On the pathogenesis of infections associated with percutaneous osseointegrated orthopaedic implants

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Cover illustration*: Staphylococcus epidermidis* biofilm, image by Furqan A. Shah.

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Till mamma och pappa

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ABSTRACT

Orthopaedic implants enable the restitution of locomotor function and improve the quality of life of many people. However, biomaterial-associated infection may occur due to the propensity of microorganisms to adhere and colonize implant surfaces. The objective was to gain knowledge on the pathogenesis of infections associated with percutaneous osseointegrated implants for lower limb amputation prostheses. The aims were to design *in vitro* methods for the evaluation of antimicrobial surface properties, evaluate a novel method for biofilm-susceptibility testing and characterising virulence factors in bacterial isolates from patients with implant-associated osteomyelitis, and to investigate extracellular vesicle (EV)-host cell and EV-bacterial cell interactions.

Results demonstrated that several methods, tailored to the specific surface modification and antimicrobial mode of action, should be applied to provide complementary information when evaluating the prophylactic and treatment effects of antimicrobial surfaces on planktonic and biofilm bacteria. The majority of clinical isolates of *Staphylococcus* spp. and *Enterococcus* spp. causing osteomyelitis were biofilm producers that required higher antimicrobial concentrations compared with non-producers. The biofilm susceptibility testing method may be useful to guide antimicrobial treatment decisions in orthopaedic implant-associated infection. All staphylococcal strains were able to produce EVs *in vitro*. A significantly higher level of cytotoxicity was induced in THP-1 monocytes by EVs compared with unstimulated controls. THP-1 cells internalised EVs and secreted proinflammatory cytokines to a greater degree than controls. Sub-inhibitory concentrations of gentamycin increased secretion of EVs and their protein content in *S. epidermidis*. EVs may play a role as survival factors by modulating cell growth and adherence to surfaces.

In conclusion, isolates from implant-associated infection reveal multiple virulence traits relevant for understanding and treating these infections. This thesis proposes EVs as a novel pathogenic mechanism of biomaterial-associated infection, requiring further research focus.

Keywords: osseointegration, amputation prosthesis, implant-associated infection, biofilm, staphylococci, extracellular vesicles, host defence, cytokines, cell death

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POPULÄRVETENSKAPLIG SAMMANFATTNING

Ortopediska implantat används i allt större utsträckning och har förbättrat livskvalitén för många människor med skador och sjukdomar i skelett, leder och muskulatur. Infektion i anslutning till insatta implantat utgör en allvarlig komplikation. En bidragande orsak till detta är att den främmande ytan möjliggör att bakterier under vissa förhållanden kan slå sig ner på ytan, föröka sig och bilda en så kallad biofilm. En motverkande kraft är om implantatet eller protesen integreras i vävnaden. Exempel på detta är om implantatet osseointegreras dvs växer samman med skelettet. Behandlingen med hudpenetrerande benförankrade proteser för lårbensamputerade har goda kliniska resultat med förbättrad rörlighet och livskvalitet. Tyvärr drabbas dessa patienter ibland av infektion i anslutning till den delen av implantatet som sitter i lårbenet. Mekanismer för hur sådan infektion uppkommer är inte kända. Det övergripande syftet med avhandlingen var att studera olika aspekter bakom sådan infektion. Delmålen var att designa *in vitro* metoder för utvärdering av antimikrobiella implantatytor, utvärdera en ny kombination av metoder för att mäta biofilmers motståndskraft mot antibiotika, karakterisera bakteriestammar isolerade från implantatrelaterad beninfektion med avseende på olika virulensfaktorer samt att undersöka interaktioner mellan extracellulära vesiklar (EVs) frisatta från stafylokocker och kroppens försvarsceller samt mellan EVs och bakterier.

Resultaten visar att vid utvärdering av antimikrobiella ytor bör flera testmetoder appliceras i syfte att få komplementär information. Kliniska isolat av Stafylokocker och Enterokocker visade olika grad av biofilmproduktionsförmåga *in vitro* med kraftigt förhöjd motståndskraft mot antibiotika. Vidare kunde alla Stafylokocker bilda EVs *in vitro* och en ökning av celldöd av THP-1 celler kunde påvisas när de behandlades med EVs jämfört med kontroll. EVs aktiverade THP-1 cellerna genom NF-κB och internaliserades i en del av cellerna. THP-1 cellerna utsöndrade proinflammatoriska cytokiner i större utsträckning jämfört med kontrollförhållanden. EVs utsöndrades av *S. epidermidis* under antibiotikapåverkan vilket ändrade dess proteinmängdsinnehåll och storlek. Dessutom visades att EVs påverkar bakterietillväxt och vidhäftande till ytor.

Sammanfattningsvis visar avhandlingen att bakterieisolat från djupa implantatassocierade infektioner uppvisar flera virulensegenskaper, inklusive biofilmsbildande förmåga, motståndskraft mot antibiotika samt frisättning av EVs som i sin tur påverkar bakteriers adhesion och tillväxt samt försvarscellers frisättning av cytokiner och celldöd. Denna kunskap kan användas i arbetet att förebygga, diagnostisera och behandla implantatrelaterade infektioner.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Zaborowska M, Welch K, Brånemark R, Khalilpour P, Engqvist H, Thomsen P, Trobos M. Bacteria-material surface interactions: methodological development for the assessment of implant surface induced antibacterial effects. *Journal of biomedical materials research. Part B, Applied biomaterials*, 2015; 103(1): 179-187.

II. Zaborowska M*, Tillander J*, Brånemark R, Hagberg L, Thomsen P, Trobos M. Biofilm formation and antimicrobial susceptibility of staphylococci and enterococci from osteomyelitis associated with percutaneous orthopaedic implants. *Journal of biomedical materials research. Part B, Applied biomaterials*, 2017; 105B(8): 2630-2640. * *Equal contribution.*

III. Zaborowska M, Vazirisani F, Shah FA, Omar O. Ekström K, Trobos M, Thomsen P. Extracellular vesicles from *S. epidermidis* and *S. aureus* isolated from bone-anchored prostheses induce cytolysis and proinflammatory cytokine secretion. *In manuscript.*

IV. Zaborowska M*, Taulé Flores C*, Vazirisani F, Thomsen P, Trobos M.
Role of extracellular vesicles from *Staphylococcus epidermidis* on antibiotic tolerance, planktonic growth, and biofilm formation under antimicrobial selective pressure. *In manuscript.* * *Equal contribution.*

CONTENTS

ABBREVIATIONS

AtlE	Autolysin E
AMP	Ampicillin
AMPs	Antimicrobial peptides
ANOVA	Analysis of variance
BAI	Biomaterial-associated infections
CBD	Calgary biofilm device
CFU	Colony-forming units
CIP	Ciprofloxacin
ClfA	Clumping factor A
CLI	Clindamycin
CLSM	Confocal laser scanning microscopy
CoNS	coagulase-negative staphylococci
CRP	C-reactive protein
ELISA	Enzyme-linked immunosorbent assay
Embp	Extracellular matrix-binding protein
EPS	Extracellular polymeric substance
ESR	Erythrocyte sedimentation rate
FA	Fusidic acid
FBGC	Foreign body giant cells
GEN	Gentamicin
IFN-γ	Interferon-gamma
IL	Interleukin
ILP	Integral leg prosthesis
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
LukAB	Leukotoxin AB
LZD	Linezolid
MALDI-TOF MS	Matrix assisted laser desorption/ionisation time-of-flight mass spectrometry
MBEC	Minimum biofilm eradication concentrations
MCP-1	Monocyte chemoattractant protein-1
MHB	Mueller Hinton broth
MIC	Minimum inhibitory concentration
MMP-9	Matrix metallopeptidase 9
MSCRAMM	Microbial surface components recognizing adhesive matrix molecule
NF-κB	Nuclear factor-kappa B

NTA	Nanoparticle tracking analysis
OD	Optical density
OPL	Osseointegrated prosthetic limp
OPRA	Osseointegrated Prostheses for the Rehabilitation of Amputees
OXA	Oxacillin
PAMP	Pathogen-associated molecular patterns
PBP	Penicillin-binding protein
PBS	Phosphate buffered saline
PGA	poly-γglutamic acid
PIA	Polysaccharide intercellular adhesin
PJI	periprosthetic joint infection
PMN	Polymorphonuclear cells
RGD	Arginine-glycine-aspartic acid
RPMI	Rosewell Park Memorial Institute
PRR	Pattern-recognition receptor
PSM	Phenol-soluble modulin
qPCR	Quantitative real-time polymerase chain reaction
RIF	Rifampin
SEAP	Secreted embryonic alkaline phosphatase
SEM	Scanning electron microscopy
SesC	fibrinogen-bonding protein
SdrG	(Fbe) fibrinogen binding protein
SXT	Trimethoprim/sulfamethoxazole
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor α
TSB	Tryptic soy broth
VAN	Vancomycin
WBC	White blood cell

1 INTRODUCTION

1.1 Orthopaedic implants

Implanted medical devices have revolutionised the treatment of musculoskeletal disorders. Today, approximately one million total-hip and total-knee replacement procedures are performed each year in the United States.[2] In Sweden, in 2017, 18,148 total-hip replacements and 14,976 primary knee replacements were performed, increasing the numbers from the preceding year.[3,4] Globally, the number of orthopaedic implants placed every year will continue to increase due to an increasingly ageing population, improved implant technology and improved surgical techniques.[5] The challenging and emerging part of implanting foreign materials are adverse tissue reactions and infections. These challenges force the research field of constantly improving different aspects of the implants such as choice of materials and surface properties. Other important aspects are preventive measures against infections since implanted foreign materials are more susceptible to bacterial colonisation due to locally compromised host defence. Rapid detection of implant associated infections is crucial because delaying treatment may result in implant loss.

1.2 Osseointegration

The ability of an implant to integrate with bone is called osseointegration. The discovery of osseointegration was made by P. I. Brånemark in 1952, when a titanium chamber was used in an *in vivo* rabbit model of bone marrow circulation.[6] The integration of an implant in the bone tissue provides biomechanical stability and enables load bearing. The dental implant was the first application of osseointegration and it has been used successfully in clinical practice for more than 40 years.[7-9] Other applications based on osseointegration include bone-anchored hearing aids,[10,11] craniofacial prostheses[12] and bone-anchored percutaneous implants for amputation treatment.

1.3 Percutaneous orthopaedic implants

Based on a technology similar to that for dental implants, the bone-anchored percutaneous implant for amputation treatment was introduced at Sahlgrenska University Hospital in the 1990s. The treatment protocol, OPRA (Osseointegrated Prostheses for the Rehabilitation of Amputees), was established

in 1999 by Rickard Brånemark and co-workers (Figure 1).[13] It consists of two separate surgical procedures. In the first surgery, the bone-implanted part, the titanium fixture, is inserted into the marrow cavity in the residual bone and the skin is re-sealed. Six months of healing without loading follow allow the fixture to integrate with bone tissue. During the second surgery, distal muscles are cut and sutured to the periosteum, leaving the protruded bone covered by a skin flap (trimmed of subcutaneous fat), which is attached to the end of the bone. The abutment is inserted through the skin, press-fit inserted into the fixture and secured with an abutment screw. After the second surgery, the rehabilitation entails a gradual increase of the load on the implant. Thereafter, the abutment provides an attachment site for an external prosthesis.

The prerequisite for the long-term function of the implant is osseointegration with no fibrous tissue encapsulation. The osseointegration of the implant prevents micro-motion and wear particle debris that may lead to implant loosening.[14] Follow-up studies reveal advantages in daily life offered by the OPRA system compared with a conventional socket prosthesis.[13,15,16] The conventional method of attaching the prosthesis to the limb is via a socket, which suspends the prosthesis from the stump by compressing soft tissue. Discomfort due to the socket, experienced as sores, rashes and pain, and the unreliability of prostheses being securely suspended have been reported in several studies.[17-21]

Figure 1: Overview of the OPRA system (Integrum AB©)

Apart from the OPRA system, two other percutaneous implant systems are clinically and commercially available: ILP (Integral Leg Prosthesis) and OPL (Osseointegrated Prosthetic Limb). These two systems were developed in

Germany and Australia respectively. The ILP is a chromium-cobalt-molybdenum alloy implant that is inserted in bone with press fit.[22] The implanted part is microporous and resembles cancellous bone to facilitate osseointegration. The external part is coated with titanium-niobium oxide to reduce soft-tissue adhesion. The OPL has two standard designs; one with an extramedullary head and one with an intramedullary head. The surface is a plasma-sprayed rough titanium coating where osseointegration is desired.[23,24]

1.4 Wound healing

Placing an implant, in soft tissue, for example, requires surgical implantation that causes tissue injury which involves cell death, the destruction of extracellular connective tissue components and the loss of blood vessel integrity.[25] Instantaneously, the process of wound healing begins; it consists of four overlapping series of events: haemostasis, inflammation, proliferation and remodelling.[26,27]

Platelets from damaged blood vessels come into contact with and adhere to collagen fibres that are exposed due to the tissue injury. Platelets are activated and this triggers the degranulation of platelets that release cytokines, growth factors and clotting factors. The coagulation cascade takes place, resulting in platelet aggregation and fibrin clot formation at the injury site that serves as a temporary barrier. The blood clot that forms re-establishes haemostasis by protecting the exposed wound site and provides a matrix for inflammatory and other cells to attach to during the wound healing process.

The growth factors and cytokines initially released by the platelets recruit inflammatory cells, such as neutrophils and monocytes, to the wound site. Neutrophils migrate through the blood vessel and are one of the first inflammatory cells to arrive at the site, with the mission of phagocytosing microorganisms and foreign particles. When their task is completed (typically within hours to days), the neutrophils undergo apoptosis. Monocytes are attracted to the wound and become activated macrophages that will release various cytokines and growth factors which initiate the formation of granulation tissue and they phagocytose the apoptotic neutrophils. Macrophages are the key cells in the transition from inflammation to repair and their presence is an indication that the proliferation phase has been initiated. Fibroblasts are now recruited and they synthesise, deposit and organise the new extracellular matrix.

The provisional wound matrix is replaced by granulation tissue consisting of new collagen fibres, other components of the extracellular matrix, macrophages, fibroblasts and blood vessels. The newly formed blood vessels are vital when it comes to sustaining the granulation tissue. Wound contraction is the complex interaction of cells, extracellular matrix and cytokines. Once the granulation tissue is formed, some fibroblasts transform into myofibroblasts and contract the wound. Collagen is continuously synthesised and catabolised at low rates, which leads to a shift from granulation tissue to scar tissue. Tissue degradation is controlled by proteolytic enzymes, such as matrix metalloproteins secreted by macrophages, epidermal cells, endothelial cells and fibroblasts.[25] The scar tissue will never regain the initial tensile strength from before; only about 70% of the strength is regained.[27]

1.4.1 Foreign body reaction

Injury, blood-material interactions, formation of provisional matrix, acute inflammation, chronic inflammation, granulation tissue formation, foreign body reaction, and fibrous tissue encapsulation are host reactions following implantation of biomaterials.[28] At the very moment of implantation, biomaterials are coated with host plasma proteins (predominantly fibrinogen and fibronectin); this coating is called the conditioning film. This conditioning film can be seen as the provisional matrix formation. Its composition is dependent on the physicochemical properties of the material and may influence inflammatory cell recruitment and subsequent adhesion to the material. The acute inflammation begins as in regular wound healing; macrophages attempt to phagocytose the foreign material. The macrophages begin to fuse and form multinucleated foreign body giant cells (FBGC) in an attempt to phagocytose the material. Foreign body giant cells, together with granulation tissue and new capillaries, are referred to as a foreign body reaction, which is the end stage of wound healing in contact with a biomaterial and distinguishes the healing process from the common wound healing process. The foreign body reaction results in fibrous tissue formation that encapsulates the implant.[28] At this stage, the macrophages and FBGCs have reduced bactericidal activity, as the cells are exhausted by trying to engulf the foreign body, which is a sign of chronic inflammation.

1.4.2 Skin and skin flora

The primary role of the skin is to act as the first line of defence and it serves as a physical barrier to prevent the entry of pathogens and foreign substances. The skin shields internal organs from trauma and provides protection from ultraviolet

irradiation when intact.[29] It is colonised by commensal bacteria that work in symbiosis with the skin. The composition of the microbial flora depends on different factors, such as age, gender, environmental conditions and location.[29] *Staphylococcus epidermidis* and other coagulase-negative staphylococci are the most abundant microbial skin colonisers. Other microorganisms present are *Corynebacterium*, *Propionibacterium* and *Brevibacterium,* as well as different fungal species.[29,30]

The skin has to be intact in order to act as a barrier. If the skin is damaged, the immediate process of wound healing and closure begins. The permanent breaching of the skin may cause the down-growth of the epithelium,[31] the keratinisation of the epidermis and the presence of a granulation ring[32] and there is constant ongoing inflammation at the site. Percutaneous orthopaedic implants breaching the skin introduce a potential pathway for microorganisms to enter the body in the junction between tissue, implant and the external environment. Loading of the system creates micro-motions between the skin and abutment which may contribute to the formation of the granulation ring.[33]

1.5 Implant-associated infections

Implant-associated infections are one of the main causes of the failure of implanted devices and account for at least 50% of all health care-associated infections.[34] In addition, these infections are difficult to diagnose and treat.[35] The infection may occur at different time points postoperatively: early (≤3 months), delayed (3-24 months), or late infection (>24 months).[36] Early infections are often initiated during surgery, whereas late infections usually have an haematogenous origin. For acute infections, the causative organism is often a virulent microorganism such as *Staphylococcus aureus*. The ability to prime oxidative response and influence apoptosis in neutrophils differed between *S. aureus* and *S. epidermidis*.[37] *Staphylococcus aureus* primed oxidative response and induced apoptosis, whereas *S. epidermidis* did not and even protected neutrophils against apoptosis. In addition, limited induced inflammatory response was observed when *S. epidermidis* adhered to surface which suggesting that *S. epidermidis* is involved in less acute clinical situation involving an implant surface.[38] Infection occurs when a bacterial inoculum reaches critical size and overcomes the local host defence. Delayed or late infections are usually caused by less virulent, opportunistic microorganisms such as *Staphylococcus epidermidis* and *Propionibacterium acnes*. The clinical signs and symptoms are implant loosening and persistent pain.

It may be challenging to distinguish between aseptic implant loosening of an implant from septic loosening caused by a low-virulent microorganism such as *S. epidermidis*.[39] The symptoms are similar, it may be hard to culture the bacteria and, there is a risk of contaminations of commensal microbes when sampling.[40] Histopathological analysis of Polymorphonuclear cells (PMN) infiltration in periprosthetic tissue was concluded to be the best method to distinguish between aseptic and septic loosening of hip prostheses.[41] In Sweden, during 1999 - 2017, revisions due to aseptic loosening of total-hip prostheses accounted for 57.5%.[3]

1.5.1 Osteomyelitis

Osteomyelitis can be described as microorganisms colonising bone tissue in association with inflammation and bone destruction.[42] The features of osteomyelitis, such as occurrence, type, severity and clinical prognosis, depend on the pathogen and its virulence, as well as the properties of the host.[43] Damage to the bone matrix and the destruction of the vasculature are observed as the infection spreads to surrounding soft tissues. Sequestra, sections of dead bone, may form and detach to form separate infectious islands. Due to the lack of vasculature, the sequestra are protected from immune cells and antimicrobials and this may lead to the chronic persistence of the infection.[44,45] The presence of an implant can cause chronic osteomyelitis, which often leads to the removal of the implant. There are several host cytokines that are important in the pathogenesis of osteomyelitis that are induced by staphylococcal infection in bone. The main inflammatory cytokines involved are tumour necrosis factor α (TNF-α), interleukin 1β (IL-1β) and interleukin 6 (IL-6). These cytokines play an important role in bone remodelling. The three cytokines stimulate the proliferation and differentiation of osteoclast progenitor cells to mature osteoclasts and they stimulate bone resorption.[44] The bacteria themselves also interact directly with bone cells. The internalisation of *S. aureus* in osteoblasts contributes to the pathogenesis of osteomyelitis, since the internalisation provides protection for the microorganisms from the host defence, as well as antimicrobial agents. In addition, there have been reports of intracellular persistence via the formation of small colony variant phenotypes that form due to adaptation and contribute to the persistence of antimicrobial tratement.[46] Ten years after implantation, the prevalence of osteomyelitis associated with percutaneous orthopaedic implants is 20%.[47] The two-year risk of implant-related osteomyelitis and implant removal due to septic causes in these femoral osseointegration patients is approximately 8% and 2%, respectively.[13]

1.5.2 Race for the surface

The presence of a foreign body causes the local depletion of the immune defence and lowers the threshold for microbial infection (it requires an at least 10,000 times lower infectious dose of the microorganism).[48] The instantaneous coating of an implant surface by host proteins provides an optimal substrate for microbial adherence and this is thought to be a critical factor for the development of implant infection. Bacterial adherence can be divided into two stages; primary unspecific reversible attachment and specific irreversible attachment.[49] The physicochemical properties, atomic structure and composition of the surface play an important role in determining which plasma proteins adhere to the surface and eventually which, the host cells or bacteria, will be able to adhere first and win the race for the surface colonising the implant.[49]

1.5.3 Diagnosis of infection and identification of causative organism

There is not one test that provides a full picture of the infection related to an implant. Instead, different clinical signs and symptoms, together with blood tests, radiography, bone scans and microbiological cultures, are able to provide an accurate diagnosis.[50] However, there are international definitions regarding diagnostic criteria and therapeutic strategies for periprosthetic joint infections (PJI).[51] A test for clinical diagnosis need to have the required performance indicators such as sensitivity and specificity. Sensitivity and specificity express the proportion of patients with a certain disease and without a certain disease, respectively, that are correctly identified in the test.

Implant-associated infections may be difficult to diagnose, as they are often caused by persistent biofilm-producing microorganisms that can escape routine diagnostics. When there is a suspicion of infection, the white blood cell (WBC) count, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) levels are usually measured. ESR and CRP are suggested as criteria for the definition of periprosthetic joint infections.[51] The CRP will be elevated directly after surgery and will regularly decline to normal levels after a few weeks. It is therefore important to measure the CRP at different time points. However, C-reactive protein measurement is not a sensitive test for chronic inflammation caused by low-virulence, biofilm-producing microorganisms. Approximately 4% of periprosthetic joint infections (PJI) in the hip and knee have a normal ESR and CRP in chronic infections.[52] Histopathological examinations of tissue biopsies taken adjacent to the implant or the implant itself are useful when diagnosing the infection. X-rays are used to detect implant loosening and abscesses. Levels of

interleukin 1 (IL-1) and IL-6 in synovial fluid has been shown to differentiate between patients with periprosthetic infection from patients with aseptic loosening.[53,54]

Intraoperative tissue samples sent for microbiological cultures are the most accurate specimens for this purpose. Because of their high sensitivity, they represent one of the most reliable methods for diagnosing implant-associated infections. However, the combination of use of several laboratory and histopathology markers of inflammation creates a better platform for distinguishing between septic and aseptic loosening of prothesis.[41]

Antimicrobial treatment before sampling, delays in sending the specimens to the laboratory, no anaerobe cultures, an inappropriate culture medium or short culture times, contamination, and sending swabs instead of biopsies may jeopardise the ability to isolate the microorganism.[36] The detection of a microorganism can be enhanced using molecular techniques, such as polymerase chain reaction (PCR), but the method is extremely sensitive that it may provide false-positive results due to sample contamination (*e.g.* skin flora). In addition, PCR does not distinguish between live or dead bacteria or provide the antimicrobial susceptibility of the pathogen.[40] Matrix assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) can be used for the rapid identification of bacterial species.[55] Alpha-defensin, a defensin peptide, is a biomarker that has shown great potential for the diagnosis of PJI.[56,57] The levels of alpha-defensin are measured from samples of synovial fluid.

Table 1: List of different tests used in the diagnosis of orthopaedic implant infections. Sensitivity and specificity shown in %. Table adapted from Widmer 2001.[50]

Test	Sensitivity	Specificity	Ref
Blood leukocyte count	75-100	98.9-100	58
C-reactive protein	58.3-100	90.3-100	58,59
Serum erythrocyte sedimentation rate	16.7-50	90.3-100	58,59
IL-6	40-95	80-87	60
Culture of intraoperative tissue	88.2-100	86-100	50,61
Culture of sonicated implant	94.1	42.8	61
Synovial-fluid leukocyte	94	88	62,63
Histopathology	25-100	>95	41,50,64
Plain radiograph	14	70	65
Alfa-defensin	97.1-97	96.6-97	66,67
PCR	86	91	68
MALDI-TOF MS	95	84	55

1.5.4 Treatments

Implant-associated infections caused by biofilm-forming microorganisms often require long-term antibiotic treatment and combinations of antibiotics. The properties of an antimicrobial agent for this purpose should include having a bactericidal effect on surface-adhering, slow-growing and biofilm-producing microorganisms.[69] Rifampin is a potent antibiotic against staphylococci with a bactericidal mode of action, as it inhibits bacterial RNA synthesis. Rifampin must always be combined with another antimicrobial in order to prevent resistance in staphylococci.[70] Algorithms to aid when choosing treatment determining which diagnostic path to take have been developed.[36,71] Treatment failures will in worst cases lead to implant removal.

1.6 Pathogenesis of orthopaedic implant infections

To establish an infection, bacteria have to orchestrate the expression of several virulence factors that determine the pathogenicity. There are different alternative pathways, to cause either a highly virulent, acute infection or a low-virulence but persistent chronic infection, depending on the infecting bacterial species, site of infection and characteristics of the host defence.

1.6.1 Routes of infection for percutaneous orthopaedic implants

There are different routes for microorganisms to enter the body and finally reach the implant site. For percutaneous orthopaedic implants, the protective skin barrier is permanently breached and is therefore vulnerable to microbiological entrance. *Staphylococcus aureus*, coagulase-negative staphylococci (CoNS) various groups of streptococci, *Enterococcus faecalis* and Enterobacter were found to colonise the abutment at the skin-penetrating site in 27 of 30 patients, but only one of these patients had a definite diagnosis of deep infection.[32] Other potential pathways of infection are contamination during surgery and haematogenous spread.[42]

1.6.2 Infectious agents

The main microorganisms that cause infections associated with orthopaedic implants are the gram-positive bacteria *S. aureus*, *S. epidermidis* and less frequently *Propionibacterium acnes*, which take advantage of the weakening of the body's defence near the implant surface. Other microorganisms that often appear in late infections are streptococci and enterococci.[72] Staphylococci account for about

66% of biomaterial-associated infections.[73] *Staphylococcus aureus* and *S. epidermidis* are the most commonly found isolates from percutaneous bone-anchored amputation prostheses, hearing aids infections and in infected knee arthroplasty according to clinical studies.[32,74,75]

Staphylococcus aureus

Staphylococcus aureus is a highly virulent microorganism with an arsenal of toxins and factors promoting evasion from the host. The cell wall is composed of a single lipid membrane surrounded by a thick layer of peptidoglycan, teichoic acid. The teichoic acids exist in two major forms, lipoteichoic acid (LTA) linked to the cell membrane and wall teichoic acids linked to the peptidoglycan. The peptidoglycan chains contribute to the rigidity of the cell wall and protects the bacteria from osmotic lysis and the teichoic acid provide a negative net charge of the bacterial cell.[76] The ability to adhere to a surface is one of the many virulence factors of *S. aureus*. The key components for successful adherence to the surface are several microbial surface components recognising adhesive matrix molecules (MSCRAMM) that facilitate the attachment to host matrix molecules such as fibrinogen, fibronectin and collagen.[77] The conditioning film formed at the implant surface serves as a substrate for *S. aureus* adherence mediated by MSCRAMM.[78] One of the MSCRAMMs present on the surface of *S. aureus* is clumping factor A (ClfA) that is the dominant fibrinogen-binding protein. Genes encoding for wall-anchored adhesins fibronectin binding proteins, *fnbA* and *fnbB*, have been reported to be present in 98% and 99% of clinical *S. aureus* isolates in orthopaedic implant-associated infections.[79] The possession of different virulence factors such as cell wall-anchored proteins and capsule polysaccharides promotes the evasion of *S. aureus* from the host defence (Figure 2).[80] Protein A is a cell wall-anchored protein that binds to the Fc region of IgG. The binding results in coating the surface of the bacterial cell with IgG molecules that are oriented in the wrong direction and are therefore functionally impaired. As a result, opsonisation does not take place and the bacteria are therefore not able to be recognised by the neutrophils or to activate the complement system.[81] Preventing opsonisation is a way for *S. aureus* to prevent engulfment, which is important in the success of infection.[81] Most *S. aureus* strains express a microcapsule layer around the cell that contributes to the resistance to phagocytosis. Cell-wall-anchored proteins interact with integrins and promote the invasion of non-phagocytic host cells. When internalised, the bacteria are able to cause host cell apoptosis or necrosis, or they can enter a semi-dormant state called small colony variants inside the cells.[82] *Staphylococcus aureus* also possesses the ability to promote

evasion via exotoxins, invade host cells or degrade components of the extracellular matrix. A group of enzymes and cytotoxins which includes hemolysins (alfa, beta, gamma, and delta), nucleases, proteases, lipases, hyaluronidase, and collagenase are secreted by nearly all *S. aureus strains.*[83] It has been shown that α-hemolysin (hla) and leukotoxin AB (LukAB) are important in biofilm persistence by promoting macrophage dysfunction and cell death.[80,84]

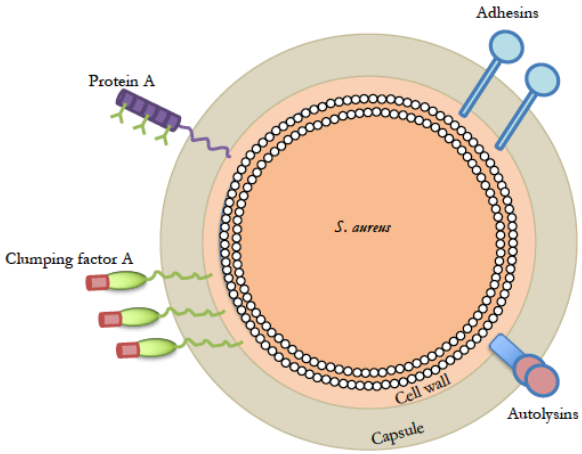

Figure 2: Cell wall components of *Staphylococcus aureus.* Figure adapted from Lowy[1]

Staphylococcus epidermidis

Staphylococcus epidermidis is much less virulent than *S. aureus,* with the ability to form a biofilm as its main virulence factor.[85] *Staphylococcus epidermidis* lacks secreted toxins in contrast to *S. aureus.*[86] However, *S. epidermidis* produces phenol-soluble modulins (PSMs) that can induce proinflammatory cytokines and have a cytolytic effect on neutrophils.[30,86] MSCRAMMs of *S. epidermidis* binds to host fibrinogen (SdrG), fibronectin (Embp), vitronectin (AtlE, Aae), and collagen (GehD).[86-88] Proteins involved in bacterial accumulations are accumulation-associated protein (Aap), extracellular matrix-binding protein (Embp), biofilm-associated protein (Bap).[89] The exopolymer poly-γglutamic acid (PGA) is important for *S. epidermidis* resistance to neutrophil phagocytosis and antimicrobial peptides.[90]

Enterococci

Enterococci are important nosocomial pathogens in the intestinal tract of humans and animals. Clinically, they pose a growing problem due to their high antimicrobial resistance, especially vancomycin resistance. Enterococcal biofilms have been observed in a number of implant-associated infections[91] and is an important virulence factor of enterococci. One of the quorum-sensing systems is *fsrABC* and it regulates the biofilm-associated genes and operons (including *bopABCD*, *ebpABC*, *gelE* and *sprE*).[92] *E. faecalis* biofilm cells revealed 101 differentially regulated genes compared with planktonic cells.[93]

Quorum-sensing system

Staphylococci have developed a density-dependent, quorum-sensing system that enables cell-cell communication. Quorum sensing is a way for bacteria to regulate pathways in order to control gene expression, to detect and respond to changes in the environment in a large population of bacteria.[94] Quorum sensing plays a vital role in biofilm formation and is therefore an important mechanism for studying infection-control strategies.[95,96] The signals of quorum-sensing systems are small molecules called autoinducers. These autoinducers need to reach a certain threshold concentration in order to activate a transcription regulator which is achieved at high cell population densities. The *agr* locus consists of the transcriptional units RNAII and RNAIII, responsible for the expression of the autoinducer peptide and the control of the expression of PSMα and PSMβ peptides respectively. Agr may influence biofilm behaviour, such as attachment, dispersal and the chronic nature of biofilm-associated infections, by activating the expression of virulence determinants such as α-toxin, surface-associated adhesins, δ-hemolysin and the autolysin AtlE.[97] The autolysins, AtlA and AtlE, involved in cell wall turnover, cell division and cell lysis are found in *S. aureus* and *S. epidermidis*.[98] The inhibition of *agr* activity leads to the increased expression of adhesin factors and the decreased expression of dispersal factors which may convert an acute infection into a chronic infection.[99]

1.6.3 Staphylococcal biofilm formation

Bacteria are able to exist in a free-floating planktonic phase or in an adhered biofilm phase, where the latter is the preferred mode of growth.[100,101] Most bacteria are biofilm opportunistic; when a surface is available, they will attach to it and recruit other free-floating bacteria to form a complex bacterial community, the biofilm.[102] Although the molecular mechanisms of biofilm formation depend on the bacterial species (there is no universal biofilm mechanism), the biofilm-

formation process is cyclic and consists of five steps (Figure 3). In the first step, planktonic bacteria reversibly adhere to a surface by physicochemical interactions, such as van der Waals forces, hydrogen bonding, electrostatic interactions and hydrophobic interactions.[103] In step two, adherence to the surface relies on bacterial adhesins (MSCRAMMs), capsule and extracellular matrix components that adhere to the proteins of the conditioning film formed on the surface and the adhesion becomes irreversible. Step three is the accumulation; intercellular connections are made between the bacterial cells. For *S. epidermidis,* polysaccharide intercellular adhesin (PIA), regulated by the *ica* operon, is expressed and it serves as the glue between the bacterial cells. Extracellular DNA (eDNA) has been shown to be important in the accumulation phase by contributing to biofilm stability.

The formation of a biofilm represents a cost-effective way of living and the bacteria create niches in different locations in the biofilm to survive and help the large population. Naturally, bacteria at the bottom of the biofilm are less exposed to nutrients and therefore become dormant. These dormant cells (persister cells) have a low growth rate and are therefore less vulnerable to cell wall-active antibiotics.[104,105]

The extracellular polymeric substances (EPS), such as polysaccharides, eDNA and supportive proteins secreted by the bacteria, help the bacteria to adhere to a surface and enables bridging between the cells. The ica operon, containing the genes *icaA*, *icaB*, *icaC* and *icaD,* encodes the enzyme that synthesises the PIA poly-N-succinyl-β -1,6-glucosamine.[106,107] PIA is the main polysaccharide in the biofilm matrix in *S. aureus* and *S. epidermidis*. The EPS maintains highly hydrated microenvironment, helps trapping nutrients, facilitates horizontal gene transfer between the bacterial cells, store energy within the biofilm, and protects them from immune cells.[69,100,108,109] Complex networks of channels within the biofilm are formed to transport nutrients and waste products in and out of the biofilm. The channels provide accessibility to essential nutrients in even the deepest regions of the biofilm.[110] There is a gradient of nutrients[111] which can cause heterogeneous gene expression throughout a biofilm.[112] The biofilm is able to respond to changing conditions by displaying variability in physiological states and has a powerful defence against antimicrobial agents and the host immune response.[104] The biofilm resembles multicellular organisms which protect the bacteria from the host's immune mechanism[104,113] Macrophages attempting to engulf biofilms become frustrated macrophages. The biofilm mode creates an

optimal environment for the horizontal gene transfer of antimicrobial resistance genes by plasmid exchange between cells.[104,114,115]

Step four is the maturation of the biofilm and tower-like formations are formed. The fifth and last step is dispersal. The bacteria may leave the biofilm when the shear forces overcome the tensile strength of the biofilm or when it is no longer profitable for the bacteria to stay due to limitations in nutrients. PSMs, controlled by the *agr* locus, cause the disruption of the biofilm matrix to form channels for the delivery of nutrients to deeper layers of the biofilm.[116] The result of channels weakens the biofilm structure causing dissemination of parts of the biofilm. The bacteria detach and become free floating again to find another surface to attach to and build a new biofilm.[108]

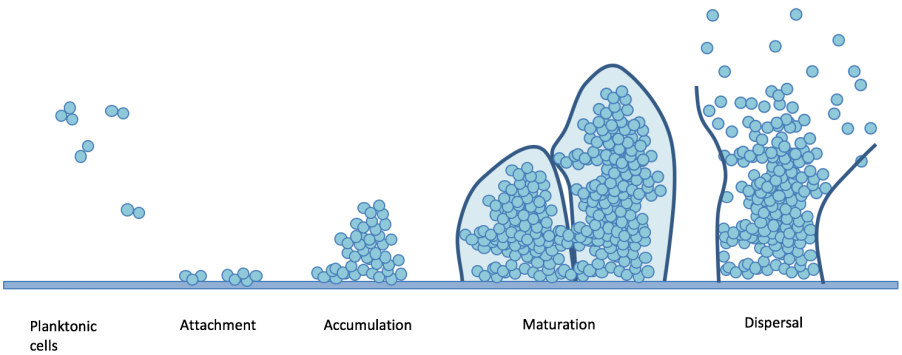

Figure 3: Schematic overview of the different stages of biofilm formation. Adapted from Otto 2009[87].

1.6.4 Biofilm antimicrobial resistance, tolerance and persistence

Antimicrobial resistance is the inherited ability of bacteria to grow at high concentrations of antibiotics, preventing the interaction with its intended target, and it is quantified by the minimum inhibitory concentration (MIC). There are three broad categories of antimicrobial resistance mechanisms: (1) inactivation of the antimicrobial agent through enzymes; (2) mutations that eliminate the molecular target for the antimicrobial agent; (3) and by reducing antimicrobial permeability.[117] One example of the first category is the staphylococcal β-lactamase, which modifies β-lactam antibiotics through hydrolysis of the β-lactam ring. The opening of the β-lactam ring prevents the binding to its penicillin-binding protein (PBP) target site. Examples of the second category are

modification to DNA gyrase (GyrA) resulting in quinolone resistance, methylation of 23s rRNA that inhibits macrolides, modification of PBP, and by acquiring the chromosomal gene *mecA* resulting in methicillin resistance.[118] The third category mainly applies for Gram-negative bacteria, which are efficient in restricting the diffusion of antimicrobial agents through their outer membrane. The negatively charged lipopolysaccharides (LPS) in their outer membrane limits the entry of hydrophobic antimicrobial agents.[118]

Tolerance to an antimicrobial agent is defined as the ability of a microorganism to survive, but neither grows nor dies, in the presence of a bactericidal antimicrobial agent. Tolerance mechanisms can prevent the bactericidal agent from using its downstream toxic effects even though the agent has bound to its target.

Biofilm antimicrobial resistance may be either acquired or intrinsic. The acquired resistance involves horizontal gene transfer of antimicrobial resistance genes by plasmid exchange between cells, or by mutations. In contrast, the intrinsic antimicrobial resistance (or tolerance) of biofilms, is a non-heritable phenotype tightly connected to the biofilm mode of growth and is multifactorial. The mechanisms that contribute to antibiotic tolerance include: restricted antimicrobial diffusion in the biofilm due to the EPS, bacterially-altered microenvironments, such as pH differences, which may antagonise antibiotic efficacy, and bacterial persister cells.[104,119] Persister cells possess lower metabolic activity and are in a dormant state, which makes them less susceptible to antimicrobials.[104,120,121] Persistence does only occur in a subpopulation of bacterial cells.[122] One strategy of treating biofilm infections is by targeting the different subpopulations of bacterial cells within the biofilm.

1.6.5 Gram-positive extracellular vesicles

Gram-negative bacteria secrete spherical and bilayered membrane vesicles with a diameter of 20-300 nm, reflect the outer membrane and periplasmic components.[123] It has been shown that they contain toxins, adhesins, enzymes, communication compounds, nucleic acids and pathogen-associated molecular patterns.[124-126] These vesicles take part in cell-cell communication, killing competitive bacteria, delivering toxins to host cells, inactivation of antimicrobials by enzymatic degradation.[127-130] Membrane vesicles of Gram-negative bacteria has also been located in the extracellular matrix of biofilm.[131] Vesicles derived from Gram-negative bacteria have been studied for more than 50 years, but only a few

studies focus on vesicles from Gram-positive bacteria, due to their lack of an outer membrane.[126] Extracellular vesicle (EVs) have been observed for several Gram-positive bacterial species, including *S. aureus*, and contain a range of cargo molecules, such as nucleic acids, proteins, lipids, viruses, enzymes and toxins.[132,133] Lee *et al.* reported the identification of 90 vesicular proteins found in *S. aureus,* where the cytoplasmic proteins were the most common, followed by extracellular and membrane proteins.[134] Data indicated that many vesicular proteins are likely to be involved in facilitating the transfer of proteins to other bacteria, as well as eliminating any competitive organisms, cellular defence, in antibiotic resistance, pathological functions in systemic infections.[134,135] An *in vivo* study performed by Gurung *et al.* has shown that *S. aureus* produces EVs that deliver bacterial effector molecules to host cells and induce morphological changes of epithelial Hep-2 cells leading to cell death.[136] It has also been suggested that α-hemolysin is released via the EVs of *S. aureus* and delivered to human host cells.[137] This indicates that EVs secreted from *S. aureus* may be associated with the development or progression of diseases. In addition, studies on EVs derived from *S. aureus* showed that they contained the β-lactamase protein BlaZ which confers penicillin resistance.[132,138] Recently, it was found that EVs are able to act as bridging factors in biofilms that produce an environment that is resistant to antibiotitcs.[124] However, to our knowledge, no studies have been performed on *S. epidermidis* and its ability to produce EVs, as well as the role of staphylococcal EVs in medical device-related infections.

1.7 Infection prevention and control strategies

There are different strategies for the control of implant-associated infections. They include the use of laminar flow in operating theatres, strict rules and routines in the operating theatre, sterile garments, preoperative antimicrobial prophylaxis and improvements in postoperative care. Another aspect of infection prevention is controlling the surface properties of the implant. The physicochemical properties of the implant surface play a major role in whether the host cells or bacteria arrive first at the surface and colonise it.[49] The research in the biomaterial field of engineering antimicrobial surfaces is extensive with different approaches.[139-141]

1.7.1 Non-adhesive surfaces

Different clinical applications require different antimicrobial approaches. One strategy is to create non-adhesive or bacteria-repellent surfaces preventing

bacteria from adhering. However, this strategy also prevents the host cells adhering and thereby prevents tissue integration that is crucial for orthopaedic implants, for example. The non-adhesive approach is applicable with contact lenses, urinary catheters and other temporary implant applications that do not require tissue integration. To achieve a non-adhesive mode of action, hydrophilic polymer coatings and polymer brush coatings are applied to the surface.[142]

1.7.2 Tissue-integrating surfaces

Applying the race for the surface theory, tissue-integrating surfaces have been developed.[49] The principle for these surfaces is to attract host cells to adhere to the surface before the bacteria arrive. This has been accomplished by using arginine-glycine-aspartic acid (RGD) peptides[143,144] as cell adhesion promoters for vascular grafts, while hydroxyapatite coatings have been used for dental and orthopaedic implants.[145]

1.7.3 Contact-killing surfaces

Killing the microbes as they approach the surface by direct interaction with the bacterial cell is achieved using a contact-killing mode of action. Silver is widely used for this mode of action, as it has broad-spectrum antibacterial properties. It has been used as an antimicrobial agent since ancient times to clean wounds and silver threads have been used as sutures. Nowadays, silver is used in medical devices such as catheters[146], wound dressings[147] and on stainless steel pins[148]. Pure metallic silver is inert and does not react with host tissue and does not kill bacteria until it is ionised, however, the exact mode of action is unknown.[149] The advantage of silver compared with antibiotics is that it is less prone to resistance development[150] and Gosheger *et al.* showed that a silver coating does not produce local or systemic side-effects in humans.[151] However, argyria has been reported in patient with burn wound treated with silver-coated dressing.[152] One drawback with the contact-killing strategy is the adhesion of host proteins (conditioning film) upon implantation, compromising the contact-killing mode of action.

1.7.4 Releasing surfaces

Antimicrobial peptides

Antimicrobial peptides (AMPs) are small, cationic components that are part of the innate immune system. They play an important role in preventing bacterial infections and possess broad spectrum antimicrobial activity against bacteria, fungi, and viruses.[153] AMPs act by either permeabilising microbial cell membranes or by translocation across the cell membrane to attack their cytoplasmic target.[154]

AMP coatings are able to make the implant surface biofilm resistant without being toxic to host cells. The development of antimicrobial resistance is therefore considered low in contrast to antibiotics.[155]

Antibiotics and antiseptics

One way of applying antimicrobials locally is by coating the implant surface with an antibiotic or antiseptic agent. Molecules such as triclosan, chlorhexidine and gentamicin have been applied to the surface and slowly release the active substance.[150] However, problem with drug-eluting surfaces has been that they tend to be fragile and it is hard to achieve the release over a longer period of time. Many of the attempts to create drug-releasing surface have not yet reached clinical use.[150]

1.8 Diagnostic tools to guide treatment

1.8.1 In vitro model testing

MIC

Routine antimicrobial susceptibility in clinical laboratories is determined by disk diffusion tests. Another method is (MIC) determination which requires serial liquid broth dilutions or an antibiotic gradient strip test. These tests rely on recovered, planktonic bacteria and serve as an important method in the treatment of many acute infections. The antimicrobial tolerance is lost once the bacteria from the biofilm revert to conditions that permit planktonic growth.[111] The MIC may therefore be misleading when testing isolated bacteria from a chronic implant-associated infection which may involve biofilms.

MBEC

The Calgary biofilm device (CBD) is a well plate with 96 identical cones attached to the lid of the microwell plate. The specially designed plate enables the formation of 96 identical biofilms on the cones when placed in wells containing bacterial inoculum solutions. After the desired incubation time, the cones can be transferred to an antimicrobial plate to determine the minimum biofilm eradication concentrations (MBEC).[156] This method has not been evaluated for clinical use, but MBEC may better reflect the antimicrobial concentrations needed to eradicate an infection caused by biofilm-producing bacteria.

2 AIMS

The main objective of this PhD thesis was to acquire a deeper knowledge of the pathogenesis of infections associated with percutaneous orthopaedic implants.

The specific aims were as follows.

1) To explore different methods that can be used to evaluate the treatment effects of biomaterial surfaces and to evaluate the antimicrobial performance of different surface treatments [Paper I]

2) To design and evaluate a novel combination of the Calgary biofilm device and a commercial susceptibility MIC plate using strains derived from patients with implant-associated osteomyelitis, as well as to determine the strains' biofilm formation abilities and biofilm antimicrobial susceptibility and to relate these properties to the clinical outcome of these patients [Paper II]

3) To determine whether staphylococci derived from implant-associated osteomyelitis produce extracellular vesicles and, if so, to characterise them (size, concentration, protein content), and finally to evaluate the expression and secretion of selected cytokines and cytotoxic effects of extracellular vesicles in a THP-1 monocytic cell line [Paper III]

4) To investigate whether sub-inhibitory concentrations of gentamicin have an impact on extracellular vesicle production in clinical *Staphylococcus epidermidis* and whether these vesicles influence bacterial growth and adhesion properties. To study whether extracellular vesicles from an antimicrobial resistant and biofilm-producing strain alter the phenotypic susceptibility and biofilm formation properties of a susceptible non-biofilm-producing clinical strain [Paper IV]

3 MATERIALS AND METHODS

In what follows, several *in-vitro* models were included using both reference and clinical strains. Experiments involving bacterial strains derived from patients with percutaneous orthopaedic implant infections were approved by the Regional Ethical Review Board in Gothenburg.

3.1 Patients

Bacterial strains analysed in the retrospective study presented in Paper II were retrieved from patients with deep infections related to percutaneous orthopaedic implants for amputees. The patient group included all eligible patients treated since the introduction of the method until the start of data retrieval. Retrieved samples were cultured at the Clinical Bacteriology Department (Sahlgrenska University Hospital, Gothenburg) and disk diffusion susceptibility testing was performed to determine which antimicrobial treatment to administer to the patients. Diagnosis, treatment and outcome information was extracted retrospectively from patient records. Infection was defined and graded by signs and symptoms of deep infection, X-ray findings and positive tissue cultures, according to a method previously described by Tillander *et al.*[157]

An outcome score (0-3) was used to group the patients according to the number of complications (relapse, re-infection and implant extraction). Treatment failure was defined as either a relapse within the study period or implant extraction due to unresponsiveness to administered antimicrobial treatment. Re-infection, caused by different microorganisms after completed antimicrobial treatment with clinical resolution, was not regarded as treatment failure. The demographics are presented in Table 2.

Table 2: Demographics and clinical outcome.

Demographics and clinical outcome	
Number of patients	11
Gender:	
Male	8
Female	3
Number of implants	11
Reason for amputation:	
Trauma	10
Tumour	1
Infection	0
Femoral amputation level:	
High	3
Mid	7
Low	1
Osteomyelitis:	
Definite[a]	8
Probable[b]	2
Possible[c]	1
Median years of age at time of diagnosis of osteomyelitis (range)	42 (22-71)
Median time in months since implantation (range)	47 (2-143)
Median number of months on antibiotics (range)	4 (1.5-8)
Implant extraction due to osteomyelitis	4

[a](+ signs/symptoms + X-ray + cultures[#]), [b](+ signs/symptoms ± X-ray + cultures[#]) and [c](+ signs/symptoms ± X-ray – cultures[#])
[#]Two or more positive bone and/or bone marrow cultures out of five, yielding indistinguishable bacteria in routine identification[157]

3.2 Bacterial cultures

3.2.1 Bacterial strains

A common reference strain of *Staphylococcus aureus* (ATCC 25923; Culture Collection, University of Gothenburg) [Papers I-IV] was originally a clinical isolate from Seattle 1945. *Staphylococcus aureus* (ATCC 29213; Culture Collection, University of Gothenburg) [Papers I-II, IV] originally obtained from a wound is a reference strain for staphylococci in MIC determinations. *Staphylococcus aureus* Xen29 (Caliper Life Sciences, Alameda, CA) possesses a stable copy of the *Photorhabdus luminescens* lux operon on the bacterial chromosome and has been used in this work for *in-situ* luminescence measurements [Paper I]. *Staphylococcus epidermidis* (ATCC 35984; Culture Collection, University of Gothenburg) [Papers I-IV] is a biofilm-producing strain originally obtained from a patient with catheter

sepsis. This strain has been used throughout this work as a reference strain for biofilm production and as an extracellular vesicle donor. *Staphylococcus epidermidis* (ATCC 35983; Culture Collection, University of Gothenburg) [Papers II] is a biofilm-producing strain originally obtained from blood and it was used in the study as a control strain for its moderate biofilm-production ability. *Staphylococcus epidermidis* (ATCC 12228; Culture Collection, University of Gothenburg) [Papers II-IV] is a non-biofilm-producing strain and it has been used as a negative control for biofilm production. *Pseudomonas aeruginosa* PAO1 (ATCC 15692; Culture Collection, University of Gothenburg) [Paper I] is a gram-negative strain originally isolated from an infected wound and it was used in this work as a reference strain for a disk diffusion test. *Staphylococcus epidermidis* DSM 18857 (Leibnitz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Germany) [Paper I] was used in primary adhesion tests.

The following strains were isolated from percutaneous implant-associated infections: *Staphylococcus epidermidis* CCUG 64518, *Staphylococcus epidermidis* CCUG 64521, *Staphylococcus epidermidis* CCUG 64523, *Staphylococcus aureus* CCUG 64514, *Staphylococcus aureus* CCUG 64516, *Staphylococcus aureus* CCUG 64520, *Staphylococcus aureus* CCUG 64522. (Culture Collection, University of Gothenburg) [Papers II-IV] and *Enterococcus faecalis* CCUG 64515, *Enterococcus faecalis* CCUG 64517, *Enterococcus faecalis* CCUG 64519, *Enterococcus faecalis* CCUG 64524, *Enterococcus faecalis* CCUG 64526 and *Enterococcus faecalis* CCUG 64527 (Culture Collection, University of Gothenburg) [Papers II]. The CoNS clinical strains were further characterised as *S. epidermidis* using the API Staph (bioMérieux SA, Marcy-l'Etoile, France). All the clinical strains have been characterised, with respect to biofilm production and antimicrobial susceptibility in both the planktonic (MIC) and biofilm (MBEC) state. Extracellular vesicles have been isolated from the clinical staphylococcal strains. All strains used in this work are listed in Table 3.

Table 3: List of bacterial strains used in this work.

Species	Strain ID	Source	In Papers	Ref
S. epidermidis	CCUG 64518	Osteomyelitis	II-IV	This work
S. epidermidis	CCUG 64521	Osteomyelitis	II-IV	This work
S. epidermidis	CCUG 64523	Osteomyelitis	II-IV	This work
S. aureus	CCUG 64514	Osteomyelitis	II-IV	This work
S. aureus	CCUG 64516	Osteomyelitis	II-IV	This work
S. aureus	CCUG 64520	Osteomyelitis	II-IV	This work
S. aureus	CCUG 64522	Osteomyelitis	II-IV	This work
E. faecalis	CCUG 64515	Osteomyelitis	II	This work
E. faecalis	CCUG 64517	Osteomyelitis	II	This work
E. faecalis	CCUG 64519	Osteomyelitis	II	This work
E. faecalis	CCUG 64524	Osteomyelitis	II	This work
E. faecalis	CCUG 64526	Osteomyelitis	II	This work
E. faecalis	CCUG 64527	Osteomyelitis	II	This work
S. epidermidis	ATCC 35984	Catheter sepsis	I-IV	[158]
S. epidermidis	ATCC 35983	Blood	II	This work
S. epidermidis	ATCC 12228	Non-infection associated isolate	II-IV	[159]
S. epidermidis	DSM 18857	Infected central venous catheter	I	This work
S. aureus	ATCC 25923	Clinical infection isolate	I-IV	[160]
S. aureus	ATCC 29213	Wound	I-II, IV	[161]
S. aureus	Xen29	Pleural fluid isolate	I	[162]
P. aeruginosa	PAO1	Wound	I	[163]

3.2.2 Bacterial inoculum preparations

All the strains were stored at -80°C in freezing media containing tryptic soy broth (TSB) and 20% glycerol. Upon use, the strains were streaked on Luria Bertini (LB) agar [Paper I] or Mueller-Hinton agar [Paper II] or 5% horse blood Columbia agar plates [Papers I-IV] and incubated over night in humidified air at 37°C. Single colonies were then added to TSB [Papers I-III] or Mueller-Hinton broth (MHB) [Papers III-IV] until optical density ($OD_{546\ nm}$) of 0.25 for *S. epidermidis* strains, 0.13 for *S. aureus* strains, 0.27 for *E. faecalis* and 0.26 for *P. aeruginosa* was reached, corresponding to approximately 10^8 CFU × mL^{-1}. Inoculum suspensions between 10^5-10^7 CFU × mL^{-1} were then prepared by diluting the OD suspensions. The bacterial concentrations in the inoculum suspensions were confirmed by plating 20 μL spots of 7-12 tenfold dilutions in saline on duplicate blood agar plates and incubated over night for 18-20 hours.

3.3 Materials

3.3.1 Substrate and coatings

The substrate for different coatings used in Paper I was commercially available machined (M) titanium (Ti) grade 5 (Ti-6Al-4V) (Elos Medtech, Pinol, Gørølse, Denmark). The substrates were cylindrical (Ø59 mm, thickness 2 mm, surface roughness R_a = 0.4 mm). M Ti is an implant material that is widely used in dental and orthopaedic applications and it therefore served as a control surface.

Cathodic vacuum arc evaporation[164] for a deposition time of 30 minutes was applied to the substrate to produce a crystalline TiO_2 coating with a pronounced anatase phase composition. Upon UV irradiation, TiO_2 has a photocatalytic antimicrobial mode of action. These TiO_2-coated disks were denoted physical vapour deposition (PVD).

The silver coating on substrates was applied using Bio-Gate (Nürnberg, Germany) surface treatment technology (HyProtect Coating). This coating is applied by a combination of chemical vapour deposition (CVD)-PVD-CVD process steps and results in a three-layer coating.[165] Oxygen is used to initiate the polymerisation of the precursor monomer (hexamethyldisiloxane) in the CVD (chemical vapour deposition) part of the process. Silver is transferred from solid phase to gas phase in the PVD (physical vapour deposition) process step. The vapour condenses onto the substrate and forms small agglomerates. In the last CVD step, the silver clusters are covered with a plasma polymer layer which results in the complete embedment of the elemental silver in a polysiloxane matrix. The Ag/SiOxCy plasma polymer applied to the substrate has a contact killing mode of action. This test surface is referred to as Ag.
The materials used in this work are listed in Table 4.

3.3.2 Scanning electron microscopy (SEM)

The coated disks were characterised by scanning electron microscopy (SEM) using a Zeiss 1550 scanning electron microscope, operating at 5 kV using an Inlens detector (Paper I).

SEM was also used to visualise EVs derived from bacteria [Papers III-IV]. The EVs were deposited onto 200 mesh Cu 01700-F formvar carbon-coated grids at a concentration of 0.5-1 $\mu g \times mL^{-1}$. After one hour, the samples were fixed in 2% paraformaldehyde for 10 minutes, washed in PBS, post-fixed in 2.5%

glutaraldehyde, washed in distilled H_2O and contrasted with 2% uranyl acetate for 15 minutes. Samples were dried, Au sputter coated (~10 nm), and examined using SEM (Ultra 55 FEG SEM, Leo Electron Microscopy Ltd, UK) in the secondary electron mode, operated at 5 kV accelerating voltage and 10 mm working distance.

Table 4: List of materials used for antimicrobial testing in Paper I.

Surface	Notation
Machined titanium	M Ti
TiO_2 coating	PVD
Silver coating	Ag

3.4 Assays for antimicrobial surface evaluations

3.4.1 Biofilm eradication

In Paper I, *S. aureus* ATCC 25923 was inoculated into a CDC biofilm reactor CBR90 (BioSurface Technologies Corporation, Bozeman, MT) containing 500 mL of TSB to form homogeneous biofilms under flow on TiO_2-coated (PVD) disks and commercially available machined titanium (M Ti). The CDC reactor was incubated at 37°C and 130 rpm under batch conditions for 24 hours. Disks were rinsed and UV irradiated (20 mW × cm^{-2}, 365 nm peak emission) for 0, 15 and 60 minutes on both sides [Paper I].

3.4.2 Primary bacterial adhesion

A proliferation assay [Paper I] was performed to analyse the ability of adhered cells to survive and proliferate on a surface. Silver (Ag) and machined titanium (M Ti) disks were incubated with *Staphylococcus epidermidis* DSM 18857 for one hour. Non-adhered cells were removed by rinsing and the disks were transferred to new well plates with minimal media and incubated for another 18 hours at 37°C. The disks were removed and the wells, containing potential daughter cells, were supplemented with TSB and incubated for another 24 hours in a plate reader in kinetic mode and optical density at 578 nm was measured [Paper I].

3.4.3 Biofilm inhibition

PVD and M Ti disks were irradiated with UV for 0, 15, 30 and 60 minutes prior to incubation with 10^5 CFU × mL^{-1} *S. aureus* ATCC 25923 to investigate whether pre-treatment would result in a reduction in adhered bacteria. The disks were then incubated at 37°C for 24 hours [Paper I].

The biofilm inhibitory effect of silver was also tested by incubating Ag and M Ti disks with 10^5 CFU × mL^{-1} *S. aureus* ATCC 25923 for 24 hours at 37°C and 150 rpm [Paper I].

3.4.4 Imprint method

Ten microlitres of 10^6 CFU × mL^{-1} *S. aureus* ATCC 25923 were deposited on PVD and M Ti disks before UV treatment (0, 15, 30 and 60 minutes). The disks were then imprinted on blood agar plates for five seconds. The disks were removed and the plates were incubated for 24 hours at 37°C. The imprint method was also applied to the Ag disks in a modified set-up. Ag and M Ti disks were incubated at 37°C for two hours with 30 µL of 10^5 CFU × mL^{-1} *S. aureus* ATCC 25923, after which the disks were imprinted on blood agar plates and incubated for 24 hours at 37°C [Paper I].

3.4.5 In situ bioluminescence

PVD and M Ti disks with 50 µL of 10^6 CFU × mL^{-1} of *S. aureus* Xen29 in LB broth were UV treated for 0, 15, 30 and 60 minutes. The disks were transferred to a white 24-well plate and the bioluminescence was measured in a plate reader. The Ag disks were evaluated in a similar way. Ag and M Ti disks were placed in a white 24-well plate with 1 mL of 10^5 CFU × mL^{-1} of *S. aureus* Xen29 in LB broth and incubated at 37°C for two hours. The disks were then rinsed with saline. Fresh LB broth was added to the disks and they were incubated for another 48 hours. The capability for biofilm formation in the adhered cells was analysed by CFU counting [Paper I].

3.4.6 Disk diffusion method

S. aureus ATCC 25923, *S. epidermidis* ATCC 35984 and *P. aeruginosa* PA01 in a concentration of 10^8 CFU × mL^{-1} were spread on Mueller–Hinton agar plates to form confluent growth. Ag, M Ti and standard gentamicin disks were placed on the agar. One additional control plate of *S. aureus* ATCC 25923 was also tested for penicillin (10 iU), kanamycin (30 µg), erythromycin (15 µg) and tetracycline (30 µg). The plates were incubated for 18 h at 37°C prior to measuring the zone of inhibition [Paper I].

3.5 Quantification of bacteria and biomass [Papers I-IV]

3.5.1 Colony forming unit (CFU) counts

The viability counting method was used to determine the number of viable cells in this study. A bacterial suspension of unknown concentration was tenfold diluted serially and plated on duplicate plates. Twenty microlitres from each dilution were added to duplicate blood agar plates and allowed to air dry before incubation (approximately 18 hours). The colonies were counted manually and used to estimate the concentration (CFU × mL^{-1}) [Papers I-IV].

3.5.2 Microtiter plate assay

The ability of a bacterium to adhere to a microtitre plate was assessed using a microtitre plate assay. A volume of 200 µL of 10^5 CFU × mL^{-1} was added to each well of a microtitre plate and incubated at 37°C for 24 hours. The plate was inverted and rinsed in three water baths to remove non-adhered bacteria. The remaining biofilm biomass (adherent cells and extracellular matrix) was stained with 0.1% crystal violet (Tris(4-(dimethylamino)phenyl)methylium chloride). The plate was rinsed in three water baths and the stain was eluted with 95% ethanol for 10-15 minutes, after which the solution was transferred to a new microtitre plate. The absorbance was measured at 600 nm in a plate reader.

The cut-off value (OD_c) was defined as three standard deviations (SD) above the mean OD of the blank (TSB):

OD_c: = average OD of blank + (3x SD of blank).

The strains were classified as previously described by Christensen *et al.*[166] and further categorised by our own biofilm biomass scoring (range 0-3) [Paper II].

3.5.3 Fluorescence quantification

The biofilm production ability and cell viability of bacteria were analysed by fluorescence. The antimicrobial effect on planktonic bacteria was determined by measuring the amount of live and dead cells by fluorescence. A volume of 50 µL of 10^6 CFU × mL^{-1} of *S. aureus* ATCC 25923 was deposited on PVD and M Ti disks and then irradiated with UV for 0, 2 and 15 minutes. A working solution of SYTO9 (staining live cells green) and propidium iodide (PI) (staining dead cells red) from a FilmTracer™ LIVE/DEAD® Biofilm viability kit (Invitrogen) was applied to each sample well and incubated in the dark for 20 minutes. The bacterial solution was transferred to falcon tubes and was sonicated (30 seconds), vortexed (one minute) and centrifuged (10,000 *g*, five minutes). The pellet was

resuspended in 1 mL of 0.9% saline solution and transferred to a black well plate. The fluorescence was measured in a plate reader using an excitation filter of 485 nm and an emission filter of 520 nm [Paper I].
In another experiment, the biofilm grown under 24 hours in a microtitre plate was stained with SYTO9 and PI for 30 minutes and the fluorescence intensity was measured in a plate reader [Paper II].

3.5.4 Congo red agar plate test

The slime production of different strains was determined using the congo red agar plate test. Strains were streaked on blood agar plates supplemented with 0.8 g of congo red per 1 L of brain heart infusion agar. A six-colour reference scale was used to determine the degree of slime production. The reference scale has been described before, where intensely black, black and almost black colonies are formed on CRA by slime-producing strains and bordeaux, red and intensely red colonies are formed by non-slime-producing strains[167] [Paper II].

3.5.5 Detection of icaA and icaD genes

Polysaccharide intercellular adhesin (PIA) is part of extracellular polymeric substances of staphylococcal biofilms and the synthesis of PIA is mediated by the *icaADBC* locus. The first two genes, *icaA* and *icaD,* perform a primary role in the exopolysaccharide synthesis.[168] Cultures of the individual clinical staphylococcal strains and reference strains were centrifuged to pellet the bacterial cells (10^8 CFU in 1.5 mL^{-1}). The DNA was extracted from the bacterial cells using a commercial Gene Elute Bacterial Genomic DNA elution kit (Sigma, St. Louis, MO, USA). A multiplex-PCR kit (Qiagen GmbH, Hilden, Germany) was used to amplify *icaA* and *icaD* with primers: 5'-TCTCTTGCAGGAGCAATCAA-icaA-Forward;
5'-TCAGGCACTAACATCCAGCA-icaA-Reverse;
5'-ATGGTCAAGCCCAGACAGAG-icaD- Forward;
5'-CGTGTTTTCAACATTTAATGCAA icaD-Reverse.[167] DNA bands of the PCR products of *icaA* and *icaD* genes were visualised with 2.2% agarose gel electrophoresis (Lonza, Rockland, ME, USA) [Paper II].

3.6 Susceptibility testing

3.6.1 MBEC assay

The Calgary biofilm device (Innovotech®) is a commercially available plate for growing identical biofilms on pegs attached to a 96-well lid that fits into a 96-well plate containing bacterial suspensions. The biofilms were grown at 37°C and under shear force created by a rotary shaker incubator (150 rpm) for 24 hours. To determine the susceptibility of bacteria grown in biofilms, the Calgary biofilm device was combined with a commercial microbroth dilution plate (Sensititre®).

3.6.2 Antibiotic selection

The microbroth dilution plate was custom made (Sensititre® CML1FNUN TREK®) with ten different antimicrobial agents commonly used for the clinical treatment of staphylococcal and enterococcal infections. The following antimicrobial agents were included: gentamicin (GEN), clindamycin (CLI), vancomycin (VAN), linezolid (LZD), ciprofloxacin (CIP), oxacillin (OXA), fusidic acid (FA), ampicillin (AMP), trimethoprim/sulfamethoxazole (SXT) and rifampin (RIF). A list of the concentrations is presented in Table 5.

Table 5: *List of antibiotics used in the custom-made Sensititre® plate*

Antibiotic	Abbreviation	Well concentrations	Group of antibiotics
Gentamicin	GEN	0.12-8, 128	Aminoglycosides
Clindamycin	CLI	1-128	Lincosamides
Vancomycin	VAN	0.5-32, 512	Glycopeptides and Glycolipopeptides
Linezolid	LZD	1-128	Oxazlodinones
Ciprofloxacin	CIP	0.5-32, 128	Fluoroquinolones
Oxacillin	OXA	1-32, 128, 512	B-lactams
Fusidic acid	FA	0.5-32, 512	Fusidic acid
Ampicillin	AMP	0.25-32, 128, 512	B-lactams
Trimethoprim/ sulfamethoxazole	SXT	1/19-32/608	Trimethoprim/ Sulfonamides
Rifampin	RIF	0.03-16, 128, 256	Rifamycins

3.7 Isolation and characterisation of extracellular vesicles [Papers III-IV]

3.7.1 Isolation of EVs

EVs from bacterial cultures were isolated and used in Paper III and Paper IV. One colony from overnight cultures on horse blood agar plates was inoculated in 100 mL of TSB (Paper III) or MHB (Paper IV). The bacterial suspensions were incubated at 37°C for 22 hours at 125 rpm. The supernatants were made cell free by centrifugation (3,000 *g*, 20 minutes, 4°C) and were then sequentially filtered through 0.45 and 0.22 μm pore-size vacuum filters. The bacteria-free liquids were concentrated using the ÄktaFlux Benchtop System (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) by ultrafiltration with a 100 kDa hollow-fibre membrane (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) (Paper III) and kept frozen until it was used. The concentrated media (Paper III) or only filtered media (Paper IV) was centrifuged at 16,500 *g* for 20 min and the supernatant was filtrated with 0.22 μm pore-size vacuum filters (Sarstedt, Nümbrecht, Germany). The supernatant was ultracentrifuged at 150,000 *g* for three hours at 4°C in a T-647.5 rotor (Sorvall wx Ultra series, Thermo Scientific, USA) to pellet the EVs. The resulting EV pellets were washed in PBS, centrifuged at 150,000 *g* for three hours at 4°C and re-suspended in PBS. Three separate batches of EVs per bacterial strain were isolated and stored at -80°C until further use.

3.7.2 Nanoparticle tracking analysis

Nanoparticle tracking analysis is a method that measures particles in the nanometer size range based on light microscopy and Brownian motion. Isolated EVs were analysed using a NanoSight LM10/LM14 instrument (NanoSight Ltd, Amesbury, UK). EVs were diluted in PBS, injected into the LM14 module and captured three times for 60 seconds, repeated three times. The recorded videos were then subjected to nanoparticle tracking analysis (NTA) using Nanosight particle tracking software 3.1 to provide nanoparticle concentrations and size distribution profiles (mean and standard error from 3´3 captures). This was performed for each EV batch for the different strains.

3.7.3 Protein quantification

Nanodrop, which is based on photometry, measured the protein concentration using the Pierce® BCA Protein Assay Kit, following the manufacturer's instructions.

3.8 Cell culture [Paper III]

3.8.1 THP-1 monocytic cell line and THP1-Blue™ NF-κB cells

The human THP-1 monocytic cell line was used for a stimulation assay in Paper III. The monocytes were propagated in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% foetal bovine serum (FBS), 1% antibiotic-antimycotic solution (10000 units × mL^{-1} penicillin, 10000 μg × mL^{-1} streptomycin, and 25 μg × mL^{-1} Amphotericin B) and 0.05 mM 2-mercaptoethanol in a 37°C humidified incubator with 5% CO_2. When the cells reached the desired cell number, they were seeded in Nunc 24-well plates at a density of 500,000 cells × mL^{-1} suspended in 1 mL RPMI 1640 medium supplemented with 10% FBS without antibiotics. The cells were stimulated with *S. epidermidis*- or *S. aureus*-derived EVs at (0, 5 and 25 μg × mL^{-1}). Monocytes with medium alone served as negative controls and LTA from *S. aureus* served as a positive control.

THP1-Blue™ NF-κB cells were originally derived from a THP-1 monocytic cell line by integrating NF-κB-inducible secreted embryonic alkaline phosphatase (SEAP) reporter construct. The NF-κB activation is assayed by determining the activity of secreted embryonic alkaline phosphatase. The cells were propagated in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS) and 100 μg × mL^{-1} Normacin™. Approximately 50,000 cells × mL^{-1} suspended in 1 mL of RPMI 1640 medium supplemented with 10% FBS without antibiotics were pipetted into 96-well plates. The cells were stimulated with *S. epidermidis* EVs from strain CCUG 64521 or *S. aureus* EVs from strain CCUG 64516 (0, 5 and 25 μg × mL^{-1}). The stimulation with heat-killed *Listeria monocytogenes* (HKLM) and endotoxin free sterile water was used as a positive and negative control, respectively. The cells were incubated in a 37°C humidified incubator with 5% CO_2 for six, 12, and 24 hours. The QUANTI-Blue™ Solution was added to a new 96-well plate in a volume of 180 μL, the cell supernatants (20 μL) were added to the QUANTI-Blue™ Solution and incubated for one to two hours and the optical density was measured at 655 nm.

3.8.2 Cell quantification

The number of cells in medium was determined using a Nucleocounter® system (ChemoMetec A/S). The cell mix was loaded in Nucleocassettes™ pre-coated

with fluorescent propidium iodide that stains the cell nuclei and then quantified in the Nucleocounter®.

3.8.3 Cell viability

Lactate dehydrogenase (LDH) is an enzyme that leaks out from the cytoplasm upon cell membrane injury. Cell viability from *in-vitro* cultures was determined by measuring the lactate dehydrogenase (LDH) content of the cell-free supernatant LDH which catalyses the conversion between lactate and pyruvate and this conversion can be evaluated spectrophotometrically by measuring the reduction of NAD^+ to NADH at 340 nm (C-laboratory, Sahlgrenska University Hospital, Sweden).

3.8.4 Gene expression

Total RNA was isolated from the samples by RNeasy Micro kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The gene panel was as follows: Interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), Interleukin-10 (IL-10), Interleukin-6 (IL-6), Toll like receptor-2 (TLR2), Toll like receptor-3 (TLR3), Toll like receptor-4 (TLR4), DNA damage-inducible transcript 4 (DDIT4), and B-cell lymphoma 2 (Bcl-2). All the samples were analysed in duplicate and the PCR was performed using the SFX96 real time PCR System (Bio-Rad Laboratories, CA, US). The relative quantification of the target gene expression was performed according to the standard curve method calculated by the ΔΔ-Cq method.

3.8.5 Cytokine release

Enzyme-linked immunosorbent assays (ELISA) were employed to evaluate the amount of secreted proteins (cytokines and chemokines) in THP-1 cell suspensions. The cell suspensions were centrifuged at 400 *g* for five minutes and supernatants were aliquoted and stored at -80°C until analysis. Commercial human ELISA kits were utilised according to the manufacturer's instructions. The optical density was measured at 540 nm with a microplate reader (FLUOstar Omega, BMG Labtech, Germany) and translated to protein levels using standard curves.

3.8.6 Confocal microscopy

The effect of EVs on primary adhesion was studied using confocal laser-scanning microscopy. The clinical *S. epidermidis* strain CCUG 64523 was added to wells (100

μL) in microslide chambers at a concentration of 5×10^5 CFU × mL^{-1} suspended in MHB. EVs derived from *S. epidermidis* ATCC 35984 were added (100 μL) to the bacterial suspensions at a concentration of 100 μg × mL^{-1}. MHB alone without EVs was added to the bacterial suspensions as a control. The slide chambers were incubated for five hours and 24 hours at 37°C. Non-adhered bacteria were rinsed with saline and adhered cells were stained with a FilmTracer™ LIVE/DEAD® Biofilm viability kit for 20 minutes at room temperature. Excess dye was rinsed with saline and mounting media and objective glass was placed on slides. The samples were analysed with a Nikon C2 confocal laser-scanning microscope (CLSM, Nikon, Tokyo, Japan).

The ability of THP-1 monocytic cells to internalise EVs was examined as follows: EVs derived from two clinical strains (*S. aureus* CCUG64520 and *S. epidermidis* CCUG64523) were stained with DiO dye (Molecular Probes, Eugene, OR, USA) for 30 minutes at 37°C. The excess dye was removed by washing using 100 kDa filters (Millipore, Massachusetts, USA) and PBS, according to the manufacturer's instructions. Cultured THP-1 cells were stained with DiI dye (5 μL was added per millilitre of cell suspension of 10^6 cells × mL^{-1}) (Molecular Probes, Eugene, OR, USA) and incubated for 15 minutes at 37°C. Cells were washed to remove excess dye and seeded in 24-well plates (5×10^5 cells × mL^{-1}). The DiO-stained EVs were added to the cells (5 and 25 μg × mL^{-1}) and incubated for four hours at 37°C. Approximately 1×10^5 cells were applied to microscopic slides using cytospin centrifugation (Shandon, Runcorn, UK). Cells were fixated with 2% formaldehyde for 15 minutes and washed twice in PBS before being mounted with Vectashield HardSet Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI). The samples were analysed with a Nikon C2 confocal laser-scanning microscope (CLSM, Nikon, Tokyo, Japan).

4 SUMMARY OF RESULTS

4.1 Paper I

This work evaluates different *in-vitro* methods for the antimicrobial efficacy testing of photocatalytic TiO_2 (PVD) surfaces and silver (Ag)-coated surfaces on both planktonic and biofilm bacterial cultures. The antimicrobial performance of the surfaces was also investigated.

Primary bacterial adhesion: After an initial adhesion of 1 hours, no detectable growth of bacterial daughter cells was detected within 24 hours of culture on silver-coated disks. In contrast, the machined titanium (M Ti) disks showed the growth of daughter cells after six to seven hours.

Biofilm eradication: The biofilm formed on PVD and M Ti disks after culture in a CDC biofilm reactor reached equal amounts of CFU (10^3-10^4 CFU × mL^{-1}). Fewer CFUs were, however, detected after UV irradiation, on both test and control surfaces. The UV irradiation itself accounted for most of the decrease in viability.

Biofilm inhibition: No long-lasting photocatalytic effect was observed by pre-treating disks with UV. Equal amounts of CFUs (10^5 CFU × mL^{-1}) were detected on both PVD and M Ti disks. However, a 70% reduction in adhered cells was observed on Ag disks compared with M Ti disks after 24 hours of incubation with *S. aureus* under flow conditions.

Imprint method: In contrast, using this method, both the UV and/or the photocatalytic effect killed *S. aureus* cells on both PVD and M Ti surfaces after 60 minutes. The Ag disks demonstrated an antimicrobial effect compared with control M Ti disks.

Fluorescence: UV treatment (15 minutes) exhibited a photocatalytic effect, with a 42% increase in dead cells on PVD disks compared with no UV treatment.

In-situ bioluminescence: Both photocatalytic and UV demonstrated bactericidal effects after just 15 minutes of UV irradiation. An 82.7% decrease in bacterial

adhesion was observed on Ag disks compared with M Ti disks, when *S. aureus* Xen29 was cultured statically on the disks for 48 hours.

Disk diffusion method: No zone of inhibition was detected with either the Ag disks or the M Ti disks.

4.2 Paper II

Currently, the administration of antibiotics to patients is based on MIC determinations of the isolated microorganism. Evidence that biomaterial-associated infections are caused by biofilm-forming bacteria with increased resistance to antibiotics implies the need for new diagnostic tools. A novel combination of the Calgary biofilm device (CBD) and a commercially available custom-made susceptibility microbroth dilution plate (Sensititre®) was developed and tested on clinical isolates isolated from osteomyelitis associated with percutaneous bone-anchored prostheses. The clinical strains were characterised based on their virulence in terms of biofilm-formation capacity and antimicrobial resistance.

The analysis of the clinical outcome revealed four relapses, six implant extractions and five cases of re-infection during the study period. Treatment failure was determined for seven of eleven patients. Strains that caused infections for six of the seven treatment failures exhibited high MBECs against the antibiotics used for the respective treatment.

One strain was characterised as a strong biofilm producer, six as moderate biofilm producers, three as weak biofilm producers and two as non-biofilm producers. This is the first time strains from osteomyelitis associated with percutaneous bone-anchored prostheses have been characterised based on their biofilm-production abilities. All staphylococcal strains were positive for both *icaA* and *icaD*. All enterococcal strains had uniformly high MBEC values and higher MBEC/MIC ratios than staphylococcal strains. MIC determination for the strains for planktonic bacteria showed that 19% were resistant to the four most common antimicrobial agents: vancomycin, linezolid, ciprofloxacin and rifampin, whereas 77% of the strains grown in biofilm were resistant to MBEC determination.

A biofilm score of > 2 was assigned to 11 of 13 clinical strains, which correlated to higher MBEC/MIC ratios for vancomycin, linezolid, ciprofloxacin, ampicillin and rifampin.

4.3 Paper III

Paper III investigates whether staphylococci isolated from human implant-related osteomyelitis have the ability to release EVs and whether the EVs elicit cytolysis, NF-κB activation and proinflammatory cytokine secretion in a THP-1 monocytic cell line.

Nanoparticle tracking analysis and scanning electron microscopy revealed that all the staphylococcal strains released EVs. The number of EVs obtained from *S. aureus* strains was significantly higher than that from *S. epidermidis* strains. In contrast, however, there was no difference in the size of the EVs between the two species.

As determined by Nucleocounter analysis, a significantly lower viability was detected for both concentrations (5 and 25 $\mu g \times mL^{-1}$) of *S. aureus* EVs compared with non-stimulated controls. The high dose of *S. aureus* EVs caused a significantly higher loss of viability in comparison with LTA. The two concentrations of *S. aureus* EVs demonstrated a significant decrease in viability in THP-1 cells in a dose-dependent manner. The high dose (25 $\mu g \times mL^{-1}$) of *S. epidermidis* EVs caused a significantly lower viability of THP-1 cells compared with the non-stimulated control group. No significant difference in Nucleocounter-determined cell viability was detected between *S. epidermidis* EV and *S. aureus* EV treated groups.

A significantly higher LDH in the supernatant (indicative of cytolysis) was measured with THP-1 cells stimulated with 5 and 25 $\mu g \times mL^{-1}$ of *S. aureus* EVs compared with negative and positive controls. EVs from *S. aureus* induced a significantly higher (≈ 2-fold) LDH release when compared with the equivalent concentrations of the *S. epidermidis* EVs. No statistically significant differences in LDH were observed between *S. epidermidis* EVs compared with negative control and LTA.

The stimulation of THP-1 cells with *S. epidermidis* and *S. aureus* EVs resulted in higher gene expression of IL-8, MCP-1, MMP-9, IL-10 and IL-6 genes compared with unstimulated controls. Whereas TLR2 and TLR4 expressions were similar to those of controls, a 20- to 280-fold significantly higher expression of TLR3 was demonstrated for both *S. epidermidis* and *S. aureus* EVs compared with unstimulated control, irrespective of the EV concentration. TLR3 gene

expression was higher compared with control. DDIT4 gene expression was significantly higher for all EV-stimulated groups compared with LTA but not with unstimulated controls. All EV-stimulated groups, irrespective of species and concentrations, revealed a significantly lower Bcl-2 expression compared to both negative control and LTA.

The secretion of IL-8 was significantly higher for groups treated with both concentrations of *S. aureus* and *S. epidermidis* EVs compared with unstimulated control and LTA. For *S. epidermidis* EV stimulation, a statistically significant dose-dependent release of IL-8 by THP-1 cells was observed. All EV-treated groups elicited a significantly higher release of MCP-1, MMP-9 and IL-10 compared with the non-stimulated control group.

Both *S aureus* and *S. epidermidis* EVs promoted higher NF-κB activation in THP-1 blue cells compared with the unstimulated control at the three examined time points (6, 12 and 24 hours). Similar level of NF-κB activation was observed for the EV-stimulated groups and the HKLM positive control. Both *S. aureus* and *S. epidermidis* EVs were detected in the THP-1 cells, indicating the internalisation of EVs into cells.

4.4 Paper IV

The effect of sub-inhibitory concentrations (0, 0.03, 0.06 μg × mL^{-1}) of gentamicin (GEN) on the secretion of EVs by a clinical *S. epidermidis* strain was studied. A significant increase in the number of EVs per CFU was observed for EV_2 (0.03 μg × mL^{-1}) and EV_3 (0.06 μg × mL^{-1}) compared with EV_1 (0 μg × mL^{-1}). In contrast, the nanoparticle tracking analysis did not reveal any significant differences in the mean sizes of the different EV groups. The total amount of protein was significantly higher for EV_3 compared with EV_1.

The three EV types (EV_1, EV_2, EV_3) isolated from the three sub-inhibitory culture conditions (0, 0.03, 0.06 μg × mL^{-1} respectively) were then added to cultures of the same strain to evaluate their effect on growth, viability and adhesion under increasing concentrations of GEN. When a dose of 5 μg × mL^{-1} of EVs was added to the strain cultured without gentamicin (0 μg × mL^{-1} GEN), the area under the growth curve (AUC) did not differ for the three EV types. However, when gentamicin was present in the culture (0.03 μg × mL^{-1} GEN), the EV_3 significantly reduced the total growth of the strain compared with the EV_1 and EV_2 types. In the same way, with 0.06 μg × mL^{-1} GEN in the culture, the AUC of the strain was significantly lower upon stimulation with EV_3 compared with Ctrl, EV_1 and EV_2. When culturing the strain with its MIC dose of GEN (0.12 μg × mL^{-1}), bacterial growth was inhibited equally for all groups.
The 60 μg × mL^{-1} dose of EVs significantly decreased the total growth of clinical *S. epidermidis* strain compared with the unstimulated Ctrl for all the gentamicin culture conditions (0, 0.03, 0.06, and 0.12 μg × mL^{-1} GEN).

The generation time was the same for all EV groups and the Ctrl when the strain was cultured in 0 μg × mL^{-1} GEN and 0.03 μg × mL^{-1} GEN, when stimulated with 5 μg × mL^{-1}. A decrease in generation time (faster doubling time) was detected for EV_1 and EV_3 compared with Ctrl for the 0.06 μg × mL^{-1} GEN culture condition. The generation time was instead increased (slower doubling time) by 60 μg × mL^{-1} EV stimulation compared with Ctrl when the strain was cultured in 0 μg × mL^{-1} GEN and 0.03 μg × mL^{-1} GEN. Since the strain did not grow in 0.06 μg × mL^{-1} GEN, when stimulated with 60 μg × mL^{-1} of all EV types, the generation time was zero; while the strain divided every 76 minutes in the absence of GEN.

Stimulation with a 5 μg × mL^{-1} dose of EVs reduced adhesion (20-82%) in the absence and presence of gentamicin (0, 0.03 μg × mL^{-1}), whereas the high dose (60 μg × mL^{-1}) of EVs only reduced adhesion in the presence of gentamicin.

EVs isolated from a biofilm-producing/GEN^R *S. epidermidis* strain were added to a non-biofilm-producing/GEN^S *S. epidermidis* recipient strain to examine whether they promoted antimicrobial tolerance and biofilm formation in the susceptible clinical strain. The overall growth (as measured by the AUC) of the clinical strain was significantly increased by adding 100 μg × mL^{-1} EVs of the biofilm-producing/GEN^R strain compared with controls, with and without GEN and especially during exponential growth. In parallel, the generation time (at maximum growth rate) was shorter (8-33 minutes faster), as well as inhibited cell adhesion, compared with the Ctrl. The viability proportion, as measured by the live/dead staining, remained similar for both the EV and the Ctrl group.

5 DISCUSSION

5.1 Diagnosis of biofilm infections

The pathogenesis of many implant-associated infections is related to the presence of microorganisms growing as biofilms. Several measures have been taken to prevent these infections in the clinical setting, including strict hygienic rules, preoperative prophylactic antimicrobials, operating theatre environment conditions, and implant surface properties.[141] The rates of *e.g.* prosthetic joint infections is estimated at about 1-2%, which is a large reduction compared with the introduction of arthroplasties in the 1960s when the infection rate was as high as 5-10%.[169] In spite of this, the difficulty involved in the diagnosis and administration of the right treatment remains a challenge.

Difficulties already occur with the sampling at the operating theatre. In some cases, bacteria are only identified until removal of the prosthesis.[170] Biopsies might fail to sample the bacteria if the biopsies are not taken at the right place.[171] In Paper II, which was a retrospective study, the sampling had not been taken systematically in accordance to a protocol regarding number of tissue biopsies, culturing of implant compartments or marrow blood samples [Paper II]. The inconsistency may of course jeopardise the accuracy of diagnosis, nevertheless, clinical history of the patients strongly suggested osteomyelitis. Equal to or more than 2/5 positive cultures was used for defining osteomyelitis. In addition, in most cases marrow blood samples were taken. The marrow blood samples were taken with a semi-invasive procedure unique for this type of implants. A small removable screw inside the fixture allows for the aspiration of marrow blood as well as minor tissue biopsies while the implant is still in place. Marrow culturing has been shown to have high diagnostic value.[172]

The next problem to face is the fact that biofilm producing bacteria are hard to culture. The culture may be less reliable in biofilm-related infectious diseases due to inadequate sampling, too short incubation times, or inadequate choice of selective media.[173] If technically possible, sonication of implant/implant parts shown to increase the sensitivity of bacterial detection.[61,174,175] In a case study performed by our group, where an extracted fixture was sonicated before culturing, showing that the implant was positive for *Escherichia coli* (5×10^4 CFU/implant) and *Enterococcus faecalis* (10^2 CFU/implant). *Escherichia coli* was the only species detected by standard tissue cultures performed by the clinical lab,

whereas *E. faecalis* was additionally detected after sonication (data not published). To distinguish between septic and aseptic loosening of prothesis it is suggested to combine several laboratory and histopathology markers of inflammation.[41]

5.2 Characterisation of clinical isolates from chronic infections

The phenotypic and genotypic biofilm properties of bacterial strains derived from osteomyelitis related to percutaneous osseointegrated orthopaedic implants have been analysed for the first time [Paper II]. Greater resistance to antimicrobials was demonstrated for bacteria growing within biofilms compared with their planktonic counterparts. This result is supported by other observations on staphylococcal strains derived from prosthetic joint infections[176] and *S. epidermidis* strains from orthopaedic implant infections.[177] Higher MBEC/MIC ratios were detected for biofilm-producing *S. aureus* and *S. epidermidis* compared with non-biofilm producers [Paper II] which is in agreement with observations concerning prosthetic joint infections.[176] The *E. faecalis* strains exhibited equally high MBEC than MIC values, although they were produced different amounts of slime (according to the CRA method). There are several ways for biofilms to increase their resistance to antimicrobials.[69,178] The ability to produce slime appeared to play an important role in the contribution of high MBECs, since slime-producing strains showed significantly higher MBEC/MIC ratios than non-slime producing strains when examining all the strains [Paper II]. A report in line with our results show reduced bactericidal activity due to extracellular slime production of *S. epidermidis* in comparison to its biofilm-negative mutant.[179] Another study investigating the importance of slime production in clinical strains of CoNS on antimicrobial activity showed that the presence of slime did not influence the activity of rifampin and very little influence on the activity of clindamycin however, vancomycin demonstrated a significant decrease in efficacy.[180] A positive relationship was observed between the amount of isolated slime and the degree of resistance to vancomycin in a study on *S. epidermidis* isolates however not to rifampin.[181] A study compared *P. aeruginosa* and *S. aureus* biofilms and their resistance to a cationic agent[182]. Interestingly, even though *S. aureus* biofilm was much weaker than the thicker and stronger *P. aeruginosa* biofilm, its formation lead to more drastic resistance than the resistance in *P. aeruginosa* biofilm. The extracellular polymeric substances played a significant role in resistance of *P. aeruginosa* while physiological changes in the biofilm cells were more likely contributing to the resistance of *S. aureus*.[182] The importance of considering species-specific factors as well as molecular specific factors in biofilm resistance

patterns was emphasised.[182] The mechanisms behind the effect of slime are not fully clear, but it has been suggested that it works as a diffusion barrier against small antimicrobial molecules.[115] The fact that the biofilms in our study were relatively young (formed under 24 hours) it could implicate that other factors than slime also contribute to the elevated antimicrobial resistance.

Two of the staphylococcal strains did not produce slime, but all of the staphylococcal strains possessed the *icaA* and *icaD* genes. The cell-cell adhesion function mediated by the ica locus appears to be conserved within the staphylococcal genus.[167,183] However, it is not necessarily expressed. A study investigated the distribution of genes associated with biofilm formation and the expression of biofilm-forming phenotype of *S. aureus* and *S. epidermidis* strains derived from total-hips or total-knees infected arthroplasties.[184] The study reported that all *S. aureus* strains and almost 70% of the *S. epidermidis* strains produced biofilm. Polysaccharide intercellular adhesin (PIA)-independent biofilms were produced by 27% of the biofilm-positive *S. epidermidis* strains, which were mediated by the accumulation-associated protein (Aap). However, *icaADBC*-independent biofilm mechanisms (deletion of the ica operon) have been described where biofilm-associated protein (Bap) was essential for both the initial adhesion and accumulation in *S. aureus* infection.[185] It has been suggested that SarA plays an important role as a master regulator controlling both *ica*-dependent and *ica*-independent biofilm mechanism.[107] The co-expression of *icaA* and *icaD* is, however, needed for the full phenotypic expression, while the expression of only *icaA* induces low enzymatic activity.[186] In our study, the expression of *ica* was not confirmed and only the presence of the *icaA* and *icaD* genes was determined. The ica locus is one of many potential targets for the prevention of biofilm-associated infections.

Significant conclusions regarding MBECs in relation to clinical outcome could not be drawn due to the small sample size [Paper II]. Nevertheless, qualitative assessments indicate that the majority of treatment failures were associated with higher MBEC to the antibiotic administered to the patient. Only one patient experienced all three categories of complications (relapse, re-infection and extraction) and the infection was caused by a strong biofilm-producing *E. faecalis* strain.

Another potential virulence trait of the clinical staphylococcal strains, the ability to produce EVs, has been explored [Paper III]. All the strains produced EVs irrespective of whether or not they were characterised as biofilm producing. The

effect of the EVs was tested on the monocytic THP-1 cell line, and on bacterial growth and adhesion under selective culture conditions [Paper III-IV].

5.3 Mechanisms in the pathogenesis of percutaneous orthopaedic implant infections

Patients with percutaneous orthopaedic implants are continuously exposed to the external skin microflora. Even though the cumulative risk of osteomyelitis over a 10-year period is 20%, the osteomyelitis does not always lead to implant removal. The treatment retention rate for percutaneous orthopaedic implants is 30%.[47]

Implant-associated infections have for long been treated as they were caused by planktonic bacteria. Infections caused by biofilm have unique characteristics, as described by Costerton and co-workers in the 1980s. The infection switches between dormant and acute periods. There may be an initial response to antimicrobials, but relapses are frequent.[187]

5.3.1 The role of biofilms in infections

Biofilm formation is a major virulence factor contributing to the chronic feature of many implant-associated infections. Antibiotic treatment alone is often inadequate to overcome these infections. Several reports on biofilm infections linked to medical devices or prosthesis have been published including orthopaedic implants[188], catheters[189], urinary catheters[91], vascular prostheses[190], prosthetic heart valves[91] and cerebrospinal fluid shunts[191], to mention some. In addition, biofilm formation is also common in infections not associated with foreign bodies such as chronic airway infections in cystic fibrosis patients[192], chronic otitis media[193], chronic sinusitis[193] and chronic diabetes wound infections[194]. Characterisation of biofilm production abilities of isolated strains in the clinical setting is rarely performed when it comes to device-related biofilms, which could potentially help guide treatments. Conversely, biofilms are often confirmed and characterized during the diagnosis of non-device-related biofilm infections, for example in chronic wounds and cystic fibrosis.[195] There are different methods that can be applied to characterise a biofilm infection.[35] Fluorescence *in situ* hybridization (FISH) is a technique based on fluorescent probes that binds to parts of chromosome with a high degree of sequence complementarity and visualised by fluorescence microscopy. It can distinguish polymicrobial aggregates and allows visualization of the strains present in the biofilm and their specific special arrangement and localization in the host tissue.[196] Polymerase chain

reaction (PCR) is another frequently used method with high sensitivity, however, it does not distinguish between live and dead bacteria, nor if bacterial cells are aggregated or forming biofilms.

There are studies reporting the *in vitro* characterisation of clinical isolates however, not for the purpose of guiding treatments. One study reported that out of 205 clinical isolates from different chronic relapse infections, 126 strains formed biofilms *in vitro* although this study used the *S. epidermidis* ATCC12228 as an incorrect positive control strain for biofilm formation.[197] Arciola and co-authors reported biofilm characteristics of strains isolated from catheter- and orthopaedic infections.[79,186,198] In our small cohort, 11 out of 13 strains were biofilm producers according to *in vitro* measurements and most of them were classified as moderate or strong biofilm producers [Paper II]. The biofilm production was heterogenous across species and strains [Paper II]. Three of the staphylococcal strains exhibited low MBECs for some antimicrobials. It appeared that one of these strains was non-biofilm producer. Microtiter plate assay is a standard method for the determination of biofilm biomass. In our study we were lacking a non-slime producing *S. aureus* reference strain. We noticed the importance of having proper positive and negative control reference strains for each of the species when classifying the degree of biofilm production of the strains.

5.3.2 EVs as a virulence factor: EV – bacterial cell interactions

Bacteria are remarkable organisms that can adapt to extreme environmental conditions to ensure the survival of their species. Antimicrobial treatment induces selective pressure and makes bacteria be subjected to stress. The selective pressure might produce subpopulations that are better suited for causing disease (fitness advantage).[199] In Paper IV, the effect of antimicrobial pressure, exerted by sub-inhibitory concentrations of gentamicin, on vesiculation was evaluated. The sub-inhibitory concentrations of gentamycin resulted in EVs of similar size and with higher protein content than those EVs isolated from control culture conditions. There is a risk that lysed cell debris contaminations contributed to the increased number EVs (according to NTA), however, when looking at the MIC dose of 0.12 $\mu g \times mL^{-1}$ gentamycin (data not published) where more lysed cells are expected, the number of EVs was in fact much lower. Other studies have encountered the same concern. Studies on *Pseudomonas aeruginosa* vesiculation under antimicrobial pressure and aquatic bacteria under UV stress[200] have similarly concluded higher vesiculation under pressure with higher OMV protein levels, which most likely reflect the vesiculation stimulation and not lysed

cells.[201,202] A critical limitation of the present work on EVs is regarding the purity [Paper III-IV]. Additional step when concentrating the EV suspension by ultrafiltration was applied in Paper III using a 100 kDa hollow fiber membrane compared to only ultracentrifugation in Paper IV. The filter might have affected EV purity. In the research field of exosomes that are secreted by mammalian cells, effort has been spent on evaluating and optimizing exosome isolation techniques to optimise the purity.[203-205] It is challenging to rapidly and efficiently isolate exosomes in large scale which also applies to the field of isolating bacterial vesicles. Ultracentrifugation is an expensive and time consuming method, does not result in high purity isolation and the vesicle yield is not very efficient[206] Factors affecting the EV isolation by ultracentrifugation are acceleration, type of rotor, time, viscosity of the sample, and storage.[207-209] Density gradient ultracentrifugation and ultrafiltration such as gel chromatography increase the purity but disadvantages are its low yield and that fractions achieved are diluted and may require concentration of the sample.[207] In our studies we were interested in investigating the cumulative effect of EVs, therefore fractioning was not performed. Further investigations on the effect of size and content of EVs separated by fractioning and how they differ is a natural next step to perform. The size of EVs varied between strains [Paper III] and one possible explanation for this observation may be that EVs can be synthesised and regulated in different ways.[126] The total protein content may vary due to size of EVs and needs to be further investigated. In both Paper III and Paper IV, the total effect of all isolated EVs has been analyzed.

OMVs derived from Gram-negative bacteria mediate the transfer and delivery of virulence factors to host immune cells and, the hypervesiculation of OMVs increases the tolerance to antimicrobials and increases the virulence of the strain.[210] The results obtained in Paper III and IV confirmed these effects, staphylococcal EVs elicited cytotoxicity and cytokine release in monocytes and at the same time influenced antimicrobial tolerance and bacterial growth.

OMVs have also been shown to be involved in the aggregation of bacterial cells and biofilm production,[131,211,212] and sub-inhibitory concentrations of antimicrobials can enhance biofilm production in *Pseudomonas aeruginosa*.[213,214] Another study showed higher levels on drug-binding proteins in biofilm-derived OMVs compared to planktonic-derived OMVs.[130] In our Paper IV, on the other hand, we noticed the opposite behaviour. The bacterial adhesion to culture plate mainly decreased with the addition of EVs. There may be several mechanisms for

this observed effect, but they were not evaluated in this study. This difference in adhesion between the EV-types may reflect different cargos, including different surface adhesins. This result goes in line with another recent study demonstrating decrease in adhesion to tissue culture plate with EVs present, which indicate that EVs adhere first to the surface making it more hydrophilic and not as accessible for the bacteria.[215] These results are supported by our observations of a drastic decrease in adherence of *S. epidermidis* to glass after 24 hours incubation when treated with *S. epidermidis* EVs from another strain [Paper IV].

The dose of 5 μg × mL^{-1} EVs was perhaps too low to contribute to any measurable difference in total growth, while 60 μg × mL^{-1} EVs dose decreased the total growth after 18 hours for all cultures compared to control. This large decrease in total growth with the 60 μg × mL^{-1} EVs dose can be explained by the significant decrease in maximum growth rate, rather than changes in viability observed by fluorescence measurements. The proportions of live cells were generally higher than the proportion of dead cells in the fluorescence measurements. Therefore, addition of 60 μg × mL^{-1} EVs isolated from the clinical *S. epidermidis* strain allowed the cells to survive at MIC concentrations, by neither growing nor dying in the presence of gentamicin, but by decreasing the maximum growth rate, demonstrating tolerance to the antimicrobial agent.

With the sub-inhibitory concentration of 0.06 μg × mL-1, no bacterial growth was detected when adding 60 μg × mL^{-1} EVs while growth was detected with 5 μg × mL^{-1} EV dose (except for EV_3). This result indicates that the EVs contains different cargos of proteins, and that depending on the concentration of these cargos they could modulate the biological effects. Although the same total amount of protein was added to the *S. epidermidis* cultures (5 or 60 μg × mL^{-1}), the total EV numbers varied between EV types (EV_2 > EV_3 > EV_1) and some specific proteins could have been packed differently into the EVs due to the different antimicrobial pressure during their secretion. EV_3 had the most amount of protein content and behaved differently than EV_1 and EV_2. For the EV_3 group, either the higher number of EVs or the enrichment of certain proteins could have influenced the observed decreased effect on bacterial growth, maximum growth rate and live/dead ratio.

Furthermore, we investigated if EVs (derived from GEN^R/$biofilm^{pos}$ strain) can alter the phenotype of a non-biofilm producing, gentamicin susceptible strain

GEN^{S}/biofilm^{neg}). EVs cultured with the clinical GEN^{S}/biofilm^{neg} *S. epidermidis* strain, promoted a significant increase in growth and faster generation time during exponential phase, as well as inhibited cell adhesion after 18 h compared to control. No shift in MIC was observed for the culture time tested, however, the EVs contributed to an increased tolerance to gentamicin and elicited a growth promoting effect. This growth promotion effect has been previously observed by our group in a different *S. epidermidis* strain and in *S. aureus* (data not published).

Taken together, the results from Paper IV, suggest that the increase in number of EVs released by *S. epidermidis* when exposed to gentamicin might have important clinical implications in the treatment of patients with implant-associated infections caused by *S. epidermidis*. Furthermore, sub-optimal treatment concentrations might affect the cargo of released EVs, which can contribute to the total growth, cell division, and adhesion of *S. epidermidis*. EVs from the clinical GEN^{S}/biofilm^{neg} *S. epidermidis* strain contributed to a decreased overall growth, slower planktonic cell division, and less adhesion when they were added in cultures of the same strain.

5.3.3 EVs as a virulence factor: EV – host defence interactions

We evaluated the effect of EVs derived from clinical osteomyelitis strains on THP-1 monocytic cell line. A dose-dependent reduction of THP-1 cell viability was observed when stimulated with *S. aureus* EVs [Paper III]. Although the high dose (25 μg × mL^{-1}) of *S. epidermidis* EVs caused a significantly lower viability of THP-1 cells compared with the non-stimulated control group as judged by Nucleocounter assay, no significant difference in LDH release was detected for *S. epidermidis* EV groups compared to control. These findings go hand in hand with the fact that *S. epidermidis* is considered much less virulent than *S. aureus*.[87] *S. aureus* is in the possession of a powerful set of toxins (α-Hemolysin (α-toxin), γ-hemolysin, leukotoxins and phenol-soluble modulins (PSMs))[80,84,216], whereas *S. epidermidis* production of toxins is limited to phenol soluble modulins, having non-cytolytic and cytolytic (PSMs) properties.[87,217]

An interesting finding was the raised gene expression of DDIT4 and the reduced expression of Bcl-2, involved in the cellular response to hypoxia and stress[218] and implicated in *e.g.* cell apoptosis and death[219]. Involvement of Bcl-2 regulated mechanisms in bacteria-induced cell apoptosis have been suggested previously.[84] However, to our knowledge, an enhanced expression of DDIT4 as an effect of bacteria/bacterial products has not previously been reported in relation to host

cell death. Taken together, our findings suggest that not only *S. aureus* EVs but also *S. epidermidis* EVs are agonists of cell injury and death. These observations provide strong motivation to further studies on this aspect of microbe-host cell interactions.

Own previous studies have shown that opsonized live *S. epidermidis* promote the expression of proinflammatory cytokines and production of reactive oxygen species in human monocytes *in vitro*.[220] Moreover, *S. epidermidis* elicit strong recruitment of PMNs, cell death and increased gene expression of TNF-α, IL-6, IL-8, TLR2 and elastase when administered at titanium implants *in vivo*.[221] Since *S. epidermidis* presents a less aggressive and more chronic nature with its pathogenicity mainly related to biofilm formation[81,222], it was interesting to observe a similar degree of NF-κB activation, gene expression, cytokine expression and cytokine secretion, attributed to *S. epidermidis* and *S. aureus* EVs, indicating a similar ability to activate a pro-inflammatory state in monocytes, at least during *in vitro* conditions.

Among the Pattern recognition receptors (PRR) expressed by cells of the innate immune system, the cell membrane bound Toll-like receptors identify molecular patterns associated with microbial pathogens and constituents released in conjunction with cell death[223] The present study explored if THP-1 cells expressed three TLRs (TLR2, TLR3, TLR4), implicated in NF-κB activation and cytokine secretion induced by gram-positive and Gram-negative bacteria/bacterial products. For example, LTA from *S. aureus* induces PMN secretion of IL-8 via CD14 and TLR2 and nuclear factor-κB activation.[224] After 24 hours culture of THP-1 cells, only TLR3 was significantly upregulated by interaction with *S. aureus* and *S. epidermidis* EVs. TLR3 recognizes double-stranded RNA usually carried by some viruses.[225] An important continuation of the present experiments is to determine the early temporal changes of EV-induced expression of TLRs in relation to the pathways of apoptosis and NF-κB /cytokines.

Proteomic studies have revealed that the *S. aureus* EVs contain lipids, proteins (toxins and adhesion molecules), β-lactamase, and other tissue destructive enzymes.[132,138,226] To our knowledge, similar studies of *S. epidermidis* EVs have not yet been performed and needs to be further investigated. LTA (5 μg × mL^{-1}) served as positive control, however, its response turned out to be weak. Another well-known inflammatory inducer, frequently used as positive control is lipopolysaccharide[227] (LPS), which is found in the Gram-negative bacterial cell

membrane, but since the EVs used were from Gram-positive origin, LTA was chosen.

Monocytes are one of the first cells to arrive to the infection site and are therefore an interesting model cell to use. Among the advantages of using a cell line is the reduced number of variables that affect the outcome. However, if EVs elicit similar effects on primary cells such as PMNs needs to be further investigated, as well as analysing several time-points other than 24 hours as endpoints for the analysis of cytokine release. The EV sample size was small, however, we have obtained all available samples originating from patients with percutaneous orthopaedic implants diagnosed with osteomyelitis. The relationship between biofilm production abilities and the number of released vesicles, their cytolytic and cytokine secretory effects could not be ruled out. Larger patient cohort with infections associated with total hip replacements is an ongoing study which will allow these comparisons.

5.4 Novel diagnostic tools and strategies for infection control

5.4.1 Evaluation of antimicrobial surfaces

There are several variables that may influence the risk of implant-associated infection making the assessment of the clinical efficacy of orthopaedic implants an extremely difficult task. As mentioned above, apart from strict hygienic rules, preoperative prophylactic antimicrobials, operating theatre environment conditions and predisposing factors of the patient, the actual implant material surface plays a major role. A variety of categories of anti-infective approaches have been implemented to achieve biomaterials with antimicrobial properties and new additional approaches are rapidly advancing. Different strategies are needed depending on the application which, in addition, means that appropriate evaluation methods are also needed and adapted to the material's mode of action.

Factors affecting the end point results when evaluating antimicrobial surfaces with different methods are culture media, rinsing steps, bacterial inoculum size[228], sterilization method[229,230]. Different methodological setups were evaluated with two different test surfaces (Ag and PVD) and control (Ti) surface [Paper I]. The test surfaces performed differently depending on the evaluation method.

The disk diffusion method is a common method for evaluating antimicrobial release of a test surface. The size of inhibition zone is a function of the ability for

the antibiotic to diffuse, the susceptibility of the strain, and its growth rate under standard experimental conditions (medium, agar, thickness, incubation temperature and disk content). The zone of inhibition may depend on the rate of diffusion of antimicrobials though the agar.[231] In contrast, the imprint method, showed an antimicrobial effect of the Ag surface, which confirmed the contact-killing mode of action (and not the release mode of action). The UV contributed to the killing of the bacteria and the photocatalytic activity was mostly short.

In vitro testing is the first step for evaluating antimicrobial surfaces, the next step towards clinical use is to test the surface *in vivo* using an animal model. The presence of the host immune defence is a very important additional factor *in vivo*, which will contribute to the final outcome. *In vitro* testing cannot predict which host proteins will adhere to the surface at the moment of implantation that will serve as a substrate for host cells and microorganisms. A difference in bacterial adhesion may be obtained depending on the adsorbed proteins.[232]

A critical limitation of the Paper I is the limited amount of test surfaces. More than two different modes of action could have been evaluated simultaneously to better show the difference between the *in vitro* tests.

The final answer whether a prosthetic surface property conveys an antimicrobial effect requires clinical trials. As previously discussed, a prerequisite for implanted orthopaedic prostheses is the development of a mechanically stable interface between the bone and the prosthesis. This interface is the scene of multiple biological events, ranging from initial inflammation to bone formation and remodeling.[233,234] The presently studied amputation prostheses are built upon the principle of osseointegration, the need for stable interfaces between implant, bone and soft tissues and between the different components of the implant system in order to enable an efficient load transfer, the sealing of the interior from the exterior and the avoidance of wear and abrasion.[13] Results demonstrate osseointegrated implants, 92% survival and improved function and quality of life after 2 years-follow-up compared to conditions before surgery.[16,235,236]

It is hypothesised that unless proper osseointegration is achieved or if osseointegration is lost, the possibilities for microorganisms to adhere to and colonize the implant surface is increased.[49] Therefore, novel *in vitro* documented antibacterial surfaces must also be assessed with respect to their ability to become integrated in bone in experimental *in vivo* conditions.[237] In addition, such concepts

should be subjected to a step-wise introduction into the clinical reality, similar to the principle suggested for the introduction of novel arthroplasties.[238]

5.4.2 Biofilm susceptibility testing

Prescribing antibiotic regimens that target acute infection when treating a chronic biofilm infection may be devastating since the regimen may potentially may not heal the infection and further induce resistance mechanisms. MIC determination is the standard procedure when treatment is administered to the patient. As it has been observed in Paper II, bacteria living in biofilms elicit >1000 higher resistance to antimicrobials than their planktonic counterpart, and a combination of antimicrobials are needed together with long treatment periods. The MIC and MBEC values differed for vancomycin, linezolid, ciprofloxacin and rifampin, the antibiotics most commonly used in the treatment of orthopaedic implant infections [Paper II]. The fact that the 13 strains exhibited much higher resistance to these antibiotics when adhered to the CBD implies their low efficacy in chronic biofilm infections associated to implants. There is a need for a new tool to better predict antimicrobial susceptibilities. The combination of CBD and Sensititre antibiotic plate tested in Paper II is a good candidate for this purpose. The method is relatively cost efficient, easy to standardize and the results are produced within 5 days. This time is extremely short considering the long average antibiotic treatment length of these patients (4 months) [Paper II].

6 SUMMARY AND CONCLUSIONS

- Several methods, tailored to the specific surface modification and antimicrobial mode of action, should be applied to provide valuable complementary information when evaluating antimicrobial surfaces with prophylactic and treatment effects against planktonic and biofilm bacteria. These methods can be employed to evaluate the first *in vitro* "proof-of-concept" in the development of new antimicrobial approaches.

- Most of the staphylococcal and enterococcal clinical strains isolated from patients with osteomyelitis associated with percutaneous bone-anchored implants had the ability to produce biofilms. In general, the biofilms required higher antimicrobial concentrations compared with non-biofilm producers. The same strain when grown as biofilms exhibited a significant increase in antimicrobial resistance compared with when it was grown planktonically.

- The majority of treatment failures were linked to high biofilm resistance towards the most commonly used antimicrobial agents in the clinic. The hereby-described diagnostic method for biofilm susceptibility testing may be useful to guide antimicrobial treatment decisions in orthopaedic implant-associated infection.

- The *S. aureus* strains produced significantly more EVs than the *S. epidermidis* strains. Both *S. aureus*- and *S. epidermidis-derived* EVs upregulated Toll-like receptor 3 (TLR3) gene expression, activated NF-κB, and promoted the gene expression and secretion of IL-8, MCP-1, MMP-9 and IL-10. Both EV types were internalized by a proportion of the THP-1 cells. Whereas EVs from both staphylococcal species upregulated the pro-apoptotic DNA-damage-inducible transcript 4 (DDIT4) and down-regulated the anti-apoptotic B-cell lymphoma 2 (Bcl-2) genes, cytolysis was preferentially induced in *S. aureus* EV-stimulated cells.

- Sub-inhibitory concentrations of antimicrobial agents stimulated *Staphylococcus epidermidis* to secrete higher number of EVs with more protein content, which strongly affected bacterial cell adherence and growth. Treating orthopaedic implant-associated infection with sub-inhibitory concentrations of antimicrobial agents could be promoting EV-release with specific virulence traits.

It is concluded that, isolates from implant-associated infection reveal multiple virulence traits relevant for understanding and treating these infections. This thesis proposes EVs as a novel pathogenic mechanism of biomaterial-associated infection, requiring further research focus.

7 FUTURE PERSPECTIVES

Major efforts are focused on the prevention, diagnosis and treatment of implant-associated infections. Strategies to minimise the occurrence of these infections requires increased understanding of both material-related and biological factors. The findings of this thesis demonstrate potential virulence traits of clinical isolates from implant-related osteomyelitis. To further investigate their role in pathogenesis of these infections it would be of interest to explore the following:

- The potential of the combination of Calgary biofilm device and Sensititre susceptibility plate in a prospective study for MBEC-guided treatment.
- Further studies on the effect of EVs derived from clinical osteomyelitis isolates on primary cells. By *e.g.* blocking of specific cell surface receptors and inhibition of internalisation processes, further understanding of the EV-host cell interactions will be provided. Further, the effects *in vivo* of the EVs will be of great interest.
- Molecular analysis of fraction isolated EVs (Proteomic, genomics, and RNA-sequencing).
- *In vitro* transfer experiments to ascertain if resistance genes can be horizontally transferred via EVs.
- The virulence effect of EVs on host cells when compared to its own parental bacterial cell (heat-inactivated).
- With a greater number of strains, evaluate if the biofilm formation degree associates with a particular EV-content or phenotype.
- *In vivo* proof of EV involvement in the pathogenesis of biomaterial-associated infection, by the isolation of staphylococcal EVs in infected tissue/implant samples from patients.

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9 REFERENCES

1. Lowy FD. Medical progress - Staphylococcus aureus infections. New England Journal of Medicine 1998;339(8):520-532.
2. Maradit Kremers H, Larson DR, Crowson CS, Kremers WK, Washington RE, Steiner CA, Jiranek WA, Berry DJ. Prevalence of Total Hip and Knee Replacement in the United States. J Bone Joint Surg Am 2015;97(17):1386-97.
3. Kärrholm J, Mohaddes M, Odin D, Vinblad J, Rogmark C, Rolfson O. Swedish Hip Register. Annual register report 2017 2018:1-195.
4. Robertsson O, W-Dahl A, Lidgren L, Sundberg M. Annual Report 2017. Swedish Knee Arthroplasty Register 2017:1-100.
5. Nemes S, Gordon M, Rogmark C, Rolfson O. Projections of total hip replacement in Sweden from 2013 to 2030. Acta Orthopaedica 2014;85(3):238-243.
6. Branemark PI. Vital microscopy of bone marrow in rabbit. Scand J Clin Lab Invest 1959;11 Supp 38:1-82.
7. Chrcanovic BR, Kisch J, Albrektsson T, Wennerberg A. A retrospective study on clinical and radiological outcomes of oral implants in patients followed up for a minimum of 20 years. Clin Implant Dent Relat Res 2018;20(2):199-207.
8. Jemt T. Single-Implant Survival: More Than 30 Years of Clinical Experience. Int J Prosthodont 2016;29(6):551-558.
9. Adell R, Lekholm U, Rockler B, Branemark PI. A 15-year study of osseointegrated implants in the treatment of the edentulous jaw. Int J Oral Surg 1981;10(6):387-416.
10. Tjellström A, Granström G. Long-term follow-up with the bone-anchored hearing aid: a review of the first 100 patients between 1977 and 1985. Ear Nose Throat J. 1994;73(2):112-114.
11. Tjellstrom A, Hakansson B. The Bone-Anchored Hearing-Aid - Design Principles, Indications, and Long-Term Clinical-Results. Otolaryngologic Clinics of North America 1995;28(1):53-72.
12. Albrektsson T, Branemark PI, Jacobsson M, Tjellstrom A. Present clinical applications of osseointegrated percutaneous implants. Plast Reconstr Surg 1987;79(5):721-31.
13. Brånemark R, Berlin O, Hagberg K, Bergh P, Gunterberg B, Rydevik B. A novel osseointegrated percutaneous prosthetic system for the treatment of patients with transfemoral amputation: A prospective study of 51 patients. *The Bone & Joint Journal* 2014;**96B**(1):106-13.
14. Howie DW, Cornish BL, Vernon-Roberts B. Resurfacing hip arthroplasty. Classification of loosening and the role of prosthesis wear particles. Clin Orthop Relat Res 1990(255):144-59.
15. Hagberg K, Hansson E, Branemark R. Outcome of Percutaneous Osseointegrated Prostheses for Patients With Unilateral Transfemoral Amputation at Two-Year Follow-Up. Archives of Physical Medicine and Rehabilitation 2014;95(11):2120-2127.
16. Palmquist A, Windahl SH, Norlindh B, Branemark R, Thomsen P. Retrieved bone-anchored percutaneous amputation prosthesis showing maintained osseointegration after 11 years-a case report. Acta Orthopaedica 2014;85(4):442-445.

17. Dillingham TR, Pezzin LE, MacKenzie EJ, Burgess AR. Use and satisfaction with prosthetic devices among persons with trauma-related amputations: a long-term outcome study. Am J Phys Med Rehabil 2001;80(8):563-71.
18. Hoaglund FT, Jergesen HE, Wilson L, Lamoreux LW, Roberts R. Evaluation of problems and needs of veteran lower-limb amputees in the San Francisco Bay Area during the period 1977-1980. J Rehabil R D 1983;20(1):57-71.
19. Legro MW, Reiber G, del Aguila M, Ajax MJ, Boone DA, Larsen JA, Smith DG, Sangeorzan B. Issues of importance reported by persons with lower limb amputations and prostheses. J Rehabil Res Dev 1999;36(3):155-63.
20. Dudek NL, Marks MB, Marshall SC, Chardon JP. Dermatologic conditions associated with use of a lower-extremity prosthesis. Arch Phys Med Rehabil 2005;86(4):659-63.
21. Hagberg K, Branemark R. Consequences of non-vascular trans-femoral amputation: a survey of quality of life, prosthetic use and problems. Prosthet Orthot Int 2001;25(3):186-94.
22. Aschoff HH, Kennon RE, Keggi JM, Rubin LE. Transcutaneous, distal femoral, intramedullary attachment for above-the-knee prostheses: an endo-exo device. J Bone Joint Surg Am 2010;92 Suppl 2:180-6.
23. Frolke JP, Leijendekkers RA, van de Meent H. Osseointegrated prosthesis for patients with an amputation : Multidisciplinary team approach in the Netherlands. Unfallchirurg 2017;120(4):293-299.
24. Al Muderis M, Khemka A, Lord SJ, Van de Meent H, Frolke JP. Safety of Osseointegrated Implants for Transfemoral Amputees: A Two-Center Prospective Cohort Study. J Bone Joint Surg Am 2016;98(11):900-9.
25. Dee KC, Puleo DA, Bizioa R. An introduction to Tissue-Biomaterial Interactions. Hoboken, New Jersey: John Wiley & Sons; 2003.
26. Diegelmann RF, Evans MC. Wound healing: an overview of acute, fibrotic and delayed healing. Front Biosci 2004;9:283-9.
27. Singer AJ, Clark RA. Cutaneous wound healing. N Engl J Med 1999;341(10):738-46.
28. Anderson JM, Rodriguez A, Chang DT. Foreign body reaction to biomaterials. Semin Immunol 2008;20(2):86-100.
29. Grice EA, Segre JA. The skin microbiome. Nature Reviews Microbiology 2011;9:244.
30. Wilson BA, Salyers AA, Whitt DD,Winkler ME. Bacterial pathogenesis: a molecular approach. Washington DC: ASM Press; 2011. 1-526 p.
31. von Recum AF. Applications and failure modes of percutaneous devices: a review. J Biomed Mater Res 1984;18(4):323-36.
32. Lenneras M, Tsikandylakis G, Trobos M, Omar O, Vazirisani F, Palmquist A, Berlin O, Branemark R, Thomsen P. The clinical, radiological, microbiological, and molecular profile of the skin-penentration site of transfemoral amputees treated with bone-anchored prostheses. Journal of Biomedical Materials Research Part A 2017;105A:578-589.
33. Johansson ML. The percutaneous implant. The effects of design, host site and surgery on the tissue response. Gothenburg: University of Gothenburg; 2018. 85 p.
34. Guggenbichler JP, Assadian O, Boeswald M, Kramer A. Incidence and clinical implication of nosocomial infections associated with implantable biomaterials - catheters, ventilator-associated pneumonia, urinary tract infections. GMS Krankenhaushygiene interdisziplinar 2011;6(1):Doc18-Doc18.

35. Hoiby N, Bjarnsholt T, Moser C, Bassi GL, Coenye T, Donelli G, Hall-Stoodley L, Hola V, Imbert C, Kirketerp-Moller K and others. ESCMID guideline for the diagnosis and treatment of biofilm infections 2014. Clin Microbiol Infect 2015;21 Suppl 1:S1-25.
36. Zimmerli W, Trampuz A, Ochsner PE. Prosthetic-joint infections. N Engl J Med 2004;351(16):1645-54.
37. Nilsdotter-Augustinsson A, Wilsson A, Larsson J, Stendahl O, Ohman L, Lundqvist-Gustafsson H. Staphylococcus aureus, but not Staphylococcus epidermidis, modulates the oxidative response and induces apoptosis in human neutrophils. Apmis 2004;112(2):109-18.
38. Nilsdotter-Augustinsson A, Claesson C, Lindgren PE, Lundqvist-Gustafsson H, Ohman L. Adherence of Staphylococcus epidermidis to extracellular matrix proteins and effects of fibrinogen-bound bacteria on oxidase activity and apoptosis in neutrophils. Apmis 2005;113(5):361-73.
39. Ince A, Rupp J, Frommelt L, Katzer A, Gille J, Lohr JF. Is "aseptic" loosening of the prosthetic cup after total hip replacement due to nonculturable bacterial pathogens in patients with low-grade infection? Clin Infect Dis 2004;39(11):1599-603.
40. Trampuz A, Osmon DR, Hanssen AD, Steckelberg JM, Patel R. Molecular and antibiofilm approaches to prosthetic joint infection. Clin Orthop Relat Res 2003(414):69-88.
41. Nilsdotter-Augustinsson A, Briheim G, Herder A, Ljunghusen O, Wahlstrom O, Ohman L. Inflammatory response in 85 patients with loosened hip prostheses: a prospective study comparing inflammatory markers in patients with aseptic and septic prosthetic loosening. Acta Orthop 2007;78(5):629-39.
42. Ribeiro M, Monteiro FJ, Ferraz MP. Infection of orthopedic implants with emphasis on bacterial adhesion process and techniques used in studying bacterial-material interactions. Biomatter 2012;2(4):176-94.
43. Brady RA, Leid JG, Calhoun JH, Costerton JW, Shirtliff ME. Osteomyelitis and the role of biofilms in chronic infection. *FEMS Immunology and Medical Microbiology* 2008;**52**(1):13-22.
44. Wright JA, Nair SP. Interaction of staphylococci with bone. International Journal of Medical Microbiology 2010;300(2-3):193-204.
45. Sia IG, Berbari EF. Infection and musculoskeletal conditions: Osteomyelitis. Best Pract Res Clin Rheumatol 2006;20(6):1065-81.
46. von Eiff C, Peters G, Becker K. The small colony variant (SCV) concept -- the role of staphylococcal SCVs in persistent infections. Injury 2006;37 Suppl 2:S26-33.
47. Tillander J, Hagberg K, Berlin O, Hagberg L, Branemark R. Osteomyelitis Risk in Patients With Transfemoral Amputations Treated With Osseointegration Prostheses. Clin Orthop Relat Res 2017;475(12):3100-3108.
48. Southwood RT, Rice JL, McDonald PJ, Hakendorf PH, Rozenbilds MA. Infection in experimental hip arthroplasties. J Bone Joint Surg Br 1985;67(2):229-31.
49. Gristina AG. Biomaterial-centered infection: microbial adhesion versus tissue integration. *Science* 1987;**237**(4822):1588-95.
50. Widmer A. New developments in diagnosis and treatment of infection in orthopedic implants. *Clinical Infectious Diseases* 2001;**33**(Suppl 2):S94-106.
51. Parvizi J. New definition for periprosthetic joint infection. *The American Journal of Orthopedics* 2011;40(12):614-615.

52. McArthur BA, Abdel MP, Taunton MJ, Osmon DR, Hanssen AD. Seronegative infections in hip and knee arthroplasty: periprosthetic infections with normal erythrocyte sedimentation rate and C-reactive protein level. Bone Joint J 2015;97-b(7):939-44.
53. Deirmengian C, Hallab N, Tarabishy A, Della Valle C, Jacobs JJ, Lonner J, Booth RE, Jr. Synovial fluid biomarkers for periprosthetic infection. Clinical orthopaedics and related research 2010;468(8):2017-23.
54. Xie K, Dai K, Qu X, Yan M. Serum and Synovial Fluid Interleukin-6 for the Diagnosis of Periprosthetic Joint Infection. Scientific reports 2017;7(1):1496-1496.
55. Carbonnelle E, Mesquita C, Bille E, Day N, Dauphin B, Beretti JL, Ferroni A, Gutmann L, Nassif X. MALDI-TOF mass spectrometry tools for bacterial identification in clinical microbiology laboratory. Clin Biochem 2011;44(1):104-9.
56. Bingham J, Clarke H, Spangehl M, Schwartz A, Beauchamp C, Goldberg B. The alpha defensin-1 biomarker assay can be used to evaluate the potentially infected total joint arthroplasty. Clin Orthop Relat Res 2014;472(12):4006-9.
57. Deirmengian C, Kardos K, Kilmartin P, Cameron A, Schiller K, Booth RE, Jr., Parvizi J. The alpha-defensin test for periprosthetic joint infection outperforms the leukocyte esterase test strip. Clin Orthop Relat Res 2015;473(1):198-203.
58. Kim SG, Kim JG, Jang KM, Han SB, Lim HC, Bae JH. Diagnostic Value of Synovial White Blood Cell Count and Serum C-Reactive Protein for Acute Periprosthetic Joint Infection After Knee Arthroplasty. J Arthroplasty 2017;32(12):3724-3728.
59. Parvizi J, Ghanem E, Sharkey P, Aggarwal A, Burnett RS, Barrack RL. Diagnosis of infected total knee: findings of a multicenter database. Clin Orthop Relat Res 2008;466(11):2628-33.
60. Saeed K. Diagnostics in prosthetic joint infections. J Antimicrob Chemother 2014;69 Suppl 1:i11-9.
61. Esteban J, Gomez-Barrena E, Cordero J, Martin-de-Hijas NZ, Kinnari TJ, Fernandez-Roblas R. Evaluation of quantitative analysis of cultures from sonicated retrieved orthopedic implants in diagnosis of orthopedic infection. J Clin Microbiol 2008;46(2):488-92.
62. Campoccia D, Montanaro L, Arciola CR. The significance of infection related to orthopedic devices and issues of antibiotic resistance. Biomaterials 2006;27(11):2331-9.
63. Trampuz A, Zimmerli W. Diagnosis and treatment of infections associated with fracture-fixation devices. Injury 2006;37 Suppl 2:S59-66.
64. Bauer TW, Parvizi J, Kobayashi N, Krebs V. Diagnosis of periprosthetic infection. J Bone Joint Surg Am 2006;88(4):869-82.
65. Rabin DN, Smith C, Kubicka RA, Rabin S, Ali A, Charters JR, Rabin H. Problem prostheses: the radiologic evaluation of total joint replacement. Radiographics 1987;7(6):1107-27.
66. Berger P, Van Cauter M, Driesen R, Neyt J, Cornu O, Bellemans J. Diagnosis of prosthetic joint infection with alpha-defensin using a lateral flow device: a multicentre study. Bone Joint J 2017;99-b(9):1176-1182.
67. Bonanzinga T, Zahar A, Dütsch M, Lausmann C, Kendoff D, Gehrke T. How Reliable Is the Alpha-defensin Immunoassay Test for Diagnosing Periprosthetic Joint Infection? A Prospective Study. Clinical orthopaedics and related research 2017;475(2):408-415.
68. Qu X, Zhai Z, Li H, Li H, Liu X, Zhu Z, Wang Y, Liu G, Dai K. PCR-based diagnosis of prosthetic joint infection. Journal of clinical microbiology 2013;51(8):2742-2746.

69. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science 1999;284(5418):1318-1322.
70. Trampuz A, Zimmerli W. Prosthetic joint infections: update in diagnosis and treatment. Swiss Med Wkly 2005;135(17-18):243-51.
71. Osmon DR, Berbari EF, Berendt AR, Lew D, Zimmerli W, Steckelberg JM, Rao N, Hanssen A, Wilson WR. Diagnosis and Management of Prosthetic Joint Infection: Clinical Practice Guidelines by the Infectious Diseases Society of America. Clinical Infectious Diseases 2013;56(1):1-10.
72. Oliveira WF, Silva PMS, Silva RCS, Silva GMM, Machado G, Coelho L, Correia MTS. Staphylococcus aureus and Staphylococcus epidermidis infections on implants. J Hosp Infect 2018;98(2):111-117.
73. Darouiche RO. Treatment of infections assoiciated with surgical implants. The New England journal of medicine 2004;350(14):1422-1429.
74. Trobos M, Johansson ML, Jonhede S, Peters H, Hoffman M, Omar O, Thomsen P, Hultcrantz M. The clinical outcome and microbiological profile of bone-anchored hearing systems (BAHS) with different abutment topographies: a prospective pilot study. Eur Arch Otorhinolaryngol 2018;275(6):1395-1408.
75. Stefansdottir A, Johansson D, Knutson K, Lidgren L, Robertsson O. Microbiology of the infected knee arthroplasty: Report from the Swedish Knee Arthroplasty Register on 426 surgically revised cases. Scandinavian Journal of Infectious Diseases 2009;41(11-12):831-840.
76. Gross M, Cramton SE, Götz F, Peschel A. Key role of teichoic acid net charge in Staphylococcus aureus colonization of artificial surfaces. Infection and immunity 2001;69(5):3423-3426.
77. Cardile AP, Sanchez CJ, Jr., Samberg ME, Romano DR, Hardy SK, Wenke JC, Murray CK, Akers KS. Human plasma enhances the expression of Staphylococcal microbial surface components recognizing adhesive matrix molecules promoting biofilm formation and increases antimicrobial tolerance In Vitro. BMC Res Notes 2014;7:457.
78. Darouiche RO, Landon GC, Patti JM, Nguyen LL, Fernau RC, McDevitt D, Greene C, Foster T, Klima M. Role of Staphylococcus aureus surface adhesins in orthopaedic device infections: Are results model-dependent? Journal of Medical Microbiology 1997;46(1):75-79.
79. Arciola CR, Campoccia D, Gamberini S, Baldassarri L, Montanaro L. Prevalence of cna, fnbA and fnbB adhesin genes among Staphylococcus aureus isolates from orthopedic infections associated to different types of implant. FEMS Microbiol Lett 2005;246(1):81-6.
80. Oliveira D, Borges A, Simoes M. Staphylococcus aureus Toxins and Their Molecular Activity in Infectious Diseases. Toxins 2018;10(6).
81. Foster TJ. Immune evasion by Staphylococci. Nature Reviews Microbiology 2005;3(12):948-958.
82. Foster TJ, Geoghegan JA, Ganesh VK, Hook M. Adhesion, invasion and evasion: the many functions of the surface proteins of Staphylococcus aureus. Nat Rev Microbiol 2014;12(1):49-62.
83. Dinges MM, Orwin PM, Schlievert PM. Exotoxins of Staphylococcus aureus. Clinical Microbiology Reviews 2000;13(1):16-+.
84. Bantel H, Sinha B, Domschke W, Peters G, Schulze-Osthoff K, Janicke RU. alpha-Toxin is a mediator of Staphylococcus aureus-induced cell death and activates caspases via the

intrinsic death pathway independently of death receptor signaling. J Cell Biol 2001;155(4):637-48.
85. Vuong C, Otto M. Staphylococcus epidermidis infections. Microbes Infect 2002;4(4):481-9.
86. Otto M. Molecular basis of Staphylococcus epidermidis infections. Semin Immunopathol 2012;34(2):201-14.
87. Otto M. Staphylococcus epidermidis--the 'accidental' pathogen. Nat Rev Microbiol 2009;7(8):555-67.
88. Fey PD, Olson ME. Current concepts in biofilm formation of Staphylococcus epidermidis. Future microbiology 2010;5(6):917-33.
89. Schommer NN, Christner M, Hentschke M, Ruckdeschel K, Aepfelbacher M, Rohde H. Staphylococcus epidermidis uses distinct mechanisms of biofilm formation to interfere with phagocytosis and activation of mouse macrophage-like cells 774A.1. Infect Immun 2011;79(6):2267-76.
90. Kocianova S, Vuong C, Yao Y, Voyich JM, Fischer ER, DeLeo FR, Otto M. Key role of poly-gamma-DL-glutamic acid in immune evasion and virulence of Staphylococcus epidermidis. The Journal of clinical investigation 2005;115(3):688-694.
91. Donlan RM. Biofilms and device-associated infections. Emerging Infectious Diseases 2001;7(2):277-281.
92. Ch'ng J-H, Chong KKL, Lam LN, Wong JJ, Kline KA. Biofilm-associated infection by enterococci. Nature Reviews Microbiology 2018.
93. Seneviratne CJ, Suriyanarayanan T, Swarup S, Chia KHB, Nagarajan N, Zhang C. Transcriptomics Analysis Reveals Putative Genes Involved in Biofilm Formation and Biofilm-associated Drug Resistance of Enterococcus faecalis. J Endod 2017;43(6):949-955.
94. Le KY, Otto M. Quorum-sensing regulation in staphylococci-an overview. Frontiers in Microbiology 2015;6.
95. Madigan M, Martinko J, Parker J. Regulation of gene expression. In: Carlson G, editor. Brock biology of microorganisms. Upper Saddle River: Pearson Education; 2003. p 224.
96. Waters C, Bassler B. Quorum sensing: Cell-to-cell communication in bacteria. Annu. Rev. Cell Dev. Biol 2005;21:319-346.
97. Boles BR, Horswill AR. Agr-mediated dispersal of *Staphylococcus aureus* biofilms. *PLoS Pathogens* 2008;**4**(4):e1000052.
98. Paharik AE, Horswill AR. The Staphylococcal Biofilm: Adhesins, Regulation, and Host Response. Microbiology Spectrum 2016;4(2).
99. Yarwood JM, Schlievert PM. Quorum sensing in Staphylococcus infections. Journal of Clinical Investigation 2003;112(11):1620-1625.
100. Flemming HC, Wingender J. The biofilm matrix. *Nature Reviews Microbiology* 2010;**8**(9):623-33.
101. Stoodley P, Dodds I, Boyle JD, Lappin-Scott HM. Influence of hydrodynamics and nutrients on biofilm structure. *Journal of Applied Microbiology Symposium Supplement* 1999;**85**:19S-28S.
102. Hall-Stoodley L, Stoodley P. Evolving concepts in biofilm infections. Cell Microbiol 2009;11(7):1034-43.
103. An YH, Friedman RJ. Concise Review of Mechanisms of Bacterial Adhesion to Biomaterial Surfaces. J Biomed Mater Res (Appl Biomater) 1998;43:338-348.

104. Hoiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O. Antibiotic resistance of bacterial biofilms. *International Journal of Antimicrobial Agents* 2010;**35**(4):322-32.
105. Lewis K. Persister Cells. In: Gottesman S, Harwood CS, editors. Annual Review of Microbiology, Vol 64, 2010; 2010. p 357-372.
106. Heilmann C, Schweitzer O, Gerke C, Vanittanakom N, Mack D, Gotz F. Molecular basis of intercellular adhesion in the biofilm-forming Staphylococcus epidermidis. Molecular Microbiology 1996;20(5):1083-1091.
107. O'Gara JP. ica and beyond: biofilm mechanisms and regulation in Staphylococcus epidermidis and Staphylococcus aureus. Fems Microbiology Letters 2007;270(2):179-188.
108. Donlan RM. Biofilms: microbial life on surfaces. *Emerging infectious diseases* 2002;**8**(9):881-90.
109. Zimmerli W, Moser C. Pathogenesis and treatment concepts of orthopaedic biofilm infections. *FEMS immunology and medical microbiology* 2012;**65**(2):158-68.
110. Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: From the natural environment to infectious diseases. Nature Reviews Microbiology 2004;2(2):95-108.
111. Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM. Microbial biofilms. Annu Rev Microbial 1995;49:711-45.
112. Archer NK, Mazaitis MJ, Costerton JW, Leid JG, Powers ME, Shirtliff ME. Staphylococcus aureus biofilms: properties, regulation, and roles in human disease. Virulence 2011;2(5):445-59.
113. Dapunt U, Hansch GM, Arciola CR. Innate Immune Response in Implant-Associated Infections: Neutrophils against Biofilms. Materials (Basel) 2016;9(5).
114. Francolini I, Donelli G. Prevention and control of biofilm-based medical-device-related infections. *FEMS Immunology and Medical Microbiology* 2010;**59**(3):227-38.
115. Donlan RM. Biofilm formation: A clinically relevant microbiological process. *Healthcare epidemiology* 2001;**33**:1387-1392.
116. Otto M. Staphylococcal infections: mechanisms of biofilm maturation and detachment as critical determinants of pathogenicity. Annual review of medicine 2013;64:175-88.
117. McKeegan KS, Borges-Walmsley MI, Walmsley AR. Microbial and viral drug resistance mechanisms. Trends in Microbiology 2002;10(10):s8-s14.
118. Hogan D, Kolter R. Why are bacteria refractory to antimicrobials? *Current opinion in Microbiology* 2002;**5**:472-477.
119. Davies D. Understanding biofilm resistance to antibacterial agents. Nature reviews. Drug discovery 2003;2(2):114-22.
120. Fisher RA, Gollan B, Helaine S. Persistent bacterial infections and persister cells. Nature Reviews Microbiology 2017;15(8):453-464.
121. Stewart PS. Mechanisms of antibiotic resistance in bacterial biofilms. International journal of medical microbiology : IJMM 2002;292(2):107-13.
122. Brauner A, Fridman O, Gefen O, Balaban NQ. Distinguishing between resistance, tolerance and persistence to antibiotic treatment. Nature Reviews Microbiology 2016;14(5):320-330.
123. Beveridge TJ. Structures of gram-negative cell walls and their derived membrane vesicles. Journal of Bacteriology 1999;181(16):4725-4733.
124. Ellis TN, Kuehn MJ. Virulence and Immunomodulatory Roles of Bacterial Outer Membrane Vesicles. Microbiology and Molecular Biology Reviews 2010;74(1):81-+.
125. Kuehn MJ, Kesty NC. Bacterial outer membrane vesicles and the host-pathogen interaction. Genes Dev 2005;19(22):2645-55.

126. Brown L, Wolf JM, Prados-Rosales R, Casadevall A. Through the wall: extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi. Nat Rev Microbiol 2015;13(10):620-30.
127. Bonnington KE, Kuehn MJ. Protein selection and export via outer membrane vesicles. Biochim Biophys Acta 2014;1843(8):1612-9.
128. Haurat MF, Elhenawy W, Feldman MF. Prokaryotic membrane vesicles: new insights on biogenesis and biological roles. Biol Chem 2015;396(2):95-109.
129. Manning AJ, Kuehn MJ. Contribution of bacterial outer membrane vesicles to innate bacterial defense. Bmc Microbiology 2011;11.
130. Ciofu O, Beveridge TJ, Kadurugamuwa J, Walther-Rasmussen J, Hoiby N. Chromosomal beta-lactamase is packaged into membrane vesicles and secreted from Pseudomonas aeruginosa. Journal of Antimicrobial Chemotherapy 2000;45(1):9-13.
131. Schooling SR, Beveridge TJ. Membrane Vesicles: an Overlooked Component of the Matrices of Biofilms. The Journal of Bacteriology 2006;188(16):5945.
132. Lee E-Y, Choi D-Y-, Kim D-K, Kim J-W, Park JO, Kim SH, Kim S-H, Desiderio DM, Kim Y-K, Kim K-P and others. Gram-positive bacteria produce membrane vesicles: Proteomics-based characterization of Staphylococcus aureus-derived membrane vesicles. Proteomics 2009;9(24):5425-5436.
133. Lee JC. Staphylococcus aureus membrane vesicles and Its potential role in bacterial pathogenesis. Journal of Bacteriology and Virology 2012;42(3):181.
134. Lee EY, Choi DY, Kim DK, Kim JW, Park JO, Kim S, Kim SH, Desiderio DM, Kim YK, Kim KP and others. Gram-positive bacteria produce membrane vesicles: proteomics-based characterization of Staphylococcus aureus-derived membrane vesicles. Proteomics 2009;9(24):5425-36.
135. Kim JH, Lee J, Park J, Gho YS. Gram-negative and Gram-positive bacterial extracellular vesicles. Semin Cell Dev Biol 2015.
136. Gurung M, Moon DC, Choi CW, Lee JH, Bae YC, Kim J, Lee Y, Seol S, Cho D, Kim S and others. Staphylococcus aureus Produces Membrane-Derived Vesicles That Induce Host Cell Death. PLoS ONE 2011;6(11).
137. Thay B, Wai S, Oscarsson J. Staphylococcus aureus a-Toxin-Dependent Induction of Host Cell Death by Membrane-Derived Vesicles. PLoS ONE 2013;8(1).
138. Lee J, Lee EY, Kim SH, Kim DK, Park KS, Kim KP, Kim YK, Roh TY, Gho YS. Staphylococcus aureus extracellular vesicles carry biologically active beta-lactamase. Antimicrob Agents Chemother 2013;57(6):2589-95.
139. Monteiro DR, Gorup LF, Takamiya AS, Ruvollo-Filho AC, de Camargo ER, Barbosa DB. The growing importance of materials that prevent microbial adhesion: antimicrobial effect of medical devices containing silver. International journal of antimicrobial agents 2009;34(2):103-10.
140. Campoccia D, Montanaro L, Arciola CR. A review of the biomaterials technologies for infection-resistant surfaces. Biomaterials 2013;34(34):8533-8554.
141. Von Eiff C, Kohnen W, Becker K, Jansen B. Modern strategies in the prevention of implant-associated infections. The international Journal of Artificial Organs 2005;28(11):1146-1156.
142. Busscher HJ, van der Mei HC, Subbiahdoss G, Jutte PC, van den Dungen JJ, Zaat SA, Schultz MJ, Grainger DW. Biomaterial-associated infection: locating the finish line in the race for the surface. Sci Transl Med 2012;4(153):153rv10.

143. Walluscheck KP, Steinhoff G, Kelm S, Haverich A. Improved endothelial cell attachment on ePTFE vascular grafts pretreated with synthetic RGD-containing peptides. Eur J Vasc Endovasc Surg 1996;12(3):321-30.
144. Zheng W, Wang Z, Song L, Zhao Q, Zhang J, Li D, Wang S, Han J, Zheng XL, Yang Z and others. Endothelialization and patency of RGD-functionalized vascular grafts in a rabbit carotid artery model. Biomaterials 2012;33(10):2880-91.
145. Hallan G, Lie SA, Furnes O, Engesaeter LB, Vollset SE, Havelin LI. Medium- and long-term performance of 11,516 uncemented primary femoral stems from the Norwegian arthroplasty register. J Bone Joint Surg Br 2007;89(12):1574-80.
146. Rupp ME, Fitzgerald T, Marion N, Helget V, Puumala S, Anderson JR, Fey PD. Effect of silver-coated urinary catheters: efficacy, cost-effectiveness, and antimicrobial resistance. Am J Infect Control 2004;32(8):445-50.
147. Leaper DJ. Silver dressings: their role in wound management. International wound journal 2006;3(4):282-94.
148. Schierholz JM, Lucas LJ, Rump A, Pulverer G. Efficacy of silver-coated medical devices. Journal of hospital infection 1998;40:257-262.
149. Melaiye A, Youngs W. Silver and its application as an antimicrobial agent. Expert Opin. Ther. Patents 2005;15(2):125–130.
150. Zhao L, Chu PK, Zhang Y, Wu Z. Antibacterial coatings on titanium implants. Journal of biomedical materials research. Part B, Applied biomaterials 2009;91(1):470-80.
151. Gosheger G, Hardes J, Ahrens H, Streitburger A, Buerger H, Erren M, Gunsel A, Kemper FH, Winkelmann W, Von Eiff C. Silver-coated megaendoprostheses in a rabbit model--an analysis of the infection rate and toxicological side effects. Biomaterials 2004;25(24):5547-56.
152. Trop M, Novak M, Rodl S, Hellbom B, Kroell W, Goessler W. Silver-coated dressing acticoat caused raised liver enzymes and argyria-like symptoms in burn patient. J Trauma 2006;60(3):648-52.
153. Bahar AA, Ren D. Antimicrobial peptides. Pharmaceuticals (Basel, Switzerland) 2013;6(12):1543-1575.
154. Mahlapuu M, Håkansson J, Ringstad L, Björn C. Antimicrobial Peptides: An Emerging Category of Therapeutic Agents. Frontiers in cellular and infection microbiology 2016;6:194-194.
155. Kwakman PHS, Zaat SAJ. Preventive measures against transcutaneous device infections. In: Moriarty TF, Zaat SAJ, Busscher HJ, editors. Biomaterials Associated infection - Immunological aspects and antimicrobial strategies. New York: Springer; 2012. p 238-242.
156. Ceri H, Olson ME, Stremick C, Read RR, Morck D, Buret A. The Calgary Biofilm Device: New technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *Journal of Clinical Microbiology* 1999;37(7):1771-1776.
157. Tillander J, Hagberg K, Hagberg L, Branemark R. Osseointegrated titanium implants for limb prostheses attachments: infectious complications. *Clinical orthopaedics and related research* 2010;**468**(10):2781-8.
158. Christensen GD, Simpson WA, Bisno AL, Beachey EH. Adherence of slime-producing strains of Staphylococcus epidermidis to smooth surfaces. Infect Immun 1982;37(1):318-26.

159. Zhang YQ, Ren SX, Li HL, Wang YX, Fu G, Yang J, Qin ZQ, Miao YG, Wang WY, Chen RS and others. Genome-based analysis of virulence genes in a non-biofilm-forming Staphylococcus epidermidis strain (ATCC 12228). Mol Microbiol 2003;49(6):1577-93.
160. Treangen TJ, Maybank RA, Enke S, Friss MB, Diviak LF, Karaolis DKR, Koren S, Ondov B, Phillippy AM, Bergman NH and others. Complete Genome Sequence of the Quality Control Strain Staphylococcus aureus subsp. aureus ATCC 25923. Genome announcements 2014;2(6):e01110-14.
161. Wikler MA, Cockerill FR, Craig WA, Dudley MN, Eliopoulos GM, Hecht DW. Performance standards for antimicrobial susceptibility testing; seventeenth informational supplement. Clin Lab Stand Inst. 2007;27:M100–S17.
162. Kadurugamuwa JL, Sin L, Albert E, Yu J, Francis K, DeBoer M, Rubin M, Bellinger-Kawahara C, Parr TR, Jr., Contag PR. Direct continuous method for monitoring biofilm infection in a mouse model. Infect Immun 2003;71(2):882-90.
163. Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, Brinkman FS, Hufnagle WO, Kowalik DJ, Lagrou M and others. Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen. Nature 2000;406(6799):959-64.
164. Lilja M, Welch K, Åstrand M, Engqvist H, Strømme M. Effect of deposition parameters on the photocatalytic activity and bioactivity of TiO2 thin films depostited by vacuum arc on Ti-6Al-4V substrates. J Biomed Mater Res B 2012;100B:1078-1085.
165. Khalipour P, Lampe K, Wagener M, Stigler B, Heiss C, Ullrich M, Domann E, Schnettler R, Alt V. Ag/SiOxCy plasma polymer coating for antimicrobial protection of fracture fixation devices. Journal of Biomedical Materials Research B: Applied Biomaterials 2010;94B(1):196-202.
166. Christensen GD, Simpson WA, Younger JJ, Baddour LM, Barrett FF, Melton DM, Beachey EH. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. *Journal of Clinical Microbiology* 1985;**22**(6):996-1006.
167. Arciola CR, Collamati S, Donati E, Montanaro L. A rapid PCR method for the detection of slime-producing strain of *Staphylococcus epidermidis* and *S. aureus* in periprosthesis infections. *Diagnostic Molecular Pathology* 2001;**10**(2):130-137.
168. Arciola CR, Campoccia D, Ravaioli S, Montanaro L. Polysaccharide intercellular adhesin in biofilm: structural and regulatory aspects. Front Cell Infect Microbiol 2015;5:7.
169. Lidgren L. Joint prosthetic infections: a success story. Acta Orthop Scand 2001;72(6):553-6.
170. Hall-Stoodley L, Stoodley P, Kathju S, Hoiby N, Moser C, Costerton JW, Moter A, Bjarnsholt T. Towards diagnostic guidelines for biofilm-associated infections. FEMS Immunol Med Microbiol 2012;65(2):127-45.
171. McConoughey SJ, Howlin R, Granger JF, Manring MM, Calhoun JH, Shirtliff M, Kathju S, Stoodley P. Biofilms in periprosthetic orthopedic infections. Future Microbiol 2014;9(8):987-1007.
172. Guerra-Caceres JG, Gotuzzo-Herencia E, Crosby-Dagnino E, Miro-Quesada M, Carrillo-Parodi C. Diagnostic value of bone marrow culture in typhoid fever. *Transaction of the royal society of tropical medicine and hygiene* 1979;**73**(6):680-683.
173. Neut D, van Horn JR, van Kooten TG, van der Mei HC, Busscher HJ. Detection of biomaterial-associated infections in orthopaedic joint implants. Clin Orthop Relat Res 2003(413):261-8.

174. Puig-Verdie L, Alentorn-Geli E, Gonzalez-Cuevas A, Sorli L, Salvado M, Alier A, Pelfort X, Portillo ME, Horcajada JP. Implant sonication increases the diagnostic accuracy of infection in patients with delayed, but not early, orthopaedic implant failure. Bone Joint J 2013;95-b(2):244-9.
175. Trampuz A, Piper KE, Jacobson MJ, Hanssen AD, Unni KK, Osmon DR, Mandrekar JN, Cockerill FR, Steckelberg JM, Greenleaf JF and others. Sonication of removed hip and knee prostheses for diagnosis of infection. N Engl J Med 2007;357(7):654-63.
176. Molina-Manso D, del Prado G, Ortiz-Perez A, Manrubia-Cobo M, Gomez-Barrena E, Cordero-Ampuero J, Esteban J. In vitro susceptibility to antibiotics of staphylococci in biofilms isolated from orthopaedic infections. *International Journal of Antimicrobial Agents* 2013;41(6):521-3.
177. Arciola CR, Campoccia D, Gamberini S, Donati ME, Pirini V, Visai L, Speziale P, Montanaro L. Antibiotic resistance in exopolysaccharide-forming Staphylococcus epidermidis clinical isolates from orthopaedic implant infections. Biomaterials 2005;26(33):6530-6535.
178. Konig C, Schwank S, Blaser J. Factors compromising antibiotic activity against biofilms of Staphylococcus epidermidis. European Journal of Clinical Microbiology & Infectious Diseases 2001;20(1):20-26.
179. Schwank S, Rajacic Z, Zimmerli W, Blaser J. Impact of bacterial biofilm formation on in vitro and in vivo activities of antibiotics. Antimicrob Agents Chemother 1998;42(4):895-8.
180. Souli M, Giamarellou H. Effects of Slime Produced by Clinical Isolates of Coagulase-Negative Staphylococci on Activities of Various Antimicrobial Agents. Antimicrobial Agents and Chemotherapy 1998;42(4):939.
181. Farber BF, Kaplan MH, Clogston AG. Staphylococcus epidermidis extracted slime inhibits the antimicrobial action of glycopeptide antibiotics. J Infect Dis 1990;161(1):37-40.
182. Campanac C, Pineau L, Payard A, Baziard-Mouysset G, Roques C. Interactions between biocide cationic agents and bacterial biofilms. Antimicrob Agents Chemother 2002;46(5):1469-74.
183. Cramton SE, Gerke C, Schnell NF, Nichols WW, Gotz F. The intercellular adhesion (ica) locus is present in *Staphylococcus aureus* and is required for biofilm formation. *Infection and Immunity* 1999;**67**:5427-5433.
184. Rohde H, Burandt EC, Siemssen N, Frommelt L, Burdelski C, Wurster S, Scherpe S, Davies AP, Harris LG, Horstkotte MA and others. Polysaccharide intercellular adhesin or protein factors in biofilm accumulation of Staphylococcus epidermidis and Staphylococcus aureus isolated from prosthetic hip and knee joint infections. Biomaterials 2007;28(9):1711-20.
185. Cucarella C, Solano C, Valle J, Amorena B, Lasa I, Penades JR. Bap, a Staphylococcus aureus surface protein involved in biofilm formation. J Bacteriol 2001;183(9):2888-96.
186. Arciola CR, Baldassarri L, Montanaro L. Presence of icaA and icaD genes and slime production in a collection of staphylococcal strains from catheter-associated infections. J Clin Microbiol 2001;39(6):2151-6.
187. Wu H, Moser C, Wang H-Z, Høiby N, Song Z-J. Strategies for combating bacterial biofilm infections. International journal of oral science 2014;7(1):1-7.
188. Arcola CR, Campoccia D, Gamberini S, Cervellati M. Detection of slime production by means of an optimised Congo red agar plate test based on a coloumetric scale in

Staphylococcus epidermidis clinical isolates genotyped for *ica* locus. *Biomaterials* 2002;**23**:4233-4239.

189. Tran PL, Lowry N, Campbell T, Reid TW, Webster DR, Tobin E, Aslani A, Mosley T, Dertien J, Colmer-Hamood JA and others. An organoselenium compound inhibits Staphylococcus aureus biofilms on hemodialysis catheters in vivo. Antimicrob Agents Chemother 2012;56(2):972-8.
190. Tollefson DF, Bandyk DF, Kaebnick HW, Seabrook GR, Towne JB. Surface biofilm disruption. Enhanced recovery of microorganisms from vascular prostheses. Arch Surg 1987;122(1):38-43.
191. Fux CA, Quigley M, Worel AM, Post C, Zimmerli S, Ehrlich G, Veeh RH. Biofilm-related infections of cerebrospinal fluid shunts. Clin Microbiol Infect 2006;12(4):331-7.
192. Hoiby N, Ciofu O, Bjarnsholt T. Pseudomonas aeruginosa biofilms in cystic fibrosis. Future Microbiol 2010;5(11):1663-74.
193. Wessman M, Bjarnsholt T, Eickhardt-Sorensen SR, Johansen HK, Homoe P. Mucosal biofilm detection in chronic otitis media: a study of middle ear biopsies from Greenlandic patients. Eur Arch Otorhinolaryngol 2015;272(5):1079-85.
194. Percival SL, Hill KE, Williams DW, Hooper SJ, Thomas DW, Costerton JW. A review of the scientific evidence for biofilms in wounds. Wound Repair Regen 2012;20(5):647-57.
195. Hoiby N, Bjarnsholt T, Moser C, Jensen PO, Kolpen M, Qvist T, Aanaes K, Pressler T, Skov M, Ciofu O. Diagnosis of biofilm infections in cystic fibrosis patients. Apmis 2017;125(4):339-343.
196. Kirketerp-Møller K, Jensen P, Fazli M, Madsen KG, Pedersen J, Moser C, Tolker-Nielsen T, Høiby N, Givskov M, Bjarnsholt T. Distribution, organization, and ecology of bacteria in chronic wounds. J Clin Microbiol 2008;46(8):2717-22.
197. Sanchez CJ, Jr., Mende K, Beckius ML, Akers KS, Romano DR, Wenke JC, Murray CK. Biofilm formation by clinical isolates and the implications in chronic infections. *BMC Infectious Diseases* 2013;13:47.
198. Arciola CR, Campoccia D, Ehrlich GD, Montanaro L. Biofilm-Based Implant Infections in Orthopaedics. In: Donelli G, editor. Biofilm-Based Healthcare-Associated Infections, Vol I; 2015. p 29-46.
199. Parsek MR, Singh PK. Bacterial biofilms: an emerging link to disease pathogenesis. Annu Rev Microbiol 2003;57:677-701.
200. Gamalier JP, Silva TP, Zarantonello V, Dias FP, Melo RCN. Increased production of outer membrane vesicles by cultured freshwater bacteria in response to ultraviolet radiation. Microbiological Research 2017;194:38-46.
201. Kadurugamuwa JL, Beveridge TJ. Natural release of virulence factors in membrane vesicles by Pseudomonas aeruginosa and the effect of aminoglycoside antibiotics on their release. The Journal of antimicrobial chemotherapy 1997;40(5):615.
202. Maredia R, Devineni N, Lentz P, Dallo SF, Yu JJ, Guentzel N, Chambers J, Arulanandam B, Haskins WE, Tao WT. Vesiculation from Pseudomonas aeruginosa under SOS. Scientific World Journal 2012:1-18.
203. Lamparski HG, Metha-Damani A, Yao JY, Patel S, Hsu DH, Ruegg C, Le Pecq JB. Production and characterization of clinical grade exosomes derived from dendritic cells. J Immunol Methods 2002;270(2):211-26.

204. Théry C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Current protocols in cell biology 2006;Chapter 3(1):Unit 3.22.
205. Zaborowski MP, Balaj L, Breakefield XO, Lai CP. Extracellular Vesicles: Composition, Biological Relevance, and Methods of Study. Bioscience 2015;65(8):783-797.
206. Liga A, Vliegenthart AD, Oosthuyzen W, Dear JW, Kersaudy-Kerhoas M. Exosome isolation: a microfluidic road-map. Lab Chip 2015;15(11):2388-94.
207. Konoshenko MY, Lekchnov EA, Vlassov AV, Laktionov PP. Isolation of Extracellular Vesicles: General Methodologies and Latest Trends. Biomed Res Int 2018;2018:8545347.
208. Chernyshev VS, Rachamadugu R, Tseng YH, Belnap DM, Jia YL, Branch KJ, Butterfield AE, Pease LF, Bernard PS, Skliar M. Size and shape characterization of hydrated and desiccated exosomes. Analytical and Bioanalytical Chemistry 2015;407(12):3285-3301.
209. Wu YT, Deng WT, Klinke DJ. Exosomes: improved methods to characterize their morphology, RNA content, and surface protein biomarkers. Analyst 2015;140(19):6631-6642.
210. Beceiro A, Tomas M, Bou G. Antimicrobial Resistance and Virulence: a Successful or Deleterious Association in the Bacterial World? Clinical Microbiology Reviews 2013;26(2):185-230.
211. Ellen RP, Grove DA, Grove DA. Bacteroides gingivalis vesicles bind to and aggregate Actinomyces viscosus. Infection and Immunity 1989;57(5):1618.
212. Baumgarten T, Sperling S, Seifert J, Von Bergen M, Steiniger F, Wick L, Heipieper H. Membrane Vesicle Formation as a Multiple-Stress Response Mechanism Enhances Pseudomonas putida DOT-T1E Cell Surface Hydrophobicity and Biofilm Formation. Applied and Environmental Microbiology 2012;78(17):6217.
213. Bagge N, Schuster M, Hentzer M, Ciofu O, Givskov M, Greenberg EP, Hoiby N. Pseudomonas aeruginosa biofilms exposed to Imipenem exhibit changes in global gene expression and {beta}-Lactamase and alginate production. Antimicrobial Agents and Chemotherapy 2004;48(4):1175.
214. Lucas RH, David ADA, Michael JM, Zhaoying Z, Roger AJ, Samuel IM. Aminoglycoside antibiotics induce bacterial biofilm formation. Nature 2005;436(7054):1171.
215. Im H, Lee S, Soper SA, Mitchell RJ. Staphylococcus aureus extracellular vesicles (EVs): surface-binding antagonists of biofilm formation. Molecular BioSystems 2017;13(12):2704-2714.
216. Grumann D, Nubel U, Broker BM. Staphylococcus aureus toxins - Their functions and genetics. Infection Genetics and Evolution 2014;21:583-592.
217. Cheung GY, Rigby K, Wang R, Queck SY, Braughton KR, Whitney AR, Teintze M, DeLeo FR, Otto M. Staphylococcus epidermidis strategies to avoid killing by human neutrophils. PLoS Pathog 2010;6(10):e1001133.
218. Shoshani T, Faerman A, Mett I, Zelin E, Tenne T, Gorodin S, Moshel Y, Elbaz S, Budanov A, Chajut A and others. Identification of a Novel Hypoxia-Inducible Factor 1-Responsive Gene, RTP801, Involved in Apoptosis. Molecular and Cellular Biology 2002;22(7):2283.
219. Malagelada C, Ryu EJ, Biswas SC, Jackson-Lewis V, Greene LA. RTP801 is elevated in Parkinson brain substantia nigral neurons and mediates death in cellular models of Parkinson's disease by a mechanism involving mammalian target of rapamycin inactivation.

The Journal of neuroscience : the official journal of the Society for Neuroscience 2006;26(39):9996.
220. Svensson S, Forsberg M, Hulander M, Vazirisani F, Palmquist A, Lausmaa J, Thomsen P, Trobos M. Role of nanostructured gold surfaces on monocyte activation and Staphylococcus epidermidis biofilm formation. Int J Nanomedicine 2014;9:775-94.
221. Svensson S, Trobos M, Hoffman M, Norlindh B, Petronis S, Lausmaa J, Suska F, Thomsen P. A novel soft tissue model for biomaterial-associated infection and inflammation – Bacteriological, morphological and molecular observations. *Biomaterials* 2015;**41**:106-121.
222. Arciola C, Campoccia D, Montanaro L. Implant infections: adhesion, biofilm formation and immune evasion. Volume 16. London: Nature Publishing Group; 2018. p 397-409.
223. Janssens S, Beyaert R. Role of Toll-like receptors in pathogen recognition. Clinical microbiology reviews 2003;16(4):637-646.
224. Lotz S, Aga E, Wilde I, van Zandbergen G, Hartung T, Solbach W, Laskay T. Highly purified lipoteichoic acid activates neutrophil granulocytes and delays their spontaneous apoptosis via CD14 and TLR2. J Leukoc Biol 2004;75(3):467-77.
225. Tatematsu M, Nishikawa F, Seya T, Matsumoto M. Toll-like receptor 3 recognizes incomplete stem structures in single-stranded viral RNA. Nature Communications 2013;4:1833.
226. Jeon H, Oh MH, Jun SH, Il Kim S, Choi CW, Kwon HI, Na SH, Kim YJ, Nicholas A, Selasi GN and others. Variation among Staphylococcus aureus membrane vesicle proteomes affects cytotoxicity of host cells. Microbial Pathogenesis 2016;93:185-193.
227. Refai AK, Textor M, Brunette DM, Waterfield JD. Effect of titanium surface topography on macrophage activation and secretion of proinflammatory cytokines and chemokines. J Biomed Mater Res A 2004;70(2):194-205.
228. Baker CN, Thornsberry C, Hawkinson RW. Inoculum standardization in antimicrobial susceptibility testing: evaluation of overnight agar cultures and the Rapid Inoculum Standardization System. Journal of clinical microbiology 1983;17(3):450-457.
229. Bretagnol F, Rauscher H, Hasiwa M, Kylian O, Ceccone G, Hazell L, Paul AJ, Lefranc O, Rossi F. The effect of sterilization processes on the bioadhesive properties and surface chemistry of a plasma-polymerized polyethylene glycol film: XPS characterization and L929 cell proliferation tests. Acta Biomater 2008;4(6):1745-51.
230. Doundoulakis JH. Surface analysis of titanium after sterilization: role in implant-tissue interface and bioadhesion. J Prosthet Dent 1987;58(4):471-8.
231. Cavenaghi LA, Biganzoli E, Danese A, Parenti F. Diffusion of teicoplanin and vancomycin in agar. Diagn Microbiol Infect Dis 1992;15(3):253-8.
232. Patel JD, Ebert M, Ward R, Anderson JM. S. epidermidis biofilm formation: effects of biomaterial surface chemistry and serum proteins. J Biomed Mater Res A 2007;80(3):742-51.
233. Palmquist A, Omar OM, Esposito M, Lausmaa J, Thomsen P. Titanium oral implants: surface characteristics, interface biology and clinical outcome. J R Soc Interface 2010;7 Suppl 5:S515-27.
234. Shah FA, Thomsen P, Palmquist A. Osseointegration and current interpretations of the bone-implant interface. Acta Biomaterialia 2018;In press.
235. Branemark R, Berlin O, Hagberg K, Bergh P, Gunterberg B, Rydevik B. A novel osseointegrated percutaneous prosthetic system for the treatment of patients with

transfemoral amputation: A prospective study of 51 patients. Bone Joint J 2014;96-b(1):106-13.
236. Thesleff A, Branemark R, Hakansson B, Ortiz-Catalan M. Biomechanical Characterisation of Bone-anchored Implant Systems for Amputation Limb Prostheses: A Systematic Review. Ann Biomed Eng 2018;46(3):377-391.
237. Svensson S, Suska F, Emanuelsson L, Palmquist A, Norlindh B, Trobos M, Backros H, Persson L, Rydja G, Ohrlander M and others. Osseointegration of titanium with an antimicrobial nanostructured noble metal coating. Nanomedicine 2013;9(7):1048-56.
238. Malchau H, Bragdon CR, Muratoglu OK. The stepwise introduction of innovation into orthopedic surgery: the next level of dilemmas. J Arthroplasty 2011;26(6):825-31.