copper resulted in a significant killing of both strains. This suggests that the addition of copper in SF, presumably via enhancing cytotoxic activity, increases the bactericidal activity of SF and, therefore, bacterial killing. Theoretically, free copper and bounded to ceruloplasmin might be insufficient to produce significant bacterial death. In this sense, the copper-additional environment may increase the bactericidal activity of SF, but, of course, other factors apart from the addition of copper would undoubtedly contribute to bacterial killing.

In our study, we only used a 10µM working concentration of copper, resulting in a significant killing of both strains. As we can see, the copper concentration used (10µM) was less than the normal range of total copper (13.3-28.3 µM), but it was higher than the normal reference of free copper (0-1.6 µM). As we know, a copper-binding protein such as ceruloplasmin is present

in SF, and exogenous copper might bind to ceruloplasmin in SF. As a result, it is challenging to assess the real fraction of free copper and its theoretical toxic threshold. It would be vital to know the exact concentration of ceruloplasmin in SF, the possible free fraction of copper that might be available in SF, and how much of the added copper would be bonded to ceruloplasmin to realize the accurate working concentration of copper and avoid possible toxicity.

Different mutant microorganisms with CopA deficiency, such as *E. coli*, *Streptococcus pneumoniae*, and *Neisseria gonorrhoeae* have been demonstrated to have inadequate copper efflux, intracellular accumulation, and increased copper sensitivity (318–320). Our data on the differences in sensitivity between USA300 WT and CopAZB-deficient USA300 were significant. Regarding USA300 WT, we showed high sensitivity to higher copper concentrations in SF after 24 hours, demonstrating a MBC-Cu of 50µM. On the other hand, USA300 CopAZB mutant strain was significantly sensitive to dying in SF with as little as 1,562 µM of copper after 24 hours. We demonstrated that *S. aureus* needs CopAZB proteins to export the copper out of its cytoplasm to survive in SF. Indeed, we showed that CopAZB protein deficiency further enhanced sensitivity to SF and increased bacterial killing with low copper concentrations.

As we know, SF may vary among humans, and its exact composition is yet to be defined. Ceruloplasmin is a serum ferroxidase responsible for more than 90% of copper transportation (322). Also, it is known as an acute-phase reactant, and its concentration in plasma may increase during inflammation or infection (348). Ceruloplasmin is a single polypeptide chain that weighs between 120 kDa and 132 kDa (108,323). Our study demonstrated a heterogeneous amount of protein, around 122 kDa, compatible with the estimated size of ceruloplasmin in SF. It seems that ceruloplasmin concentration varies among different samples. Although it might be responsible for

the presence of copper in SF, we could not confirm the exact concentration of ceruloplasmin, neither of bonded copper nor free fraction.

Like in most of the daily and labour activities, the COVID-19 pandemic highly impacted our thesis. Unfortunately, part of our project was interrupted due to COVID-19 restrictions. To better elucidate the pathogenesis of *S. aureus* and the antimicrobial properties of SF, our original thesis included the development of an animal model. We planned to create an animal model, including operating rats, implanting 3D printed implants and creating an infection to study the pathogenesis of *S. aureus* in PJIs. Additionally, some other experiments and analyses were also cancelled due to COVID-19 restrictions. Some tests were delayed and could not be done on time. Those analyses included further tests for different strains of *S. aureus* and, finally, the Western Blot for the precise identification and quantification of ceruloplasmin and copper in SF.

To conclude, this study highlights the vital importance of exogenous copper and the CopAZB proteins as possible antimicrobial tools against *S. aureus*, although more evidence is required to further confirm our findings. Due to the prevalence of *S. aureus* PJIs, it is possible that treatment alternatives considering the use of exogenous copper or therapeutics targeting CopAZB protein would be effective in treating *S. aureus* infections. Here, we demonstrated that 10μM of copper effectively killed both *S. aureus* strains in different human SF samples. Thereby, our study supports the use of low copper concentrations as an alternative for treating *S. aureus* infections; however, more evidence is necessary to define the efficacy, safety, and toxicity level of copper in animal models before a possible application in humans. Finally, we showed that human SF from knees and hips from osteoarthritic patients have heterogenous bactericidal activity against different S. aureus strains, which require CopAZB protein, and probably other related factors to efflux copper from the cytoplasm and resist SF killing. Thus, we propose using exogenous copper and/or

CopAZB protein as possible therapeutic alternatives to the continued efforts to decrease the incidence and improve treatment options of *S. aureus* infections.

Chapter 6

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Chapter 7

Appendices

7.1 Appendix 1: Synovial fluid study: Ethics approval

LAWSON
HEALTH RESEARCH INSTITUTE

LAWSON FINAL APPROVAL NOTICE

LAWSON APPROVAL NUMBER: R-18-698

PROJECT TITLE: Synovial Fluid Composition of Patients Undergoing Total Knee Arthroplasty for Severe Osteoarthritis

PRINCIPAL INVESTIGATOR: Dr. Edward Vasarhelyi

LAWSON APPROVAL DATE: Friday, 8 February 2019

ReDA ID: 5328

Overall Study Status: Active

Please be advised that the above project was reviewed by Lawson Administration and the project:

Please provide your Lawson Approval Number (R#) to the appropriate contact(s) in supporting departments (eg. Lab Services, Diagnostic Imaging, etc.) to inform them that your study is starting. The Lawson Approval Number must be provided each time services are requested.

Dr. David Hill
V.P. Research
Lawson Health Research Institute

Western Research

Date: 21 November 2018

To: Dr. Edward Vasarhelyi

Project ID: 111952

Study Title: Synovial Fluid Composition of Patients Undergoing Total Knee Arthroplasty for Severe Osteoarthritis

Application Type: HSREB Initial Application

Review Type: Delegated

Meeting Date / Full Board Reporting Date: 04/Dec/2018

Date Approval Issued: 21/Nov/2018

REB Approval Expiry Date: 21/Nov/2019

Dear Dr. Edward Vasarhelyi

The Western University Health Science Research Ethics Board (HSREB) has reviewed and approved the above mentioned study as described in the WREM application form, as of the HSREB Initial Approval Date noted above. This research study is to be conducted by the investigator noted above. All other required institutional approvals must also be obtained prior to the conduct of the study.

Documents Approved:

Document Name	Document Type	Document Date
111952 - Letter of Information and Consent 15-Nov-2018	Written Consent/Assent	15/Nov/2018

Documents Acknowledged:

Document Name	Document Type	Document Date
References	References	04/May/2018

No deviations from, or changes to, the protocol or WREM application should be initiated without prior written approval of an appropriate amendment from Western HSREB , except when necessary to eliminate immediate hazard(s) to study participants or when the change(s) involves only administrative or logistical aspects of the trial.

REB members involved in the research project do not participate in the review, discussion or decision.

The Western University HSREB operates in compliance with, and is constituted in accordance with, the requirements of the TriCouncil Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2); the International Conference on Harmonisation Good Clinical Practice Consolidated Guideline (ICH GCP); Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Products Regulations; Part 3 of the Medical Devices Regulations and the provisions of the Ontario Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

Please do not hesitate to contact us if you have any questions.

Sincerely,

Patricia Sargeant, Ethics Officer (ext. 85990) on behalf of Dr. Philip Jones, HSREB Vice-Chair

Note: This correspondence includes an electronic signature (validation and approval via an online system that is compliant with all regulations).

7.2 Appendix 2: Synovial fluid study: Letter of Information and Consent Form

London Health Sciences Centre

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Letter of Information and Consent Form

Synovial Fluid Composition of Patients Undergoing Total Knee Arthroplasty for Severe Osteoarthritis

Principal Investigator:
Dr. Edward Vasarhelyi

Co-Investigator:
Dr. David Heinrichs

You are being invited to participate in a research study designed for patients who will receive a primary total knee replacement under Dr. Edward Vasarhelyi's care. This letter of information describes the research study and your role as a participant. The purpose of this letter is to provide you with the information you require to make an informed decision about participating in this research. Please read this form carefully.

Study Purpose

Periprosthetic joint infection (infection in the joint after hip or knee replacement) occurs in around 1% of patients, and can be a challenge to treat for both patients and surgeons. It is still not clearly understood why infections can be more difficult to treat in some people than others. The goal of this study is to investigate the composition of (what makes up) the synovial fluid (fluid in the joint) of individuals undergoing total knee replacement. We hope that a better understanding of the environment in which the bacteria that causes infection grows, can lead to better treatments for infection in the future.

Procedure

If you decide to participate in this study your surgeon will take a sample of the fluid in your knee (synovial fluid) prior to beginning your knee replacement procedure. The sample will be taken after anaesthesia has been administered, when you are in the OR. Your sample will be stored in a tube and will be taken to Dr. Heinrich's lab on the Western University campus. Samples collected for the study will be analyzed in the lab in order to investigate the composition of the synovial fluid (what it is made up of). We will also use the samples to grow bacteria (one commonly associated with infections after joint replacement surgery) in order to better understand how the bacteria grows in the joint. No personal information will be attached to your sample; the tubes will be labeled with a number (ie. sample 1, 2, 3, etc.) and your sex and age. Study samples will be kept only until the analysis is complete, which we anticipate will be no more than 1 year from the date of collection.

Approximately 50 patients will be asked to participate in this study.

Risk

There are no additional risks of this study outside of the standard risks associated with a total knee replacement procedure.

Benefits

Participation in this study will provide no known benefit to you. Information learned from this study may help lead to improvements in treatments for periprosthetic infections in the future.

Compensation

There will be no compensation for your participation in this study.

Voluntary Participation

Your participation in this study is voluntary. You may refuse to participate or discontinue your participation at any time without affecting the care being provided to you. Should you choose to withdraw; no further information will be collected. The data you have contributed to that point will be used to help answer our research question. Once the synovial fluid sample has been collected and sent to the lab, the sample cannot be withdrawn.

Confidentiality

All information will be kept confidential to the best of our ability. No identifiers will be tied to your sample. There is always a remote chance that your information, including consent forms or the participant log, could be breached by someone without permission to your information. The chance that this information will be accidentally released is minimal. In any publication, presentation or report, all results will be de-identified and any information that would reveal your identity will not be published.

You will be given a copy of this letter of information and consent form once it has been signed. You do not waive any legal rights by signing the consent form. Representatives of The University of Western Ontario Health Sciences Research Ethics Board may contact you or require access to your study-related records to monitor the conduct of the research.

Qualified representatives of the Lawson Quality Assurance Education Program may look at your medical/clinical study records at the site where these records are held, for quality assurance (to check that the information collected for the study is correct and follows proper laws and guidelines).

London Health Sciences Centre

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Synovial Fluid Composition of Patients Undergoing Total Knee Arthroplasty for Severe Osteoarthritis

Principal Investigator: Dr. Edward Vasarhelyi

Informed Consent Form

Agreement of Participating Subject

I have read the accompanying letter of information, have had the nature of the study explained to me and I agree to participate. All questions have been answered to my satisfaction.

_____

Print Participant's Full Name

_____

Participant's Signature Date

_____

Name of Person Obtaining Consent

_____

Signature of Person Obtaining Consent Date

☐ The person signing below acted as a translator or witness for the participant during the consent process and attests that the study as set out in this form was accurately translated/communicated and has had any questions answered.

_____ _____ _____

Print Name of Translator/Witness Signature Date

_____

Language

Chapter 8

Curriculum Vitae

Name: Fernando Diaz Dilernia

Post-secondary Education and Degrees:

- Undergraduate medical education, Medical Doctor

  School of Medicine, Buenos Aires University, Buenos Aires, Argentina

  03/2005 – 03/2013

- Residency training, Orthopedics and Traumatology

  Italian Hospital of Buenos Aires, Buenos Aires, Argentina

  06/2013 – 05/2019

    - Certified Orthopedic Surgeon, Buenos Aires, Argentina
        - Italian Hospital of Buenos Aires, 05/31/2018
        - National Health Department, 07/27/2018
        - Argentinian Association of Orthopedics and Traumatology, 11/16/2019

- Adult Hip Reconstructive and Preservation Surgery Clinical Fellowship

  Italian Hospital of Buenos Aires, Buenos Aires, Argentina

  06/2019 – 05/2020

    - Certified Hip Surgeon, Italian Hospital of Buenos Aires, 05/31/2020

- Adult Hip and Knee Reconstructive Surgery Clinical Fellowship

  Division of Orthopedic Surgery, Department of Surgery, Schulich School of Medicine & Dentistry, Western University, London, Ontario, Canada

  08/2020 – Present

- Western University student, MSc in Surgery Program

  Schulich School of Medicine & Dentistry, Western University, London, Ontario, Canada

  09/2020 – Present

Honours and Awards:

- Resident's Research Award. Nally F, Rossi L, **Diaz Dilernia F**, Stagnaro J, Slullitel PA. Which prosthetic system restores hip biomechanics more effectively? Comparison among three systems. Current Orthopedic Practice. July 2014
- Best Poster Presentation. Nally F, Rossi L, **Diaz Dilernia F**, Stagnaro J, Slullitel PA, Zanotti G, Comba F, Buttaro M, Piccaluga F. Which prosthetic system restores hip biomechanics more effectively? Comparison among three systems. 10th Annual Meeting of the Latin-Americans Hip and Knee Surgeons. Cartagena, Colombia. August 2014
- Best Resident's Podium Presentation. Nally F, Rossi L, **Diaz Dilernia F**, Stagnaro J, Slullitel PA. Which prosthetic system restores hip biomechanics more effectively? Comparison among three systems. 51st Annual Congress of the Argentinian Association of Orthopedics and Traumatology. Buenos Aires, Argentina. December 2014
- Best Poster Presentation. **Diaz Dilernia F**, Zaidenberg E, Gallucci G, De Carli P, Boretto J. Foreign-body reaction and osteolysis in dorsal lunate dislocation repair with bioabsorbable suture anchor. 41st Annual Congress of the Argentinian Association of Hand Surgery. Buenos Aires, Argentina. October 2015
- Best Podium Presentation. **Diaz Dilernia F**, Estefan M, Gallucci G, De Carli P, Boretto J. Results and complications of the vascularized fibular graft in segmental

bone defects in the upper limb. 41st Annual Congress of the Argentinian Association of Hand Surgery. Buenos Aires, Argentina. October 2015

- Best Resident's Podium Presentation. Slullitel PA, Stagnaro J, **Diaz Dilernia F**, Zaidenberg E, Camino G, Piuzzi N, Revah M. Comparison of clinical and radiological outcomes between total ankle arthroplasty and arthrodesis for the treatment of post-traumatic ankle osteoarthritis. 52nd Annual Congress of the Argentinian Association of Orthopedics and Traumatology. Buenos Aires, Argentina. December 2015
- Best Podium Presentation. Ranalletta M, Rossi LA, Sirio A, **Diaz Dilernia F**, Bertona A, Maignon GD, Bongiovanni SL. Return to Sports and Recurrences After Arthroscopic Anterior Shoulder Stabilization in Martial Arts Athletes. 2nd Annual Congress of the Argentinian Association of Arthroscopy and 2nd Latin-American Arthroscopy, Knee and Sports Association. Buenos Aires, Argentina. June 2016
- Best Podium Presentation. Nally F, Zanotti G, Buttaro M, **Diaz Dilernia F**, Garcia Mansilla I, Comba F, Piccaluga F. Osteonecrosis of the femoral head: Average 5.5-year Results Comparing Core Decompression, adding Autologous Bone Graft or Stem Cells. 12th Annual Meeting of the Latin-Americans Hip and Knee Surgeons. Cartagena, Colombia. August 2016
- Best Poster Presentation. Llano L, **Diaz Dilernia F**, Buttaro MA, Comba F, Zanotti G, Piccaluga F. Unaddressed arterial injuries in revision total hip arthroplasty: mortality outcomes of a low-prevalence complication. 55th Annual Congress of the Argentinian Association of Orthopedics and Traumatology. Rosario, Santa Fe, Argentina. December 2018

- Resident's Research Award. Second Place. **Diaz Dilernia F**, García Mansilla A, Llano L, Buljubasich M, Oñativia JI, Buttaro M. Second Place Award: Residents or hip surgeons for the treatment of displaced femoral neck fractures. A 10-years survivorship rate analysis. Current Orthopedic Practice. July/August 2019
- Special Mention, Podium Presentation. **Diaz Dilernia F**, Garcia Mansilla A, Llano L, Buljubasich M, Oñativia JI, Zanotti G, Comba F, Piccaluga F, Buttaro M. Who restores hip biomechanics more effectively after a femoral neck fracture? Comparison of total hip arthroplasties performed either by hip surgeons or orthopedic residents. Podium presentation. 25th Annual Congress of the Argentinian Association for the Study of the Hip and Knee. Buenos Aires, Argentina. September 2019
- Best Podium Presentation. **Diaz Dilernia F**, Garcia Mansilla A, Albani A, Zanotti G, Comba F, Piccaluga F, Buttaro M. Initial results of a metaphyseal taper fit cementless femoral stem: a minimum 2-years follow- up. Podium presentation. 25th Annual Congress of the Argentinian Association for the Study of the Hip and Knee. Buenos Aires, Argentina. September 2019

Related Work Experience:

- Assistant Professorship, Department of Pathology
  School of Medicine, Buenos Aires University, Buenos Aires, Argentina
  2009 – 2012
- Orthopaedics and Traumatology Residency
  Italian Hospital of Buenos Aires, Buenos Aires, Argentina
  06/2013 – 05/2019
- Adult Hip Reconstructive and Preservation Surgery Clinical Fellowship

Italian Hospital of Buenos Aires, Buenos Aires, Argentina

06/2019 – 05/2020

- Adult Hip and Knee Reconstructive Surgery Clinical Fellowship

  Division of Orthopedic Surgery, Department of Surgery, Schulich School of Medicine & Dentistry, Western University, London, Ontario, Canada

  08/2020 – Present

- Reviewer
  - The Bone & Joint Journal (BJJ), 2019 – Present
  - Journal of Orthopedic Surgery and Research (JOSR), 2019 – Present
  - Journal of Hip Surgery (JHS), 2020 – Present
  - Arthroplasty Today (ARTD), 2020 – Present
  - BMJ Case Reports (BMJ), 2020 – Present

Publications:

1. Nally F, Rossi L, **Diaz Dilernia F**, Stagnaro J, Slullitel PA, Buttaro M. Which prosthetic system restores hip biomechanics more effectively? Comparison among three systems. Current Orthopaedic Practice. July/August 2015 - Volume 26 - Issue 4 - p 382–386. DOI: 10.1097/BCO.0000000000000242. Resident Research Award
2. **Diaz Dilernia F**, Zaidenberg EE, Gamsie S, Taype Zamboni DE, Carabelli GS, Barla JD, Sancineto CF. Gluteal Compartment Syndrome Secondary to Pelvic Trauma. Case Rep Orthop. 2016;2016:2780295. doi: 10.1155/2016/2780295. Epub 2016 Aug 8.
3. **Diaz Dilernia F**, Fresneda M, Deré JJ, Zicaro JP, Costa Paz M, Yacuzzi C. [The International Society of Arthroscopy, Knee Surgery and Orthopaedic Sports Medicine (ISAKOS) classification for meniscal tears: An intra and interobserver reliability

analysis]. [Article in Spanish] Revista de la Asociación Argentina de Traumatología del Deporte. 2016. Volumen: 23, Número 1. ISSN 0329-0301

4. Slullitel PA, Camino Willhuber G, Piuzzi NS, Stagnaro J, **Diaz Dilernia F**, Revah M, Zaidenberg E, Santini Araujo G, Sotelano P, Carrasco M. [Comparison of clinical and radiological outcomes between total ankle arthroplasty and arthrodesis for the treatment of post-traumatic ankle osteoarthritis]. [Article in Spanish] Revista Latino Americana de Cirugía Ortopédica. March 2017. DOI: 10.1016/j.rslaot.2017.02.005
5. Estefan M, **Diaz Dilernia F**, Gallucci G, De Carli P, Boretto J. [Early experience in a High Complexity Hospital with the vascularized fibular graft in segmental bone defects of the upper limb]. [Article in Spanish] Revista de la Asociación Argentina de Ortopedia y Traumatología. July 2017. DOI: 10.15417/598
6. Ranalletta M, Rossi LA, Sirio A, **Diaz Dilernia F**, Bertona A, Maignon GD, Bongiovanni SL. Return to Sports and Recurrences After Arthroscopic Anterior Shoulder Stabilization in Martial Arts Athletes. Orthop J Sports Med. 2017 Sep 13;5(9):2325967117725031. doi: 10.1177/2325967117725031. eCollection 2017 Sep.
7. Oñativia IJ, Slullitel PA, **Diaz Dilernia F**, Gonzales Viezcas JM, Vietto V, Ramkumar PN, Buttaro MA, Piuzzi NS. Outcomes of nondisplaced intracapsular femoral neck fractures with internal screw fixation in elderly patients: a systematic review. Hip Int. 2018 Jan;28(1):18-28. doi: 10.5301/hipint.5000532.
8. Nally FJ, Zanotti G, Buttaro MA, **Diaz Dilernia F**, Mansilla IG, Comba FM, Piccaluga F. THA conversion rate comparing decompression alone, with autologous bone graft or stem cells in osteonecrosis. Hip Int. 2018 Mar;28(2):189-193. doi: 10.5301/hipint.5000552. Epub 2017 Sep 10.

9. Slullitel PAI, **Diaz Dilernia F**, Stagnaro J, Revah M, Rojas L, Posadas-Martinez ML, Buttaro MA, Slullitel GA. Are there any risk factors for developing complications with the use of retrievable vena cava filters in orthopedic surgery? Rev Fac Cien Med Univ Nac Cordoba. 2018 Jun 11;75(2):119-127. doi: 10.31053/1853.0605.v75.n2.17746.

10. Oñativia JI, Slullitel PA, García Mansilla A, **Diaz Dilernia F**, Buttaro M, Comba F. Is hip arthroscopy useful for the treatment of borderline dysplasia? A case-controlled study. Orthopaedic Journal of Sports Medicine. 2018 Dec; 6(12 suppl5): 2325967118S00205. Published online 2018 Dec 28. DOI: 10.1177/2325967118S00205 (Abstract)

11. Slullitel PA, Llano L, García-Ávila C, **Diaz Dilernia F**, Piccaluga F, Buttaro M, Zanotti G, Comba F. Unaddressed arterial injuries in revision total hip arthroplasty: mortality outcomes of a low-prevalence complication. Int Orthop. 2020 Jan;44(1):23-29. doi: 10.1007/s00264-019-04358-2. Epub 2019 Jun 20.

12. Llano L, **Diaz Dilernia F**, Taype D, Sancineto C, Barla J, Carabelli G. Cement leakage into the hip joint during TFN-A cement augmentation in revision surgery of an extra-capsular hip fracture. Trauma Case Rep. 2019 Jun 20;22:100212. doi: 10.1016/j.tcr.2019.100212. eCollection 2019 Aug.

13. **Diaz Dilernia F**, Costantini J, Nicolino TI, Sanchez MDL, Carbo L. Unusual Listeria monocytogenes hematogenous infection in total knee replacement treated with one-stage revision surgery. Arthroplast Today. 2019 Jul 22;5(3):296-300. doi: 10.1016/j.artd.2019.06.005. eCollection 2019 Sep.

14. **Diaz Dilernia F**, García Mansilla A, Llano L, Buljubasich M, Oñativia JI, Buttaro M. Second Place Award: Residents or hip surgeons for the treatment of displaced femoral neck fractures. A 10-years survivorship rate analysis. Current Orthopaedic Practice. July/August 2019. Volume 30. Number 4. p 296–03. DOI: 10.1097/bco.0000000000000780. Resident Research Award.

15. **Diaz Dilernia F**, Slullitel PA, Oñativia JI, Comba FM, Piccaluga F, Buttaro MA. Impaction Bone Grafting or Uncemented Modular Stems for the Treatment of Type B3 Periprosthetic Femoral Fractures? A Complication Rate Analysis. J Arthroplasty. 2019 Sep;34(9):2051-2057. doi: 10.1016/j.arth.2019.04.047. Epub 2019 May 14.

16. **Díaz Dilernia F**, Estefan M, Zanotti, G, Comba, F, Piccaluga F, Buttaro M. Simultaneous Bilateral Femoral Neck Fracture Due to a Tonic-Clonic Seizure and High-Dose Steroid Therapy. Arthroplast Today. 2020 Jul 14;6(3):513-516. doi: 10.1016/j.artd.2020.05.013. eCollection 2020 Sep.

17. Slullitel PA, Oñativia JI, García Mansilla A, **Diaz Dilernia F**, Buttaro M, Zanotti G, Piccaluga F, Comba F. Is hip arthroscopy useful in the treatment of borderline dysplasia? a case-control study. [Article in English, Spanish]. Rev Esp Cir Ortop Traumatol. Sep-Oct 2020;64(5):326-334. doi: 10.1016/j.recot.2020.04.006. Epub 2020 Aug 10.

18. **Diaz Dilernia F**, García Mansilla A, Llano L, Buljubasich M, Oñativia JI, Slullitel PA, Zanotti G, Comba F, Piccaluga F, Buttaro M. Who Restores Hip Biomechanics More Effectively after a Femoral Neck Fracture? Comparison of Total Hip Arthroplasties Performed by Either Hip Surgeons or Orthopaedic Residents.

Arthroplast Today. 2020 Aug 29;6(4):736-741. doi: 10.1016/j.artd.2020.07.027. eCollection 2020 Dec.

19. Godoy-Monzon D, **Diaz Dilernia F**, Piccaluga F, Cid Casteulani A, Turus L, Buttaro M. Conversion total hip arthroplasty with a proximally modular, distal fixation reconstruction prosthesis following cephalomedullar nail failure. Hip Int. 2020 Sep;30(1_suppl):26-33. doi: 10.1177/1120700020937952.

20. Latorre MR, Sánchez Saba JE, Abrego MO, **Díaz Dilernia F**, De Cicco FL, Bilbao F. [Pure Medial Subtalar Dislocation: Case Report and Literature Review]. [Article in Spanish]. Rev Asoc Argent Ortop Traumatol. Feb 2021;86(1):83-90. doi: 10.15417/issn.1852-7434.2021.86.1.1099

21. **Diaz Dilernia F**, García Mansilla A, Llano L, Buljubasich M, Oñativia JI, Slullitel PA, Zanotti G, Comba F, Piccaluga F, Buttaro M. Response to Letter to the Editor, "Who Restores Hip Biomechanics More Effectively After a Femoral Neck Fracture? Comparison of Total Hip Arthroplasties Performed Either by Hip Surgeons or Orthopedic Residents". Arthroplast Today. 2021 Feb 3;7:208. doi: 10.1016/j.artd.2020.12.013. eCollection 2021 Feb.

22. Lucero CM, **Díaz Dilernia F**, Zanotti G, Comba F, Piccaluga F, Buttaro M. Rapidly progressive osteoarthritis of the hip secondary to a subchondral insufficiency fracture of the acetabulum: A case report. Rev Asoc Argent Ortop Traumatol 2021;86(2):XXXX. doi: 10.15417/issn.1852-7434.2021.86.2.1090

23. Larrague C, Campelo D, **Díaz Dilernia F**, Bosio S, Maenza R, Puigdevall M. [Isolated elbow dislocation in pediatric patients: non-operative treatment and complications associated with an infrequent pathology. Series of 4 cases]. [Article in

Spanish]. Arch Argent Pediatr. 2021 Apr;119(2):e133-e137. doi: 10.5546/aap.2021.e133.

24. De Cicco FL, Llano L, **Diaz Dilernia F**, Carabelli G, Taype D, Barla J, Sancineto C. [Intramedullary nail for the surgical treatment of unstable fractures of the femur in previously amputated patients]. [Article in Spanish]. Rev Fac Cien Med Univ Nac Cordoba. 2021 Mar 12;78(1):57-63. doi: 10.31053/1853.0605.v78.n1.28020.

25. Novillo M, **Díaz Dilernia F**, García Barreiro G, Posadas-Martinez ML, Comba F, Buttaro M. [Are lateral view radiographs necessary to properly classify femoral neck fractures? Intra and interobserver analysis using Garden's classification system]. [Article in Spanish]. Rev Fac Cien Med Univ Nac Cordoba. 2021 Mar 29;78(1):41-44. doi: 10.31053/1853.0605.v78.n1.30732

26. **Díaz-Dilernia F**, García-Mansilla AM, Albani-Forneris A, Slullitel PA, Zanotti G, Comba F, Piccaluga F, Buttaro M. Preliminary outcomes of the cementless UNITED hip system for primary total hip arthroplasty at a minimum 2-year follow-up. Eur J Orthop Surg Traumatol. 2021 Jun 12. doi: 10.1007/s00590-021-03038-5.