Fracture-related infection: perspectives of an orthopaedic-trauma surgeon

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Fracture-related infection (FRI) is one of the most feared complications after musculoskeletal injuries since it has not only important clinical consequences, but also a significant socioeconomic impact.

Even though FRI is one of the oldest disease entities, it has only recently been defined. Therefore, its global burden is still largely unknown and can only be estimated as shown in Figure 1.

These infections mainly result from bacteria entering the wound during the injury or surgical procedures. In contrast to periprosthetic joint infections (PJI) they are rarely caused by bloodstream infection. The pathogenic bacteria cause disruption of normal bone healing by diverting the host responses from a bone-healing course to an inflammatory and antibacterial course.

The key in treating FRIs is a multi-disciplinary team approach of orthopaedic surgeon, plastic surgeons, infectious disease specialists and various other disciplines in order to achieve bone healing, restoration of a competent soft-tissue envelope, eradication of the infection and re-establishment of mobility and health-related quality of life (HRQOL).

Since FRIs are often associated with prolonged hospital stays, repeated surgeries, and extended antibiotic treatments, they are not only associated with significant impairments of physical and mental HRQOL, but also with a huge global burden for the healthcare systems.

To improve patient outcomes on a global scale, research efforts in the field of infection prevention, diagnosis and novel treatment modalities deserve prioritization. [1, 2]

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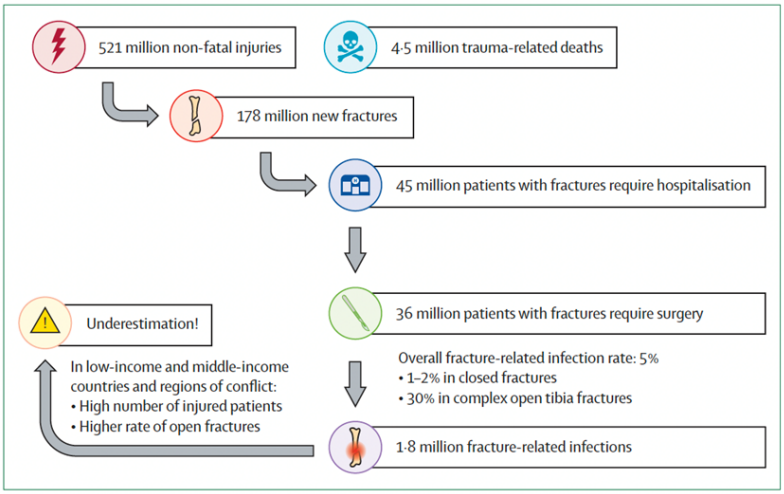

Figure 1: The global burden of fracture-related infection from [1]

Prevention and treatment of implant-associated infection: an ID Physician perspective

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INTRODUCTION:
Implant-associated infections (IAI) are steadily increasing in numbers due to the increased number of arthroplasties and other orthopaedic implants in our aging population to regain mobility. These infections are difficult to treat because bacteria form a biofilm on the surface of the implant. A biofilm is defined as a sessile community of microbial cells (=bacteria) which (i) are attached to a substratum, interface, or each other; (ii) are embedded in a matrix of (partially self-produced) extracellular polymeric substances; and (iii) exhibit an altered phenotype in regard to growth, gene expression, and protein production compared to planktonic cells (floating single cells) (**Fig. 1**). Some bacteria in chronic biofilms also persist intracellularly.

TREATMENT: Eradication of biofilm bacteria in IAI – in contrast to planktonic bacteria in soft-tissue infections without an implant – are challenging. and need adequate surgical debridement as well as prolonged antibiotic treatment, independently of antibiotic susceptibility testing in vitro, with the few compounds available being active against sessile bacteria. It is recommended to use antibiotics which penetrate the biofilm and have intracellular activity. A subgroup of bacteria within the biofilm behaves as persisters – defined as bacteria which survive the antibiotic treatment. They need to be treated for a long time or with high antibiotic concentration to reach the biofilm eradication concentration. To limit the systemic toxicity of the antibiotic, antibiotics are applicated within a carrier at the site of infection (e.g calcium sulphate carrier, polymethyl methacrylatecement carrier, plasma rich fibrin (PRF), other products in development). A novel treatment strategy (1), which permits preserving the prosthesis and with shortened antibiotic treatment length, would be highly advantageous.

PREVENTION: Current prevention strategies for IAI include an antibiotic prophylaxis shot 0 – 60 minutes before the orthopedic surgery and a skin antisepsis before skin incision. As a fact, standard skin antisepsis to prevent surgical site infections is ineffective to eradicate all skin-colonizing bacteria at time of surgery. There are innovative approaches – such as photodynamic therapy (2) - to inactive skin colonizing bacteria and to sterilize the operating field. Another prevention strategy is the use of different wash out solutions for potential early intraoperative contaminated wounds. Some compounds focus on destabilizing early formed biofilms and bactericidal killing of bacteria.

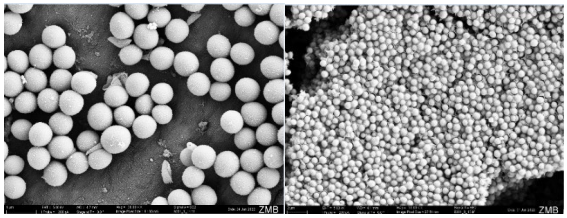

Fig. 1*: Planktonic bacteria (Staphylococcus aureus) (left) and bacteria (S. aureus) within a biofilm (unpublished SEM pictures).*

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Clinical Outcome Predictors and Rates of Infection Following Open Tibia Fractures in Latin America

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INTRODUCTION: Open tibia fractures remain a significant healthcare issue and source of fracture-related infections worldwide, including Latin America. Previous work in this region demonstrated that the severity of fracture correlated significantly with health-related quality of life (HRQOL). This study sought to evaluate factors, including injury severity, delayed treatment, and method of fracture stabilization that were associated with decreased HRQOL scores and infection rates following open tibia fractures in Latin America.

METHODS: Sixteen trauma centers in seven Latin American countries enrolled patients (aged ≥18 years) with isolated AO/OTA type 42 open tibial diaphyseal fractures between 2018-2022. Demographic and medical history, injury characteristics, radiographs, management, and a 12-Item Short Form Health Survey (SF-12) were collected at enrollment, 6, 12, 26, and 52-weeks post-operatively. A secondary analysis was performed to examine the modified Radiographic Union Scale for Tibial Fractures (mRUST) scores, complications (including superficial and deep infection rates), and return to work following injury.

RESULTS: 288 patients completed the initial enrollment data and follow-up through one-year (68.1% response rate). The mRUST score was a significant predictor of the SF-12 Physical Component Summary (PCS) score (estimate: 1.2, 95% CI (0.83 – 1.56)), with the one-year post-injury mean score being 14.9 ± 2.3, demonstrating that fracture healing correlated significantly with improved HRQOL outcomes. Additionally, 58.0% of patients returned to work at six months, and the majority (82.2%) returned to work at one-year post-injury, with GA Type I, II, and IIIA fractures showing the highest rates of healing. The mean mRUST score at one-year did not attain a score reflecting union for GA Type IIIB and Type IIIC fractures. The greater the severity of fracture type, the higher the rates of nonunion and other complications (Table 1). The most frequent complications reported were nonunion (6.9%), reoperation (6.3%), superficial infection (5.9%), and deep infection (3.5%).

Table 1. Complications Stratified by Gustilo-Anderson Fracture Type

	N (%) *288 (100)*	N=36	N=173	N=64	N=13	N=2
Gustilo-Anderson Classification	Total	GA Type I	GA Type II	GA Type IIIA	GA Type IIIB	GA Type IIIC
Nonunion	20 (6.9)	1 (2.8)	8 (4.6)	8 (12.5)	3 (23.1)	0 (0)
Reoperation	18 (6.3)	0 (0)	7 (4)	6 (9.4)	4 (30.8)	1 (50)
Superficial infection	17 (5.9)	0 (0)	15 (8.7)	2 (3.1)	0 (0)	0 (0)
Deep infection	10 (3.5)	0 (0)	2 (1.1)	3 (4.7)	4 (30.8)	1 (50)
Malunion	9 (3.1)	0 (0)	6 (3.5)	2 (3.1)	1 (7.7)	0 (0)
Delayed wound healing	5 (1.7)	0 (0)	1 (0.6)	1 (1.6)	3 (23.1)	0 (0)
Implant failure	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Other	4 (1.4)	0 (0)	1 (0.6)	3 (4.7)	0 (0)	0 (0)

DISCUSSION & CONCLUSIONS: These results provide insights into factors affecting clinical outcomes following open tibia fractures in Latin America. Fracture healing correlated significantly with improved PCS scores, with the average mRUST scores approaching healing at six months and achieving full healing at one-year for GA Type I, II, and IIIA fractures. Infection rates were comparable with those reported in higher-resourced environments, despite additional delays in presentation.

ACKNOWLEDGEMENTS: The authors would like to acknowledge the Asociación de Cirujanos Traumatólogos de las Americas (ACTUAR) network of orthopaedic trauma surgeons interested in building research capacity in the Latin American region.

Antibody response to periprosthetic joint infection – Insights from a pilot study

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INTRODUCTION: Periprosthetic joint infections (PJIs) remain the most serious complication of arthroplasty, and one that needs to be avoided at all costs. The difficulty in infection assessment, together with delayed or missing pathogen identification, constitute a considerable challenge in everyday clinical practice, often leading to delays in optimal antimicrobial therapy. We hypothesize that the patient's pathogen-specific antibody response during PJI can provide clinically relevant information regarding the infection course.

METHODS: A prospective matched cohort pilot study was conducted with 13 PJI patients and 11 aseptic (Non-PJI) patients as control, where patient material and clinical data were collected at multiple standardized time points. We developed a custom quantitative bead-based serological assay for the simultaneous measurement of antibody responses against 32 common PJI-pathogens (Infection Array; IA). To study long-term and acute antibody production, pathogen-specific antibody binding was measured in both the patients' sera (N=267) and in cell culture supernatants of their peripheral mononuclear blood cells (medium enriched for newly-synthesized antibodies; MENSA; N=528), respectively.

RESULTS: Our host-oriented IA approach provided novel insights into the kinetics of pathogen-specific antibody response during PJI. Whereas serum antibody titers remained unchanged in 82% of Non-PJI patients over the course of the study, they declined over the course of treatment in 62% of PJI patients, predominantly against staphylococci and streptococci. Using the highly sensitive MENSA approach, we were able to trace the acute pathogen-specific antibody secretion by plasmablasts of the peripheral blood, and observed transient antibody production against numerous pathogens, mainly staphylococci, streptococci and *C. acnes*, in 92% of PJI patients, but also in all Non-PJI patients. Notably, *C. albicans* triggered an acute antibody production in 92% PJI patients, often within 5 – 11 days after surgery, and in 64% of Non-PJI patients. This suggests that the immune system is constantly warding off infectious agents, so that many invasive episodes do not manifest with clinical symptoms.

DISCUSSION & CONCLUSIONS: IA is a valuable high-resolution serodiagnostic tool to study the immunoproteomic footprint of infectious pathogens during the course of PJI, revealing new facets of the pathophysiology of PJI. This host-oriented approach may therefore complement the standard diagnostic portfolio in PJI, help to monitor the efficacy of PJI treatment for optimal patient follow-up, and guide clinicians to timely interventions and adaptation of the treatment strategy.

ACKNOWLEDGEMENTS: We thank our study participants for their involvement; Erika Friebe, Susanne Neumeister, Fawaz Al Sholui and Sabine Prettin, for their dedicated work; Susanne Kühl for her organizational support; all orthopedic consultant surgeons, residents and nurses for their help in sample retrieval; and Dr. Silva Holtfreter for helpful discussions.

Phenotyping bacteria in the in-vivo environment

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INTRODUCTION: Most infections take place within three-dimensional host tissues with intricate anatomy and locally varying host physiology. The positioning of pathogen cells within this diverse environment significantly affects their stress levels, responses, fate, and contribution to the overall progression of the disease and treatment failure. However, due to the technical difficulties in locating micrometer-sized pathogen cells within cm-sized host organs, this area of research has been relatively unexplored.

METHODS: We have developed imaging techniques to locate individual bacteria cells in centimetre-sized mouse organs and human biopsies. We also determine markers of viability and replication rate including fluorescence in-situ hybridization for detecting the 16S rRNA. In parallel, we use ultrasensitive mass spectrometry to assess the abundance of bacterial proteins in the infected tissues. We use these methods to assess the impact of antimicrobial chemotherapy on bacterial physiology and survival.

RESULTS: Our results show the localization of bacterial pathogens in infected tissues both within host cells and extracellularly. Replication rates seem to vary widely with a large part of the bacterial populations replicating rather slowly. Growth-limiting stresses include reactive oxygen species and iron starvation. Antimicrobial chemotherapy appears rather ineffective against this large subpopulation.

DISCUSSION & CONCLUSIONS: Imaging and mass spectrometry of infected tissues reveals relevant host microenvironments and bacterial subsets with crucial impact on therapy outcome.

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The changing joint environment: bacterial responses

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INTRODUCTION: Diseases such as osteoarthritis and joint replacements result in alterations in the synovial fluid/wound fluid present in the joint. We have been investigating how the presence of blood, wound fluid, or synovial fluid alters *Staphylococcus aureus* (*S. aureus*) adhesion, antibiotic tolerance, and virulence factor production.

METHODS: Overnight cultures of methicillin-sensitive *S. aureus* ATCC 25923 and methicillin-resistant *S. aureus* (MRSA) and luminescent MRSA reporters (Michael Otto (NIAID, NIH)) in tryptic soy broth (TSB) were diluted into various conditions. *Adhesion*: Sterilized Ti rods were submerged in TSB or physiological fluids at 10^5 CFU/ml, and incubated (37°C, 95rpm) for 24 hours. Adherent bacteria were recovered after trypsin digestion, and, using serial dilutions, plated on 3M Petrifilm aerobic count plates. *Metabolism*: MSSA (10^5 CFU/mL) in TSB or pSynF (synthetic synovial fluid: 3 mg/ml hyaluronic acid, 12 mg/ml fibrinogen, and 10 mg/ml albumin in TSB) were incubated for 1.5 h, 37°C, 180 rpm, and after addition of 10 µL of resazurin (alamarBlue™) fluorescence (λex/em = 540/590 nm) was measured hourly for 6 h (Tecan). Metabolic activity is expressed as net fluorescence (bacteria well fluorescence – blank well fluorescence). *Luminescence*: MRSA USA300 LAC psmα-lux (10^4 CFU/ml) were incubated in TSB, human serum, human synovial fluid (SynF) or pSynF, 37ºC, 225rpm. Luminescence was read at intervals out to 48 hours and normalized to individual absorbance measurements (600 nm).

RESULTS: We asked if changes in fluid composition post-operatively were altering adhesion, antibiotic tolerance, bacterial phenotype, and interactions with the joint environment. Two examples of the dependence on fluid are shown. In Figure 1, adhesion onto titanium rods is measured in TSB, human serum, 50% human serum (to model wound fluid), synthetic pSynF, equine synovial fluid (EqSynF, i.e., "normal" SynF) and human SynF (hSynF, "diseased human SynF). Adhesion was greatest when cultured in TSB and least in 50% human serum. Interestingly, adhesion was approximately equivalent for the human serum and human SynF, both of which were less than that measured in the "normal" equine SynF.

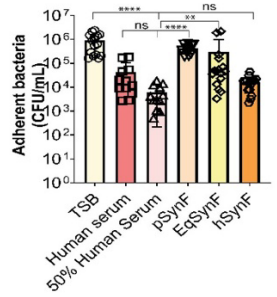

*Fig. 1. 24 h S. aureus adhesion in different fluids. Equine SynF (eq) represents uninflamed SynF whereas human (h) SynF is from a diseased joint. Inoculum, 10^5 CFU/mL; n = 12-16; not significant (ns), ** p ≤0.01 **** p ≤ 0.0001.*

We next measured metabolic activity in TSB and pSynF (Fig. 2, top). Metabolic activity was markedly depressed in the SynF which was mirrored in decreased *accessory gene regulatory* (*agr*) transcription, as indicated by phenol soluble modulin α (psmα) expression (Fig. 2 bottom). Serum, SynF and pSynF all caused large decreases in psmα.

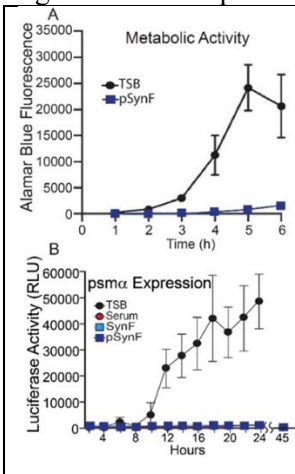

Fig. 2. MRSA metabolism and virulence factor expression is suppressed with different fluids (A) metabolic activity (alamar blue) in TSB or synthetic SynF (pSynF) and (B) agr-driven phenol soluble modulin (PSM)-α expression using human SynF, serum, pSynF, and TSB. Differences between TSB and other values are significant based on multivariant ANOVA. n=3-4

DISCUSSION & CONCLUSIONS: Treating periprosthetic joint infections requires consideration of the many different environments that transiently occur during the post-operative period. We have shown subtle but real differences in bacterial response to these changing fluids. Exploiting differences which are indicated here may give us new opportunities to combat bacterial contamination.

cGAS-STING Pathway in Orthopaedic Infections

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INTRODUCTION: Biofilm formation on the implant surface is a major cause for bacterial persistence and chronicity of orthopaedic infections as the biofilm matrix shields the bacteria against most antibiotics and the host immune system. Furthermore, the biofilm environment is discussed to shift the immune response towards anti-inflammation and tolerance. The success of immunotherapy in cancer treatment encourages its investigation as treatment option in other chronic diseases such as orthopaedic infections [1].
In a recent study we found that the cGAS-STING pathway was differentially activated between *Staphylococcus aureus* planktonic and biofilm environments [2] which makes this pathway an interesting candidate for further investigation as a potential immunotherapeutic target in orthopaedic infections (Fig. 1).

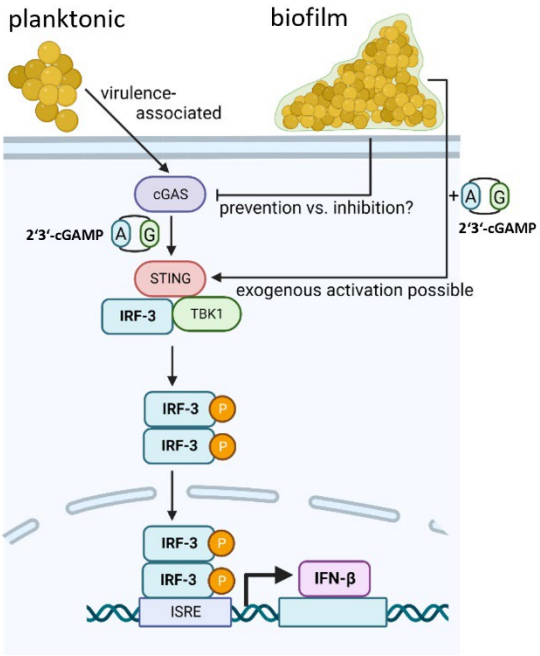

Fig. 1: cGAS-STING pathway activation in planktonic versus biofilm environments (created with BioRender.com).

Aim of our current project is to clarify the underlying mechanism behind the inhibitory effect of a biofilm environment on cGAS activation, if exogenous administration of the cGAS product 2'3'-cGAMP is sufficient to restore this missing response and whether this can strengthen the immune activity against biofilms.

METHODS: We generate conditioned media (CM) from biofilm cultures of *Staphylococcus aureus* and *Staphylococcus epidermidis* and stimulate macrophages in these biofilm environments with or without the addition of either the cGAS agonist Y-form DNA or the cGAS product 2'3'-cGAMP.

RESULTS: cGAS mRNA and protein levels seem to be slightly reduced by the biofilm CM. The effects of the biofilm environments on cGAS enzyme activity are under current investigation. Addition of 2'3'-cGAMP can induce a type 1 interferon response and increase cytokine expression upon stimulation with biofilm CM. Furthermore, addition of 2'3'-cGAMP leads to an increased surface expression of TLR-2 and co-stimulatory molecules CD80/86.

DISCUSSION & CONCLUSIONS: So far, our data indicates that exogenous administration of the cGAS product 2'3'-cGAMP might be a potential approach to circumvent the inhibitory effect of a biofilm environment on cGAS pathway activation and to support an effective immune response against orthopaedic infections.

ACKNOWLEDGEMENTS: We thank Sanya Middha and Ann-Katrin Niedermayer for their support with the experiments.

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Comparative genomics and the evolution of pathogenic staphylococci

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INTRODUCTION: Staphylococci are common commensal bacteria in human and animal epidermal microbiomes but can cause serious infections requiring antimicrobial chemotherapy. The emergence of antimicrobial resistance (AMR) is a major problem, and methicillin-resistant S. aureus and S. epidermidis (MRSA) are a significant global health challenge. Mobile genetic elements are key to the spread of AMR, including the staphylococcal cassette chromosome mec (SCCmec) that houses the mecA resistance gene. However, little is known about the forces that lead to the emergence of pathogenic lineages and how they adapt to transition from the primary niche to colonies systemic tissues and indwelling.

METHODS: Here we use new bioinformatics approaches to identify genetic elements associated with pathogenicity and AMR to understand the evolution of these important pathogens. Using genome-wide association studies (GWAS) we characterise the genes that underly the emergence of pathogenic Staphylococcus epidermidis from a background of harmless ancestors. We then investigate the horizontal gene transfer, accounting for linkage disequilibrium, and identify alleles that covary with the acquisition of SCCmec.

RESULTS: Integrated pathogenicity and AMR associated elements with phylogenetic analyses, we catalogue the chronology of potentiating genome adaptation. Finally, we move towards an integrated bioinformatics approach for understand the gene networks that underly complex phenotypes such as pathogenicity.

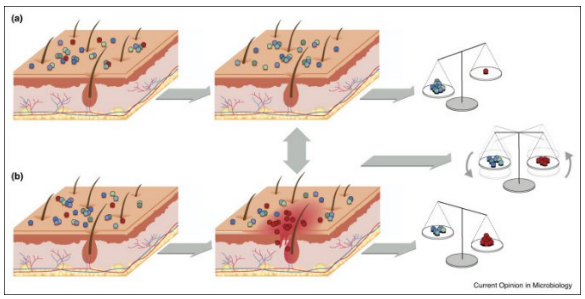

Fig. 1 Theoretical model of the evolutionary trade-off between co-colonizing commensal skin bacteria.

DISCUSSION & CONCLUSIONS: The potential for adaptations to one niche to provide a benefit in another is described and this paradigm is extended to include genetic diversity and competition among individual strains. Considering assemblages of strains in fluctuating immune environments with complex micro-niche structure, a scenario is presented in which commensal organisms can be primed for invasive disease should the opportunity arise.

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https://www.sciencedirect.com/science/article/pii/S1369527422000157#fig0005

Bone appétit: Bacterial pathogenesis during *Staphylococcus aureus* osteomyelitis

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INTRODUCTION: Osteomyelitis is most commonly caused by the bacterial pathogen *Staphylococcus aureus* and is a paradigm for treatment-recalcitrant infectious disease. Our laboratory seeks to investigate bacterial and host mechanisms that underlie the pathogenesis of osteomyelitis, and to uncover determinants that lead to antibiotic treatment failure.

METHODS: Using a murine model of post-traumatic osteomyelitis, we have investigated the *S. aureus* genes required for survival and virulence in bone using both targeted and genome-wide assays. To determine how specific staphylococcal virulence factors trigger pathologic bone remodeling, we have used micro-computed tomography, histology, and fluorescent imaging of bacterial reporter constructs. To model antibiotic treatment failure during osteomyelitis, we have created a delayed antibiotic treatment protocol that recapitulates the treatment recalcitrance observed in human infections. Using this protocol and integrated molecular imaging, we seek to investigate bacterial physiology *in situ* and define bacterial and host factors that limit antibiotic efficacy.

RESULTS: We have defined key *S. aureus* virulence factors and metabolic pathways that contribute to bacterial survival in bone and infection-related bone destruction. Using a delayed treatment model, we have demonstrated that *S. aureus* rapidly develops antibiotic tolerance *in vivo* (**Fig. 1**), and we are investigating the role of key bacterial genes in establishing an antibiotic tolerant niche during osteomyelitis. Finally, we have developed new protocols for discovery-based molecular imaging of bone using imaging mass spectrometry (IMS) of cryosectioned specimens (**Fig. 2**). IMS has delineated proteins, lipids, and small metabolites that define the host-pathogen interface during osteomyelitis and will enable identification of the molecular composition of barriers to antibiotic localization *in vivo*.

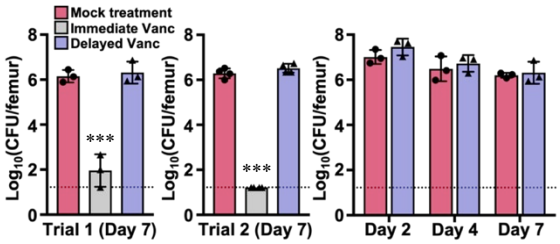

Fig. 1: **Antibiotic tolerance during *S. aureus* osteomyelitis.** Mice subjected to *S. aureus* osteomyelitis were treated with sterile PBS (mock) or 30mg/kg vancomycin (vanc) twice daily. Treatment was initiated at the time of surgery (mock and immediate vanc) or 24h after inoculation (delayed vanc). **A**) Bacterial burdens in mock or vanc-treated mice at d7 post-infection. ***p<0.001 by 2-way ANOVA with Tukey multiple comparisons test. **B**) Trial #3 with femurs harvested on the indicated day post-infection.

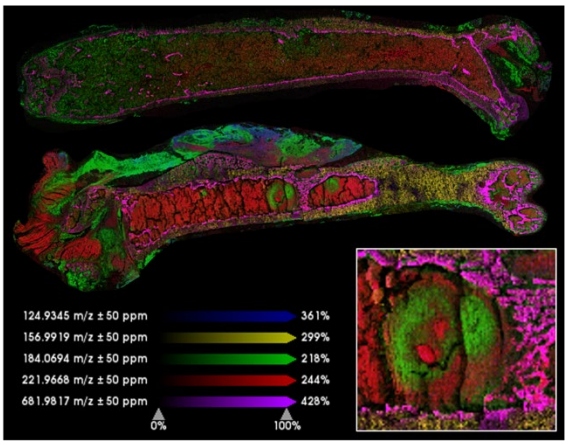

Fig. 2: Imaging mass spectrometry of mock (top) or *S. aureus* infected bone (bottom) at d14 post-inoculation. Inset shows *m/z* values localizing to a bone marrow abscess.

DISCUSSION & CONCLUSIONS: We have developed tools to identify bacterial and host factors that contribute to the pathogenesis and treatment recalcitrance of osteomyelitis.

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Characterization of *S. aureus*-Induced Bone Deformation Using Fluorescence and Raman Imaging

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INTRODUCTION: Biophotonic imaging techniques can provide in-depth information on biological samples, such as bone samples. While some methods, such as microscopic analysis of bone samples after histopathological staining, are already established in routine diagnostics, other powerful methods, such as label-free Raman imaging, are currently being developed on the research level. This presentation will focus on the application and potential of fluorescence and Raman imaging for in-depth analysis of bone samples, targeting *S. aureus*-induced bone deformation.

METHODS: A mouse model of hematogenous osteomyelitis [1] was used together with healthy control mice. A systematic study on bone sample preparation protocols revealed optimal decalcification protocols to achieve best image quality with evolving biophotonic and classical histopathological methods [2]. Fluorescence staining was used to highlight cell nuclei (DAPI), actin-cytoskeleton (I555-Phalloidin) and *S. aureus* (specific antibody, AF488) in a *S. aureus* infected pelvis bone (Figure 1, left). Bacteria were quantified using an in-house algorithm [3]. Raman spectra did not require any labelling. False colour images were generated using N-FINDR unmixing (Figure 1, right).

RESULTS: Exemplarily, we discuss a case in which osteomyelitis had manifested itself with a macroscopically visible bone deformation in the pelvis six weeks post infection. Using fluorescence imaging and label-free Raman spectroscopy all signs of a chronically florid tissue infection with osseous and soft tissue changes as well as with different inflammatory infiltrate patterns could be detected. Large lesions dominated the investigated tissue samples. *S. aureus* bacteria were found to form abscesses and were distributed in high numbers in the lesion, where they could occasionally be detected intracellularly. In addition, *S. aureus* was found in much lower numbers in surrounding muscle tissue, in trabecular bone tissue and only occasionally in bone marrow. The Raman spectroscopic imaging revealed a metabolic state of *S. aureus* with reduced activity which is in agreement with small cell variants found in other studies.

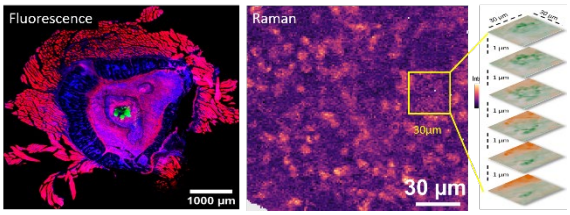

Fig. 1: Bone tissue slice imaging. Left: fluorescence showing muscle tissue (red), trabecular bone (blue), S. aureus (green) and lesion. Right: Raman showing cell nuclei (yellow) and S. aureus (green). Adopted from [4].

DISCUSSION & CONCLUSIONS: Biophotonic methods, such as fluorescence and Raman imaging are powerful and complementary methods to characterise bone infections, including inflammatory host tissue reactions and bacterial adaptation.

ACKNOWLEDGEMENTS: We thank Y. Ozegowski, S. M. Coldewey for mouse handling, C. Ebert for first experiments and L. Carvalho for discussion. We highly acknowledge financial support by the EU via IMAGE-IN (Grant No. 861122), the BMBF via LPI (13N15713) and via MESINFLAME (01EC1901B). We further thank ThIMEDOP (IZN 2018 0002), Leibniz ScienceCampus InfectoOptics Jena, DFG for RTG 2723 & JBIL.

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CCL20/CCR6 axis is essential to limiting the disease severity in *Staphylococcus aureus* osteomyelitis

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INTRODUCTION: Implant-associated bone infections remain a significant healthcare burden. *Staphylococcus* species are responsible for 75% of all osteomyelitis cases, with *S. aureus* being the primary infecting pathogen[1]. The most devastating outcome of *S. aureus* osteomyelitis is multiple organ failure followed by death due to sepsis, and the underlying immune mechanisms are largely unknown. In a clinical pilot study, we found that serum CCL20 chemokine levels were significantly elevated (~5 fold) in *S. aureus* osteomyelitis patients and even higher (~100 fold) in patients that died due to *S. aureus* osteomyelitis induced sepsis. CCL20 signals monogamously through its receptor CCR6, and the ligand-receptor pair is responsible for the chemotaxis of dendritic cells (DC), effector/memory T cells, and B cells. It is also involved in recruiting both the proinflammatory IL-17-producing helper T cells (Th17) and immunosuppressive regulatory T cells (Treg) to sites of inflammation. The role of CCL20/CCR6 axis in *S. aureus* osteomyelitis is currently unknown. We hypothesize that *osteoblast-derived CCL20/CCR6 axis is critical to mounting an immune response against S. aureus, and lack of CCL20 or its receptor CCR6 will lead to increased susceptibility to S. aureus osteomyelitis*.

METHODS: *In vitro studies:* Murine calvarial MC3T3-E1 cells, primary bone-marrow-derived osteoblasts, and macrophages harvested from C57BL6 mice were differentiated into osteoblasts and macrophage subsets (M0, M1 (IFN-γ (50ng/ml)), and M2 (IL-4 (20ng/ml)) and subjected to MRSA USA300 LAC infection (MOI 0, 1, 10, and 50) for 24 hours. Post-infection culture supernatants were harvested and assessed for CCL20 levels via ELISA.
In vivo osteomyelitis studies: All animal experiments used University-approved IACUC protocols. Mice were subjected to transtibial implant-associated osteomyelitis using bioluminescent MRSA (USA300 LAC::*lux*) strain. We used CCL20$^{-/-}$ and CCR6$^{-/-}$ knockout mice (procured from Jax Labs) of age 8-12 weeks to evaluate the role of CCL20/CCR6 axis and compared *in vivo* data to wildtype (WT) C57BL/6 age-matched mice. We performed longitudinal assessments of disease severity as a measure of: 1) body weight, 2) Bioluminescence assay (BLI), 3) ex vivo terminal assessment of CFUs of the tibia and internal organs, and 4) histopathology (H& E and Brown-Brenn stain) and micro-CT (bone osteolysis and reactive bone formation). Immunofluorescence was performed to examine the influence of CCL20/CCR6 axis on immune cell (T-cell and macrophage) recruitment to the site of *S. aureus* infection in the bone niche.

RESULTS: *In vitro* studies revealed that macrophages (M0 and M2 subtypes) and, for the first time, osteoblasts secrete CCL20 following *S. aureus* infection. *In vivo*, longitudinal BLI measurement of bacteria revealed increased early planktonic *S. aureus* load in CCL20$^{-/-}$ and CCR6$^{-/-}$ compared to WT mice. Terminal ex vivo CFU assessments (14 days post-op) confirmed a significant increase in soft tissue CFU in CCL20$^{-/-}$ mice and an increase in bone and implant/pin CFUs in CCR6$^{-/-}$ mice compared to WT animals. We observed reduced staphylococcal abscess communities (SAC) formation in CCR6$^{-/-}$ mice compared to WT. Interestingly, Micro-CT revealed increased bone loss in CCR6$^{-/-}$ mice. Overall, CCL20$^{-/-}$ and CCR6$^{-/-}$ mice exhibited higher disease severity than WT C57BL6 mice. To decipher the mechanism behind the observed phenotype, we performed IHC, which showed increased recruitment of CCR6+ T cells (Th17 subtype) and macrophages adjacent to the SACs only in the wildtype mice and not in CCL20$^{-/-}$ and CCR6$^{-/-}$ mice.

DISCUSSION & CONCLUSIONS: The current work underscores the crucial role of the CCL20/CCR6 axis in recruiting CCR6+ T-cells and macrophages. We also propose a potential feed-forward mechanism where CCR6 could contribute to increased CCL20 production. These findings suggest that the CCL20/CCR6 axis is essential for limiting *S. aureus*-induced osteomyelitis. Understanding this role could pave the way for new therapeutic interventions in the treatment of *S. aureus* osteomyelitis.

ACKNOWLEDGEMENTS: This work was made possible by a Discovery Pilot Grant from NIH P30 AR069655 with additional funding from the AO Trauma Clinical Priority Program, NIH (P50 AR07200), and AAI Careers in Immunology fellowship.

Osseointegration of Hydroxy Bisphosphonate-Conjugated Sitafloxacin (HBCS) during Skeletal Growth and Fracture Healing in a Mice Model

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INTRODUCTION: Eradication of methicillin-resistant *Staphylococcus aureus* (MRSA) infection of bone requires the elimination of distinct biofilms, including bacteria within osteocyte-lacuna canalicular networks (OLCN) and Staphylococcus abscess communities (SAC).[1] To overcome the limited efficacy of standard of care (SOC) vancomycin therapy, we developed hydroxy bisphosphonate-conjugated sitafloxacin (HBCS) (BV63072), to achieve "target-and-release" drug delivery proximal to the bone infection. We showed that MRSA within the OLCN can be killed by systemic HBCS, and also demonstrated its potential to eradicate MRSA osteomyelitis as an adjuvant to SOC therapy.[2] Based on this, we proposed a Phase 1 clinical trial of HBCS adjuvant therapy in fracture patients to the FDA, who requested pre-clinical data on the effects of HBCS on skeletal growth and fracture healing. To address this, we evaluated HBCS vs zoledronic acid (ZA, zoledronate, Zometa™) in a murine closed femur fracture model, and evaluated drug effects on radiographic, histologic, and biomechanical healing vs historical WT controls[3]. We also evaluated developmental endochondral and intramembranous bone formation and remodeling of distal femur and proximal tibia.

METHODS: All animal studies were performed with IACUC approved protocols. We utilized a closed, stabilized, mid-diaphyseal right femur fracture mouse model. Group 1: ZA 0.1mg/kg i.p. (single dose); Group 2: HBCS (BV63072) 3.0mg/kg/48hr i.p. until sacrifice on day 14, 21 or 28. Longitudinal X-rays were obtained at day 0 and every 7 days post fracture. Calcein was injected 6 days and 1 day before sacrifice to assess mineral apposition rate (MAR) and bone formation rate (BFR). Mice were euthanized on day 14 or 21 via heart perfusion with lead chromate-based Microfill (MV120) under anesthesia, and the femurs and tibiae were harvested and processed for micro-CT (n=5) and histology (n=5). Femurs and tibiae were harvested from mice sacrificed on day 28, and assessed by micro-CT and biomechanical testing (n=12), as previously described.[3]

RESULTS: **1)** MicroCT determined that HBCS increases fracture callus volume and vascular volume similar to ZA treatment: No differences in longditudinal X-rays or micro-CT assessments were detected between HBCS vs ZA treatments. However, both drug treatments significantly increased fracture callus and vascular volume vs. historical WT controls. HBCS (callus on day 14:18.82±9.45mm^3, day 21:17.36±5.1mm^3; vesse on day 14: 6.36±4.05 mm^3, day 21: 3.24±1.96mm^3) and ZA (callus on day 14:15.26±8.1mm^3, day 21:16.95±5.88 mm^3; vessel on day 14: 4.35±3.24mm^3, day 21:3.63±1.90mm^3) compared with WT (callus on day 14: 6.47±0.83mm^3, day 21:7.48±0.92 mm^3; vessel on day 14:0.92±0.26mm^3; day 21: 1.15±0.43mm^3)($p<0.005$). **2)** Biomechanical testing confirmed HBCS significantly increased yield torque (YT) and rotation at yield (RY). Compared with WT (YT:6.2±1.3N.mm; RY: 0.007±0.001rad/mm)($p<0.005$). HBCS(YT:17.7 ±4.47N.mm; RY:0.041±0.038rad/mm) were similar to ZA treatment (YT:24.4±4.72 N.mm; RY:0.033±0.028rad/mm) at 28 days ($p>0.05$). **3)** Histology confirmed both HBCS and ZA treatment had no effects on MAR and BFR in cortical bone and trabecular bone. Interestingly less BMR, but similar or lower TRAP+ osteoclasts were observed with HBCS vs ZA in the long bone metaphysis.

DISCUSSION & CONCLUSIONS: We found that HBCS significantly increased fracture callus and vascular volume and biomechanical fracture healing vs. WT. This suggests that the well-known effects of zoledronate on fracture healing are independent of its effects on osteoclasts. HBCS may be a dual-acting drug for infected fracture healing, and further studies to elucidate the mechanism of these enhanced fracture healing effects of ZA and HBCS are warranted.

ACKNOWLEDGEMENTS: This work was supported by the NIH awards R21AR081050, P30 AR069655 and P50 AR072000.

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Novel Approaches to Study Biofilm Biology in the Context of Prosthetic Joint infections

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The ability of microorganisms (bacteria and fungi) to form complex threedimensional structures known as biofilms is of tremendous importance in the context of prosthetic joint infections (PJI). Upon contact with an implant, microorganisms can attach to its surface, proliferate and ultimately form mature biofilms. Besides these surface-attached biofilms, suspended biofilm aggregates also occur in the infected joint environment, including in the synovial fluid. Biofilm-associated microorganisms are highly tolerant to antibiotics and eradication of a PJI without relapse is difficult. In most cases, management of PJI requires surgical intervention combined with empirical and targeted antibiotic therapy. After reduction of the microbial load by debridement, empirical treatment can be used, but should be de-escalated to targeted eradication or suppression therapy as quickly as possible.

Studying PJI-related biofilms *in vitro* has been hampered by the lack of *in vitro* models that accurately mimick the microenvironment found at the site of infection. Such models are important -as it is increasingly being appreciated that the microenvironment plays a crucial role in shaping the behaviour (including the antimicrobial susceptibility) of biofilm-associated microorganisms- but are currently lacking.

In this presentation I will talk about the work we are currently doing in my research group regarding the optimisation of biofilm-based susceptibility testing, using a newly developed synthetic synovial fluid (SSF) model. In addition, I will share the latest results pertaining to the combined use of the SFF model with isothermal microcalorimetry to diagnose biofilm-associated PJI in a culture-independent way.

ACKNOWLEDGEMENTS: I want to thank FWO-Vlaanderen and the European Society for Clinical Microbiology and Infectious Diseases (ESCMID) for funding PJI-related work in the Laboratory of Pharmaceutical Microbiology at Ghent University.

Uncovering mechanisms of host-pathogen interactions using CRISPR interference

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INTRODUCTION: *Streptococcus pneumoniae* and *Staphylococcus aureus* are major human pathogens responsible for enormous global morbidity and mortality. Despite this, these Gram-positive bacteria make up part of the commensal nasopharyngeal flora. How these bacteria switch from this commensal to pathogenic state and cause disease is unclear and very likely involves plasticity in expression of its virulence factors. For instance, variation in capsule production has been linked to different clinical outcomes, but the exact relationship between heterogeneity and pathogenesis is not well understood due to complex natural regulation. We also lack a complete overview of conserved genes present in most strains that are essential for virulence and could represent promising antibiotic targets and vaccine candidates.

METHODS: Here, we generated genome-wide sgRNA libraries targeting every gene in several clinical strains of *S. pneumoniae* and *S. aureus*. By CRISPRi-seq, gene essentiality in vitro and in vivo was established for some of these strains.

RESULTS: Using genome-wide in vitro CRISPRi-seq screens, a set of conserved essential *S. pneumoniae* and *S. aureus* genes were identified. Using genome-wide in vivo CRISPRi-seq screens, we identified bacterial genes important for infection leading to the discovery of a potent pneumococcal vaccine that acts through airway CD4+ T helper 17 cells.

DISCUSSION & CONCLUSIONS: The unbiased genetic approach of using CRISPRi-seq opens up new avenues for discovery of virulence-associated antibiotic and vaccine candidates heretofore overlooked by classical approaches.

ACKNOWLEDGEMENTS: Work in the Veening lab is supported by the ERC and the Swiss National Science Foundation.

Titanium Coated with Anti-DNABII Monoclonal Antibody Disrupts Bacterial Biofilm

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INTRODUCTION: Targeting bacterial biofilms could enhance prevention and treatment of orthopaedic infections. It is reported that *i)* extracellular DNA (eDNA) within the biofilm forms a lattice structure that is crucial to the structural integrity of biofilms; *ii)* the stability of this lattice structure is achieved by the binding and stabilization of the eDNA with eubacterial DNABII proteins; *iii)* removal of DNABII-eDNA binding through targeted monoclonal antibody (mAb) against the DNABII DNA-binding domains results in immediate (~minutes) collapse of biofilm [1]. In this study, we coated titanium (Ti) material with anti-DNABII mAb and evaluated the ability of this coating to disrupt bacterial biofilm.

METHODS: Ti mAb coating: Ti wires (0.6 mm diameter x 7 mm length) were treated with 10% (v/v) aminopropyltriethoxysilane followed by 4% glutaraldehyde. Wires were then incubated with 1 mg/ml anti-DNABII mAb overnight at room temperature. Residual free aldehydes were blocked with 0.15 M Tris buffer, pH 7.5. In vitro biofilm disruption assay (transwell chamber assay). Xen36 *Staphylococcus aureus* was incubated for 24 hr in an 8-well chambered coverglass (lower chamber). Ti wires with anti-tip anti-DNABII mAb coating (n=3) and negative control mAb coated wires (n=3) were then placed in upper chambers, separated from the lower chambers, with a semipermeable membrane for 2 hr (**Figure 1A**). In the lower chamber, biofilms were then stained with FM1-43FX and fixed. Quantification of biofilm biomass was performed using a Zeiss 800 scanning confocal laser microscope and COMSTAT2 software. Statistical analysis: Difference between groups was evaluated with unpaired student's t-test; $p \leq 0.05$ considered significant. Data represented by mean $\pm$ SD.

RESULTS: Biofilm biomass was significantly reduced in the anti-DNABII mAb coated Ti wire group as compared to the negative control mAb Ti wire group (**Figure 1B**). Quantification of biofilm biomass revealed a >90% reduction in biofilm biomass in the anti-DNABII mAb coated Ti wire group (1.57 $\pm$ 0.29 $\mu m^3/\mu m^2$) vs. the negative control mAb coated Ti wire group (17.0 + 3.91 $\mu m^3/\mu m^2$); p = 0.002 (**Figure 1C**).

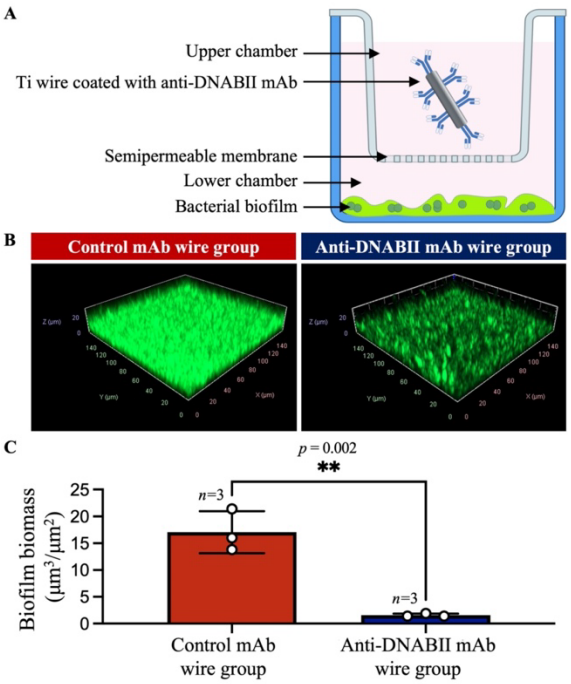

Figure 1: Ti wires with anti-DNABII mAb coating reduce bacterial biofilm mass by >90% over 2 hr. A. Anti-DNABII mAb coated Ti wires or negative control mAb coated Ti wires were placed in the dual transwell chamber assay. **B.** Representative images of biofilm in the lower chamber, as imaged by confocal laser scanning microscope, following incubation of wires for 2 hr in the upper chamber. **C.** Quantification of biofilm biomass in the lower chamber following incubation of wires for 2 hr in the upper chamber.

DISCUSSION & CONCLUSIONS: Anti-DNABII mAb was covalently bound to the Ti wire surface through silane based coating methodology. The mAb coating reduced bacterial biofilm mass >90% over 2 hr. This disruption and collapse of biofilm was mediated through blocking the DNABII protein binding site to eDNA and promoting an equilibrium shift of DNABII from biofilm [1].

ACKNOWLEDGEMENTS:
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REFERENCES:

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Carboxymethyl cellulose hydrogel containing phage cocktail and vancomycin for topical delivery against fracture-related infections

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INTRODUCTION: The rise of methicillin-resistant *Staphylococcus aureus* (MRSA) infections presents a significant challenge in treating fracture-related infections (FRI), with increasing concern over antibiotic resistance and the limitations of current therapeutic strategies. In the search for effective alternatives, phage therapy has garnered attention. By integrating a specifically tailored phage cocktail with vancomycin within the hydrogel, this research aims to explore a novel strategy for the direct treatment of MRSA infections in FRI, evaluating its potential to reduce bacterial load, and ensure sustained phage activity.

METHODS: Using a murine model for FRI, the study tested a Carboxymethyl cellulose (CMC) hydrogel containing a phage cocktail (MRSA3-R14 and COL-R23, 10^9 PFU/mL) enhanced for antibiofilm activity and vancomycin against MRSA infections. Four groups were included: The untreated group, the CMC hydrogel group, the systemic vancomycin group, and the combination group containing CMC hydrogel and systemic vancomycin. The evaluation centered on bacterial load reductions, phage distribution, the emergence of phage resistance, and the development of neutralizing antibodies.

RESULTS: The combination group showed a superior antibacterial effect compared to other three groups, achieving reductions of 0.99 log10 CFU in bacterial load compared to untreated controls, 0.81 log10 CFU versus the vancomycin group, and 0.80 log10 CFU versus the CMC gel group (all $p < 0.05$) at surgical site. Active phage was detected in the tissue of CMC hydrogel group at the point of euthanasia, indicating sustained activity. A higher titer of phage was retrieved in bone for the CMC hydrogel group compared to the combination group.

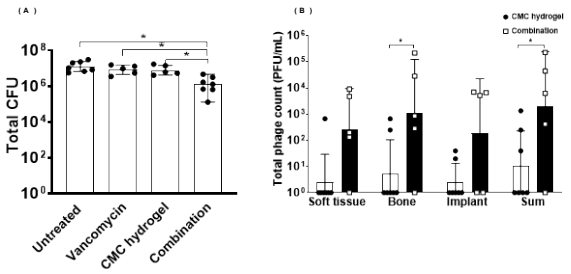

Figure 1. Evaluation of phage therapy against MRSA: Bacterial load of the surgical site (A) and distribution (B).

DISCUSSION & CONCLUSIONS: Our study explored a new method to fight MRSA infections in bone fractures using CMC hydrogel that carries a phage cocktail and vancomycin. This hydrogel can be applied once, making treatment easier and more effective in reducing the infection. It also keeps the phage active longer at the site of surgery, without leading to phage resistance. Early results are promising, but more studies and clinical trials are needed to fully confirm its effectiveness for wider use.

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An *in vitro* model to study implant-associated biofilms using a GelMA-based 3D microenvironment

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INTRODUCTION: Biofilm research has grown exponentially over the last decades, arguably due to their contribution to hospital acquired infections when they form on foreign body surfaces such as catheters and implants, however, little of the basic research in labs has been translated to actual therapeutic approaches. This low bench-to-bedside translatability is caused by the use of over-simplistic *in vitro* models that overlook the properties of the host microenvironment, the physiological conditions in those environments where biofilms thrive *in vivo*, as well as the biofilm-host interactions. In this study, we utilize gelatin methacryloyl (GelMA), a well-established extracellular matrix proxy for various tissues, and highly porous 3D printed medical-grade polycaprolactone (mPCL) scaffolds, to mimic *in vivo* bacterial infection at the implant/tissue interface.

METHODS: *S. aureus* was inoculated in 5%, 10% and 15% GelMA hydrogels with Young's modulus ranging between 1-100kPa and their growth was followed over 21 days. To study *S. aureus* growth at the gel (tissue proxy)/implant surface, a bacterial suspension was injected inside the top of a GelMA-embedded mPCL scaffold and cultured for 7 days. Confocal microscopy was used to follow population changes using Live/Dead (Syto9/PI) staining and matrix deposition using DAPI/ConA in synchrony. In addition, bacterial viability and metabolic activity were assessed using CFU counting, and PrestoBlue assays. Finally, uniaxial compression and microindentation were used to investigate the mechanical changes undergone by GelMA constructs as a result of bacterial growth and biofilm formation.

RESULTS

As depicted in Fig.1A, bacteria embedded in GelMA form micrometre-sized clusters that progressively expand, eventually developing into a layer of bacterial cells with secreted matrix components at the construct's edge after 21 days. Interestingly, bacteria embedded in 10% and 15% GelMA had a significantly higher metabolic activity in comparison to the softer gels, suggesting a key role to mechanical cues, nutrient gradients and water content in *S. aureus* growth and biofilm formation. Furthermore, when *S. aureus* were exposed to an implant surface in GelMA (Fig.1B), they infiltrated and migrated through the implant surface, suggesting biofilm formation within 7 days and providing proof on concept that these models mirror the implant/tissue interface.

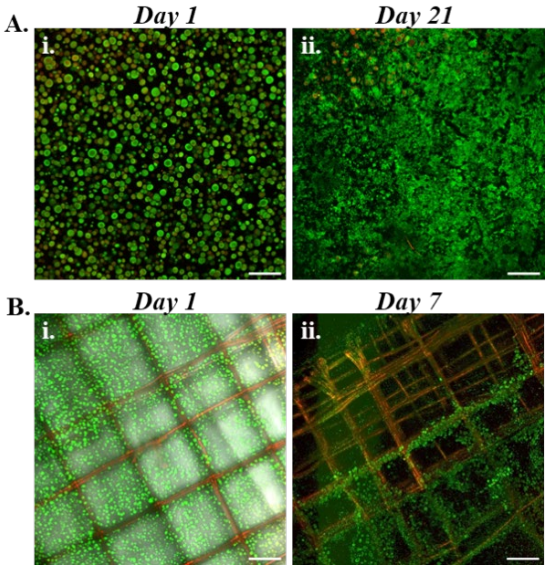

Fig. 1: Live/Dead staining of S. aureus within A) 10% GelMA and B) 3D printed mPCL scaffold/GelMA constructs. Green: Viable bacteria; Red: Dead bacteria, mPCL fibers. Scale bars: 100 µm.

DISCUSSION & CONCLUSIONS:

We demonstrate the feasibility of biofilms dynamic growth in a GelMA-based 3D microenvironment in x, y, and z directions, proposing a substitute to traditional models where biofilms grow on 2D substrates at solid/liquid interfaces. New generation *in vitro* models that mimic biofilm dynamics *in vivo* will open test potential strategies for preventing or treating infections, potentially guiding the design of new implant materials that resist biofilm formation and/or the optimization of existing protocols for implants.

Bacterial outer membrane vesicles for targeted treatment of bacterial infections

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INTRODUCTION: Bacterial infections play an important role in human diseases. Especially bacterial infections on medical devices and of internal organs are characterized by a rapid onset and severe consequences, making them a significant threat to human health. Insufficient drug accumulation at the site of infection and inadequate intracellular delivery pose important obstacles, impeding the efficacy of infection treatment. New treatment strategies to enhance the therapeutic effect of current antibiotics need to be developed. Naturally-derived biological nanocarriers such as outer-membrane vesicles (OMVs) are promising for drug-delivery, but low harvesting yield constitute an obstacle. Here we have developed OMVs loaded with iron-oxide magnetic nanoparticles, an antibiotic and a sonosensitizer for the treatment of meningitis using an external magnetic field.

METHODS: PEGylated magnetic iron-oxide nanoparticles (MNPs) were used to increase their uptake by *Escherichia coli*, which increased their OMVs secretion. An *E. coli* strain in which the synthesis of inflammatory lipid A acyltransferase was inhibited was used to prepare non-inflammatory OMVs. OMVs were loaded with ceftriaxone (CRO) and meso-tetra-(4-carboxyphenyl) porphine (TCPP) and magnetically driven into *in vitro* biofilms and across the blood brain barrier for sonodynamic treatment of bacterial meningitis.

RESULTS: The OMVs could be magnetically harvested at 60-fold higher yields than achieved by ultracentrifugation. The OMVs containing MNPs accumulated throughout the whole biofilm in an applied magnetic field in contrast to OMVs without MNPs which only accumulated on the outside of the biofilm. Thus, magnetic non-inflammatory OMVs bear potential for use as a drug carrier with the advantage that they can be magnetically targeted to an infection site. Validation of magnetic OMV use in an animal model is only useful if animals enter a study in an equally sick conditions as humans when they decide to go to a hospital. An infection that proceeds extremely fast to a life-threatening condition for which the use of magnetic OMVs could be useful is meningitis. In addition, meningitis is extremely difficult to treat due to the fact that antimicrobials need to cross the blood-brain barrier. In a mouse model with an antibiotic resistant *E. coli* meningitis, antibiotic and sonosensitizer loaded magnetic OMVs improved survival rates and clinical behavioural scores of infected mice after magnetic targeting and ultrasound application. Recurrence did not occur for at least two weeks after arresting treatment.

DISCUSSION & CONCLUSIONS: Magnetic OMVs are ideal nanocarriers for drugs and can be targeted to the infection site in a magnetic field. This was demonstrated in an *in vivo* murine meningitis model.

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Antimicrobial Chiral Polymers and Carry-Free Nanodrugs

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INTRODUCTION: Microbial resistance, including drug-resistant bacteria, intracellular bacteria, biofilms, and other forms, is a major global challenge. In order to address microbial resistance, chiral poly(amino acid) nanoparticles (FA NPs) have been proposed as drug delivery systems (DDS), which can precisely target biofilm bacteria and intracellular bacteria, achieving sterilization and infection treatment. In depth research has shown that the recognition of *D*-aminoalanine terminals on FA by bacterial membrane protein PBP4 is the key targeting mechanism. On the other hand, carrier-free nanodrugs (NDs) are used to overcome multi-drug-resistant (MDR) bacteria. Since antibiotic resistance mainly comes from changes in bacterial membrane permeability and efflux, NDs have been proven to break through bacterial membrane barriers and effectively reverse the resistance of MDR bacteria.

METHODS: The DDS is fabricated by encapsulating sitafloxacin sesquihydrate (Sita) into FA NPs, denoted as Sita@FA NPs; and encapsulating rifampicin (Rif) into a mannose decorated FA NPs, denoted as Rif@FAM NPs. For carrier-free systems, antibiotic tobramycin (Tob) and borneol 4-formylbenzoate (BF) were directly assembled into Tob-BF nanodrugs (TB NDs) based on dynamic *Schiff base*.

RESULTS: FA NPs exhibit excellent disintegration of biofilm. It can effectively insert into bacterial peptidoglycan (PG) *via* PG binding protein 4 (PBP4). Then FA NPs trigger detachment of amyloid-like fibers that connect to PG and reduce the number of polysaccharides and proteins in extracellular polymeric substances, leading to disassembly of biofilm. Based on this feature, Sita@FA NPs achieve complete elimination of *Staphylococcal* biofilm in mice, while antibiotic Sita only reduces the total bacterial count by approximately 3 lgCFU. Furthermore, the mannose units on Rif@FAM NPs guide the macrophage-specific uptake and intracellular accumulation. Then the detachment of mannose in acidic phagolysosome *via Schiff base* cleavage exposes the *D*-aminoalanine moieties, which steer the NPs to track and target intracellular bacteria through PG binding. This cascade-targeting Rif@FAM NPs exhibit on-site drug delivery and excellent intracellular bacterial clearance (1.36 lgCFU).

For carrier-free systems, since some amino groups of Tob were exposed on the TB NDs surface to realize positive charge of nanocarriers, TB NDs achieved self-loading and self-delivery. Thus, TB NDs could increase bacterial uptake and intracellular accumulation by enhancing permeability. It showed distinct synergistic bactericidal efficacy in both *in vitro* and *in vivo* assays, particularly in promoting the recovery of clinical xenograft MDR *Staphylococcus aureus* infection in mice (0.33 lgCFU), clearly superior to Tob (7.29 lgCFU), BF (6.30 lgCFU) and physically mixed Tob+BF (3.77 lgCFU, with equal dose to TB NDs).

DISCUSSION & CONCLUSIONS: Overall, FA NPs emphasize the need for carriers to break through bacterial resistance barriers and target bacteria precisely, whether they are bacteria within biofilms or intracellular bacteria. TB NDs highlights the potential of carrier-free NDs for overcoming bacterial drug resistance and eradicating MDR bacterial infection.

ACKNOWLEDGEMENTS: The authors thank the National Natural Science Foundation of China (52273118 and 22275013) for financial support.

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Repetitive combined doses of bacteriophages and gentamicin protect against *Staphylococcus aureus* implant-related infections in *Galleria mellonella*

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INTRODUCTION: Bacteriophages ('phages') are viruses that infect and replicate inside bacteria and finally release from the host through lysis. Recently, we demonstrated the efficacy of the *Staphylococcus aureus*-specific phage 191219 *in vitro*. However, in the *Galleria mellonella* biofilm implant-infection model, a single dose of phages did not improve the survival rate of the larvae.

Here, we aim to evaluate the effects of repetitive doses of the combination of the *S. aureus*-specific phage 191219 and gentamicin in the prevention and treatment of hematogenous and early-stage biofilm implant-related infections in *Galleria mellonella* larvae, respectively.

METHODS: To mimic hematogenous infection, *G. mellonella* larvae were implanted with a K-wire and infected with *S. aureus* EDCC 5055 after 24h. Subsequently, phages, gentamicin, or a combination of both, were administered. For the early-stage biofilm implant infection model, the K-wires were pre-incubated in an *S. aureus* suspension for 30 min before implantation. After 24h, the larvae received a single dose of phages, gentamicin or their combination. In both models, the larvae also received daily doses of phages and/or gentamicin up to 5 days. The effect was determined by monitoring the survival rate and by quantitative culture of the bacteria, in the tissue of larvae as well as on the implant surface, after 2 days of repetitive administration of phages and/or gentamicin.

RESULTS: In the hematogenous infection model, a single combined dose of phages and gentamicin, and repetitive injections with gentamicin alone or in combination with phages resulted in significantly improved survival rates. In the early-stage biofilm infection model, only repetitive combined administration of phages and gentamicin led to a significantly increased survival. Additionally, a significant reduction in number of *S. aureus* bacteria was observed in the larvae after receiving repetitive doses of phages, gentamicin or their combination in both infection models.

DISCUSSION & CONCLUSIONS: Based on our *in vivo* results, a single dose of the combination of phages and gentamicin is sufficient to prevent a hematogenous *S. aureus* implant-related infection, whereas gentamicin needs to be administrated daily for the same effect. To treat early-stage biofilm *S. aureus* implant-related infection, repetitive doses of the combination of phages and gentamicin are required.

Synthesis and antibacterial activity of LL-37-derived antimicrobial peptides as potential treatment of orthopaedic infections

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INTRODUCTION: Open fractures and joint prostheses are particularly susceptible to bacterial infections, with biofilm-related infections and septic nonunions being the most critical consequences[1]. These are exacerbated by the inefficiency of antibiotics and the rising issue of antibiotic resistance. There is a pressing need for targeted treatments for orthopaedic bacterial infections. LL-37, a human cathelicidin, is known for its antimicrobial properties[2]. In this research, new antimicrobial peptides (AMPs) synthetically derived from LL-37 were generated and tested for both their potential toxicity and antimicrobial effectiveness.

METHODS: Fmoc solid-phase peptide synthesis (SPPS) was used for the rapid generation of truncated analogues of LL-37. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were assessed to screen the AMPs against 18 bacterial strains relevant to orthopaedic infections, both ATCC and clinical strains including 3 *S. epidermidis*, 7 *S. aureus*, 4 *P. aeruginosa,* and 4 *E. coli* strains. Combinations of AMPs and antibiotics were also tested by Checkerboard assay to assess synergy. Selected AMPs were tested for the development of resistance by the target bacteria. *In vitro* cytotoxicity on NIH-3T3 (mouse fibroblast cells) and haemolytic activity was assessed.

RESULTS: Six LL-37-derived AMPs were produced: KR-12, FK-13, FK-16, GF-17, 17BIPHE2 and SK-24 (Fig. 1A). Among these, FK-16 and GF-17 exhibited the lowest MIC and MBC against the majority of tested strains (Fig. 1B). GF-17 showed lower MIC values compared to FK-16 ($P<0.05$, Fig. 1B). The AMPs demonstrated minimal effectiveness against *P. aeruginosa* strains, leading to their exclusion from subsequent analyses. There was no observed synergistic effect with antibiotics, and the tested strains did not develop resistance. FK-16 exhibited cytotoxic effects at concentrations above 150 µg/mL and haemolytic activity at levels exceeding 75 µg/mL. In contrast, GF-17 displayed cytotoxicity at concentrations over 75 µg/mL and haemolytic activity at concentrations greater than 18.75 µg/mL.

A

Name	Sequence	Name	Sequence
KR-12	KRIVQRIKDFLR	17BIPHE2	GFKR{D-LEU}VQR{D-LEU}KDF{D-LEU} RNLV
FK-13	FKRIVQRIKDFLR	GF-17	GFKRIVQRIKDFLRNLV
FK-16	FKRIVQRIKDFLRNLV	SK-24	SKEKIGKEFKRIVQRIKDFLRNLV

B

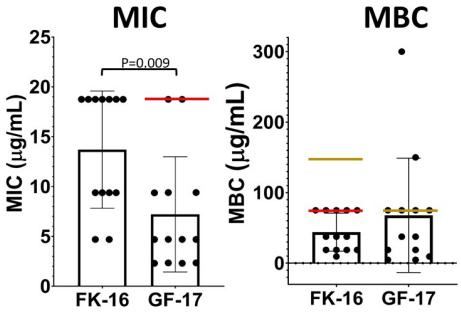

Fig. 1. A) AMP sequences. B) Antimicrobial activity of FK-16 and GF-17. Yellow line: cytotoxicity threshold. Red line: haemolytic threshold.

DISCUSSION & CONCLUSIONS: FK-16 and GF-17 emerge as highly promising candidates for combating bacterial pathogens primarily associated with orthopaedic infections. Their cytotoxic and haemolytic activities manifest only at relatively high concentrations, suggesting a favourable safety profile. The potential of these findings is further underscored by the prospect of validating these results through *in vivo* toxicity tests. Looking ahead, incorporating these new synthesized AMPs into materials commonly used in surgical settings (hydrogels, cements, etc.) could revolutionize infection control strategies in orthopaedic surgeries.

ACKNOWLEDGEMENTS: This work has been supported by Fondazione Regionale per la Ricerca Biomedica (Regione Lombardia), project ID 3414083 AMPROject.

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Bone fracture non-union

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Although a bone fracture is not usually life-threatening, it can significantly affect the patient during the healing process, which can last for weeks to months. Complications such as mal-union, delayed-union, non-union, or infection can arise, leading to further operations, prolonged hospital stays, sick leave, increased costs, and a socioeconomic burden.

Complications development depends on various factors, such as the severity of the trauma (e.g. closed vs. open fracture, with or without soft tissue damage), possible comorbidities (e.g. diabetes, metabolic disorders), individual factors (age, sex, genetic factors), and the quality of medical care. In the worst-case scenario, the bone may not heal, resulting in a non-union. Based on the classification from Weber and Cech, non-unions (named pseudarthrosis in the original work) can be divided in two types: hypertrophic and atrophic non-union[1]. Hypertrophic non-union is caused by a lack of mechanical stability, while atrophic non-union is primarily caused by a lack of biology. Vascularization is important for successful healing and impairment can be a cause for non-unions. However, studies examining non-union tissue in both human and animal models have yielded conflicting results.

Infection is a further complication during fracture healing that can result in non-union. The risk of infection is greater in open fractures with severe soft tissue trauma. Furthermore, the use of an implant increases the risk of infection as it changes the local environment and the inflammatory host reaction. Bacteria can survive in the body and cause an infection at a later time point.

Two factors are crucial for the successful healing of bones: adequate mechanical boundary conditions and host biology. These factors are interdependent and cannot be fully separated. Cells sense mechanical cues, resulting in mechano-transduction and biological changes, such as cell differentiation (see Fig. 1).

Multiple signalling pathways and the immune system have been identified to play a relevant role in bone healing, although the biological processes involved are complex and not yet fully understood.

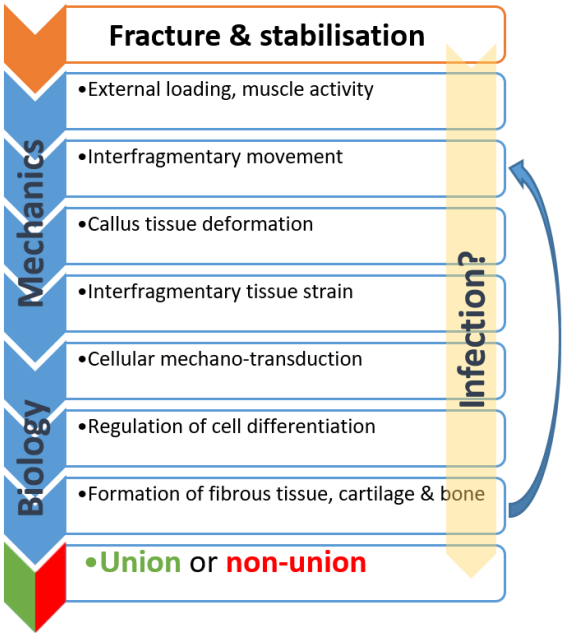

Fig. 1 Interaction of mechanical and biological factors during bone healing. Adapted from[2]

Therapeutic approaches for non-unions depend on the type and cause of the condition. Optimal mechanical stability should be obtained if this is not appropriate and cause the impaired healing. Further strategies try to improve the biological situation, although the clinical results using e.g. BMPs or PRPs are limited. Research is ongoing to improve the treatment options for non-union.

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How Infection Changes Non-Union Treatment

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Infected non-unions have 2 interrelated orthopaedic problems; (a) deep bone infection and (b) a failure of fracture healing. Various strategies exist, which treat:

(a) the fracture then the infection definitively, (e.g. exchange IM nailing)
(b) the infection definitively then the fracture, (e.g. excision of the non-union and secondary bone transport or Masquelet technique)
(c) both at the same time, (e.g. acute shortening)
(d) neither specifically (e.g. amputation) (Simpson AH & Tsang JS Injury).

The overriding objective in most patients is to obtain union and return of function with minimal morbidity. Certain procedures require long periods of non-weight bearing or long periods with external fixation and are associated with considerable morbidity.

It is important to determine which factors indicate a low morbidity rather than a high morbidity procedure may be suitable for the patient and how this could be change if novel treatments are developed.

Fracture first strategies tend to be easier for patients, as treating infection in a united bone is simpler than in unstable bone and treating malunion is simpler in a bone free of infection.

In general, with infected non-unions it is better to avoid nailing a pristine previously un-instrumented canal. However, if the primary fracture treatment was intramedullary nailing then the canal has already been contaminated and exchange nailing will not spread the infection to new areas. Even with infected tibial non-unions, 66% heal after one or two exchange nailing procedures (Tsang ST, Simpson AH et al. BJJ). The morbidity is low and even if a second exchange procedure is necessary, the time to return of function is likely to be lower than Ilizarov procedures. However, in a third of cases, the fracture remains ununited. Better, techniques of predicting which patients will heal with the low morbidity procedures would change treatment protocols. In addition, novel treatments that control infections while enabling the fracture to unite and faster reliable ways of filling bone defects after resection of an infected non-union would decrease the morbidity for patients and lead to new treatment strategies.

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The diagnosis of low-grade infection in presumed aseptic femoral or tibial shaft nonunion by membrane filtration of sonication fluid

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INTRODUCTION: Treatment algorithms for the treatment of fracture nonunion diverge based on the presence or absence of bacterial infection. However, discerning between septic and aseptic nonunion is challenging because low-grade infections lack clinical signs of infection and present similarly to aseptic nonunion. Low-grade infections are often caused by low-virulent bacteria capable of forming biofilms on the implant surface. These biofilms can be disrupted by sonication. In a previous study, membrane filtration of sonication fluid with quantification of colony forming units (CFU) was shown to be a useful adjunctive diagnostic tool for identifying septic nonunion [1]. Therefore, the aim of this investigation was to evaluate the diagnostic value of this method in differentiating low-grade infected nonunion from aseptic cases, which is the fundamental difficulty in clinical practice.

METHODS: A prospective, multicenter clinical study enrolled 77 patients with femoral or tibial shaft nonunion and suspected aseptic nonunion. During nonunion revision, tissue was sampled for culture and histopathology and the implant for sonication. Sonication involved the entire intramedullary nail or locking plate, with subsequent analysis of the sonication fluid conducted through membrane filtration and quantification of colony forming units (CFU) using previously established methods [1]. Patients were followed for 12 months. Definitive diagnosis of 'aseptic' or 'low-grade infected' nonunion was made according to the diagnostic criteria for fracture-related infection, taking into account the follow-up period. The diagnostic performance of CFU count was assessed by receiver operating characteristic (ROC) curve and Youden index, while sensitivities and specificities were calculated by two by two tables using SPSS.

RESULTS: Fifty-five nonunion patients were diagnosed as aseptic and 22 with underlying low-grade infection. The ROC curve had an area under the curve (AUC) of 0.85 (95% CI 0.76-0.94; Fig. 1). The optimal CFU cut-off value that maximized the Youden index (0.53) was 11.1 CFU per 10 ml sonication fluid. Using this cut-off, low-grade infected nonunion could be diagnosed with 64% sensitivity and 89% specificity. Comparatively, tissue culture had a sensitivity and specificity of 77% and 96%, respectively, while histopathology only had values of 9% and 87%, respectively.

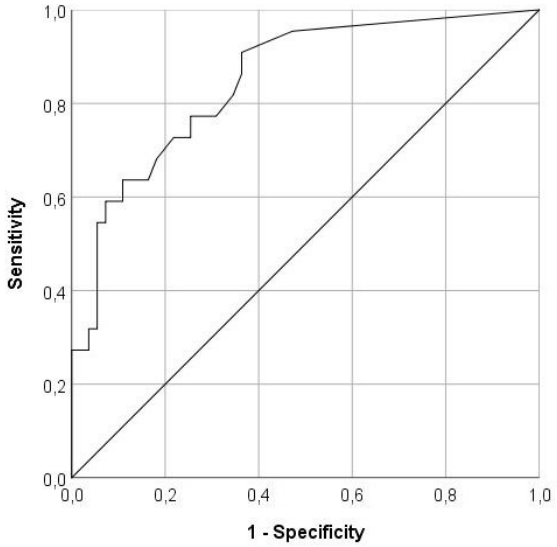

Fig. 1: ROC curve for the discrimination of aseptic and low-grade infected nonunion on the basis of CFU per 10 ml sonication fluid.

DISCUSSION & CONCLUSIONS: With an AUC exceeding 0.8, sonication of the entire implant with subsequent membrane filtration of the sonication fluid and CFU quantification demonstrated an excellent discriminatory ability distinguishing aseptic from low-grade infected nonunion. Therefore, this method may be a promising adjunct to tissue culture for detecting infections in patients with suspected aseptic nonunion.

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Increased number of Th1 cells and monocytes is associated with infected non-union in patients with long bone fracture.

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INTRODUCTION: Bone fracture non-union is classified as a failure of bone healing at least 6 months after fracture fixation. Confirming infection as the underlying cause is challenging in case of subclinical infection. Preoperative blood testing would be valuable in diagnosing infectious causes and facilitate early initiation of appropriate treatment. The aim of this study was to characterize peripheral blood mononuclear cells (PBMCs) from patients with septic and aseptic non-union and compare with patients with uneventful healing.

METHODS: Patients were recruited from eight level-one trauma centres in Germany, after appropriate ethical approval. Blood from healed (n=18; HEAL), septic non-union (n=21; S) and aseptic non-union (n=24, AS) patients was taken before surgical revision for routine implant removal or treatment of septic or aseptic non-union respectively. PBMCs were immunophenotyped using high-dimensional mass cytometry with a total of 43 markers. Targeted proteomics was performed on plasma samples using Olink 96-inflammation-panel.

RESULTS: T regulatory cells were increased in both AS (p=0.0118) and S (p=0.0478) compared to HEAL (Figure 1). Furthermore, monocytes and Th1 cells were elevated in S compared to both HEAL (p=0.0004, $p\leq$0.0001) and AS ($p\leq$0.0001; p=0.0023). Activation marker CD38 was decreased in CD4+ T cells in AS compared to HEAL (p=0.0005) and AS (p=0.0332). Exhaustion (PD-1, OX-40 and ICOS) and activation markers (HLA-DR and CD69) showed no significant differences in CD4+ and CD8+ T cells. Proteomics revealed increased IL18 expression in S compared to HEAL (p=0.0090). IL6 was increased in S compared to AS (p=0.0062) and HEAL ($p\leq$0.0001) as well as in AS compared to HEAL (p=0.0014). IL10 abundance was increased in S compared to AS (p=0.0469).

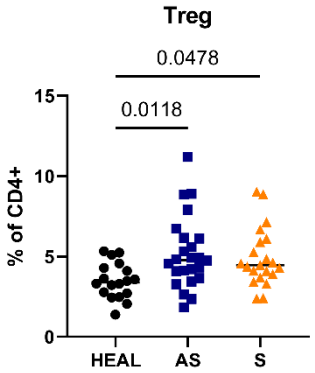

Figure 1: A) CD4+ T regulatory cells (HEAL: n=18; AS: n=24; S: n=21). Statistical analyses performed using one-way ANOVA with Tukey's multiple comparisons test or non-parametric with Kruskal-Wallis test.

DISCUSSION & CONCLUSIONS: In summary, S patients show an elevated number of monocytes and Th1 cells and a high expression of pro-inflammatory cytokines IL6 and IL18. These findings reflect a state of infection and therefore suggests correlations between protein abundances and elevated cell number in the different groups.
In addition, T helper cell subsets seem to have a fundamental role in non-union patients. These findings bring us one step closer to the final goal of providing distinct biomarkers to distinguishing between non-union patients.

Translation of infection-focused technologies to the clinic

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INTRODUCTION: During the translation of scientific results into commercially viable products researchers face challenges and questions of a different nature compared to traditional scientific work. Aspects such as manufacturability, market size, supply chain, regulatory constraints and many others must be considered in the translational journey.

METHODS: Many factors influence and determine the success of a product in medical industry. While not all can be controlled and influenced by an inventor or businessperson, they should be known and taken into account when embarking on the translational journey to transform scientific insights into commercial products.

- Does my technology/product address and solve a clinical problem?
- Is the clinical problem a frequent and/or severe problem?

Answering these questions will help understand whether the own solution really addresses a market need and whether it is desirable to customers. In other words, whether there is a product-market fit.

The question about product-market fit must be answered through interviews with potential customers, handling studies and a profound knowledge of the market. In the majority of cases, the design must be refined through multiple design iterations to achieve product-market fit. It is crucial to understand that a product without product-market fit faces a very high likelihood of failing.

In the medical world and, more specifically, in infection-focused technologies two fundamental aspects, which also relate to product-market fit, must be understood and defined early on in the development:

1. Is the product intended for the treatment or for the prevention of an infection?

This will determine the intended use of the product, the target patient population and the required pre-clinical and clinical strategies to pursue regulatory approval.

2. Is the anti-infective effect of the product the primary intended action or is it an ancillary effect to a medical device?

This is the most important aspect in determining the regulatory classification and approval pathway: medical device or drug product.

In addition to the obvious technical and scientific expertise, these aspects imply that a team or company aiming to develop and commercialize a product must have deep understanding of the clinical problem being addressed, of all paths used to solve it today, of the regulatory and IP landscape and of the customer needs.

A team aiming to commercialize a medical product must be multidisciplinary and ready to challenge its decisions at any time if the circumstances require a re-evaluation of the project.

Changing clinical or socio-economical conditions can make a product which seemed revolutionary a short time ago suddenly become a product with no market potential or with no clinical problem left to solve. Therefore, teams must be ready to recognize risks and opportunities and willing to adapt to changing requirements.

While the basis for a successful product is an effective solution to a relevant clinical problem, investors will give the competence and multidisciplinary expertise of the team equal value.

A few simple takeaways for scientists and engineers willing to embark on the translational journey are:

- A successful product must have a clear product-market fit.
- Be prepared to change and adapt to your environment.
- A good team covers many disciplines and is composed of people with different backgrounds and points of view, open to discussion and learning.

Material technology to tackle AMR; the NWA DARTBAC project

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Prosthetic joint infection (PJI) is a major complication in the orthopedic field that is difficult to treat due to the formation of bacterial biofilms. With the rising incidence of antimicrobial resistance (AMR), new antibacterial coating technologies not based on antibiotics are needed to prevent bacterial adherence and biofilm formation on implant surfaces.

The Dutch Antimicrobial Resistance Technology development and Biofilm Assessment Consortium, or DARTBAC for short is an international alliance of more than 26 scientific institutes, companies and entrepreneurs is to provide material technology solutions to the growing AMR problem. Additionally, also increasing AMR awareness is one of its primary tasks. Thirdly, we aim to develop optimized diagnostic strategies that allow faster and more accurate pathogen detection.

The latest progress in development and there results in various technology readiness levels of antimicrobial coatings, peptides, bioactive glass, additive manufacturing techniques and radiotherapeutics technology will be presented. Additionally, and short overview of molecular imaging techniques used for bacterial biofilm imaging will be discussed. Detailed information on biofilm formation and composition over time is essential for a fundamental understanding of the underlying mechanisms of biofilm formation and its response to antimicrobial and anti-biofilm technology.

Spondylodiscitis and implant-related spinal infections: Current challenges and needs from a surgeon's perspective

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INTRODUCTION: Spinal infections (SI), including discitis, spinal osteomyelitis and epidural abscesses, and post-operative spinal implant infections (PSII) are areas of significant clinical interest. They account for 2-7% of musculoskeletal infections and occur between 1 and 5 per 100,000 people per year in adults [1]. Alarmingly, the number of spondylodiscitis cases has recently increased by 40-50% to 14 per 100,000, with a mortality rate of around 20% [2]. The PSII rates are also worrying, with some studies reporting rates of up to 20% [3]. Although conservative treatment of spondylodiscitis is possible, the need for surgical intervention is often unclear. Implant-related infections often require surgical intervention due to biofilm formation. The pressing issue remains that these infection rates are excessively high, leading to a call for improved treatment and prevention approaches.

DISCUSSION & CONCLUSIONS: This talk will highlight the current dilemmas in the diagnosis and treatment of SI and PSII: Are we really experiencing an increase in incidence or are our diagnostic methods merely being refined? It also addresses the key issue of delayed diagnosis, which often occurs 2 to 6 months after the first symptoms, and highlights the potential of nucleic acid amplification assays (NAAAs) and next-generation sequencing (NGS) for early detection. The decision on surgical intervention or conservative treatment is another complex challenge. In addition, preventive measures and the role of new technologies such as machine learning and artificial intelligence in combating these infections will be discussed. The aim of this talk is not only to present the pressing problems, but also to propose actionable solutions and promote discussion on innovative prevention strategies.

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Can Sub-clinical Infection be the cause for disc degeneration and back pain? – Evidences from Metagenomic, Proteomic and Metabolomics studies

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INTRODUCTION: Subclinical infection has been postulated to play a role in disc degeneration recently and has garnered the interest of researchers worldwide. Our study evaluates the potential role of bacteria in DD and low back pain using a pan-omics approach.

METHODS: Nucleus pulposus from the MRI normal Lumbar intervertebral disc (IVD) of brain-dead voluntary organ donors (ND) were controls and discs of patients undergoing surgery for disc herniation and disc degeneration (DD) (cases) were utilized for the study.

Metagenomic analysis: Genomic DNA from discs was subjected to 16SrRNA sequencing for profiling the diversity of human disc microbiome in health and disease.
Proteomic Analysis were performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Further data analysis was performed using Uniprotdb, Pantherdb, Proteome discoverer and STRING network. Authentication of bacterial presence was performed by PCR amplification of 16S rDNA. Manual Curation of Host-defense response proteins (HDRPs) were done followed by functional characterizations.
Metabolome analysis: Untargeted metabolite profiling was carried out using HPLC-MS/MS platform. After mapping against HMDB, ChEBI, SigMol, Siderophore, ecdmb, and PaMet databases, the identified bacterial metabolites were studied by MSEA pipeline (Metaboanalyst) and statistical significance derived.

RESULTS:

Metagenomic analysis revealed a rich bacterial presence in both control and DD discs, identifying 424 different species (355-ND, 346-DD). Varying biodiversity and abundance between healthy and diseased discs were documented with protective bacteria being abundant in normal discs, and putative pathogens abundant in DD.

Proteomic analysis identified 56 specific proteins for *C. acnes* and 17 for *S.epidermidis*. Also, 67 infection-specific HDRPs, unique or upregulated, such as Defensin, Lysozyme, Dermcidin, and Phospholipase-A2, were identified confirming presence of infection. Species-specific primers for *C. acnes* confirmed presence of C. acnes in control and DD discs.

Metabolomic analysis showed 39 bacterial specific metabolites (9- primary metabolites related to bacterial growth and 30- Secondary). Principle Component Analysis and Orthogonal Partial Least Square-Discriminant Analysis (OPLS-DA) showed distinct clustered patterns between control and disc degeneration. (S)-14-Methylhexadecanoic acid, Cutibacterium specific metabolite was found only in DD. There was also upregulation of Autoinducer- 2, an important “Quorum sensing molecule” involved in bacterial cross-talk.

DISCUSSION & CONCLUSIONS: Our study documents existence of a microbiome within healthy IVD, and a different microbiome within DD, indicating potential role for dysbiosis in pathogenesis of DD. Studies on control and degenerated discs using pan-omics approach prove not only the bacterial presence, but its multiplication and interactions with host.

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EXPLORING GRAM-POSITIVE BACTERIA WITHIN THE INTERVERTEBRAL DISC: WHAT IS THEIR POTENTIAL INFLUENCE?

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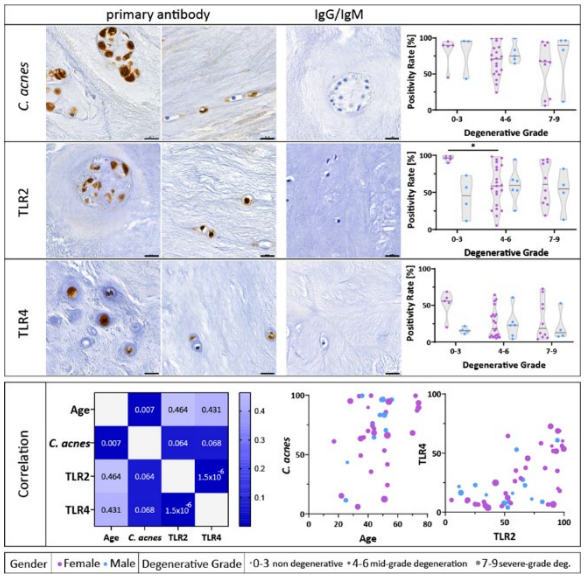
INTRODUCTION: Cutibacterium acnes (*C.acnes*) and other microbes have been identified in intervertebral disc (IVD) tissue using 16S DNA Sequencing and microbial cultures. This study examined the presence of C. acnes and other microbes in IVD tissue, exploring whether they're native or from perioperative contamination. We used immunohistochemistry (IHC) to detect Gram-positive bacteria including *C. acnes* and *S. aureus* in non-herniated human IVDs. Together with the expression of pattern recognition receptors (TLR2, TLR4, NOD 1&2, NLRP3) and the pyroptosis marker Gasdermin D. Co-culture experiments with whole bovine IVDs and *S. aureus* under static and dynamic loading conditions were conducted to assess bacterial infiltration and its interaction with the disc's environment and influence on disc phenotype.

METHODS: IHC was performed on 75 human IVD samples for Gram-positive bacteria, *S. aureus*, *C. acnes*, TLR2, TLR4, NOD 1&2, NLRP3, Gasdermin D, IL-1β, IL-6, ADAMTS4 and MMP3. Cell detection and classification were performed using QuPath. Furthermore, NP cells were treated with Lipopolysaccharide (LPS) (5-50µg/ml) and Peptidoglycan (PGN) (5-50µg/ml) in monolayer and alginate beads for up to 72 hours, followed by secretome analysis using Luminex. Bovine IVDs were cultured under static and dynamic load in the presence of *S.aureus.* Following culture discs were fixed, embedded to wax and IHC used to determine presence of bacteria and NP cell phenotype as per human IVD testing. Statistical analysis included Kruskal-Wallis, Dunn's multiple comparison test, and Pearson correlation.

RESULTS: IHC on human samples revealed Gram-positive bacteria exclusively within cells, with *C. acnes* positivity ranging from 5-99% and correlating with patient age (r=0.41, p= 0.007). TLR2 positivity ranged from 5-99% and TLR4 from 3-72%, showing a strong correlation (r= 0.62, p= 1.5e-006). Females with mid-degenerative grades exhibited significantly decreased TLR2 expression compared to those without degeneration signs. Native NP cells showed immunopositivity for NOD2 and catabolic factors, the quantification is ongoing. Examination of bovine samples is currently in progress. Treatment of NP cells with bacterial components resulted in an increase of several catabolic cytokines including IL-1, TNF, IL-6 and IFN-γ alongside increased production of chemokines, neurotrophic and angiogenic factors associated with IVD degeneration.

Figure 1(Top) Immunohistochemical staining for C. acnes,



TLR2 and TLR4 demonstrated their presence in the human disc tissue with the according IgG control. The positivity rate of TLR2 is significantly lower in females showing mid-grade degeneration compared to non-degenerative phenotype females. (Bottom) A matrix showing the p-values of a Pearson-correlation study. C. acnes showed to be correlated with age. A strong correlation was detected between TLR2 and TLR4

DISCUSSION & CONCLUSIONS: In conclusion, this study confirms Gram-positive bacteria presence in non-herniated human disc samples and highlights their potential role in triggering a catabolic response in disc cells.

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Comparison of the diagnostic efficacy of blood and tissue metagenomes in primary spinal infection

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INTRODUCTION: Early, rapid and accurate diagnosis is critical for appropriate spinal infection (SI) treatment. The application of metagenomic next-generation sequencing (mNGS) promotes the development of the diagnosis of spinal infections[1]. In a previous study, we have reported the high sensitivity of tissue mNGS allows it to identify the pathogen of primary SI[2]. However, the diagnostic value of blood mNGS for primary SI remains unknown. In this study, blood and tissue mNGS were both performed to identify pathogens of primary SI, and their diagnostic efficacy was compared with blood culture and tissue culture.

METHODS: In this study, blood and tissue specimens obtained via surgery or CT-guided puncture from 21 patients with suspected primary SI, were subjected to histopathology, bacterial culture and mNGS. The comprehensive analysis of clinical manifestations, imaging, blood tests and histopathological results was set as the reference to calculate the sensitivity and specificity of mNGS and culture. The diagnostic performance of blood and tissue bacterial culture as well as blood and tissue mNGS was evaluated and compared.

RESULTS: The sensitivity of blood mNGS was 9.52%, which was higher than that of blood culture (5%) (P =0.9486) without significance. It was found that the sensitivity of tissue mNGS (95%) and culture (45%) were significantly higher than that of blood mNGS (9.52%) and blood culture (5%). Compared with blood culture and mNGS, tissue culture and mNGS identified the pathogen of primary SI more accurately.

Table 1. Diagnostic efficacy in patients with primary spinal infection.

Source	Blood		Tissue	
Methods	Culture	NGS	Culture	NGS
PPV	100%	22.22%	100%	100%
NPV	5%	5%	8.33%	50%
Sensitivity	5%	9.52%	45%	95%
Specificity	100%	12.5%	100%	100%

Table 2. P value of sensitivity between four different diagnostic methods.

P value		Blood		Tissue
		Culture	NGS	NGS
Tissue	Culture	0.0384	0.1099	0.0134
	NGS	<0.0001	<0.0001	/
Blood	NGS	0.9486	/	<0.0001

DISCUSSION & CONCLUSIONS: The study demonstrated that blood mNGS and culture did not show good performance in pathogen identification of primary SI. The sensitivity of blood mNGS and culture was significantly lower than that of tissue mNGS and tissue culture. Thus, blood sample might not be suitable for the diagnosis of primary SI. The current sample size is relatively small and further studies should enroll more patients to generate more accurate findings.

ACKNOWLEDGEMENTS: Not applicable.

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Micro- and nanorobots for biofilm eradication

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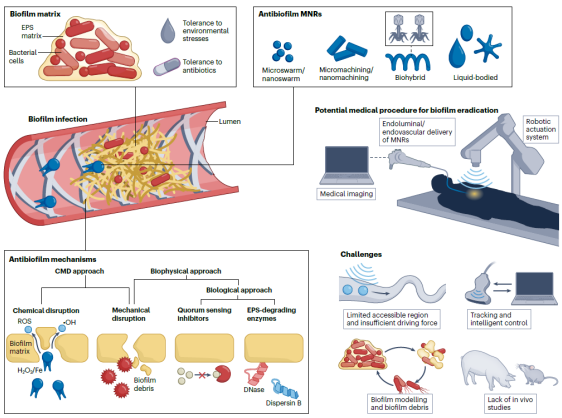
Aiming for effective biofilm eradication, various biofilm-targeting strategies have emerged, including EPS dissolution, biofilm dispersal, bacterial quorum sensing inhibition and bacterial metabolic interference1. Studying these strategies advances our understanding of the complex biofilm life cycle[1].

Micro- and nanorobots (MNRs) present a promising approach for navigating within the body and eliminating biofilm infections. Studies to understand the complexity of the biofilm matrix could offer potential strategies for using MNRs to disrupt the transition from planktonic cells to biofilm formation. Leveraging these insights, the potential applications of MNRs in clinical practice could include clearing infections in stents and prosthesis in the hollow organs, such as the gastrointestinal and hepatobiliary-pancreatic systems, pulmonary system and urinary system, as well as clearing and disrupting biofilm on implants in the musculoskeletal system and intravascular devices, such as implantable devices in the heart or blood vessels, to name a few. The minimal invasiveness of clearing biofilm infection using MNRs can help to improve patient compliance to treatment. However, special equipment to deliver and retrieve these devices from the body should be carefully considered. Thus, biodegradable MNRs are preferred, but still, their in vivo clearance needs to be well studied to ensure their biocompatibility and safety[2].



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Making Antibiotics Great Again: Phage resistance correlation with resensitivity to antibiotics in pan-resistant *P. aeruginosa*

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INTRODUCTION: Phages can be utilized for difficult-to-treat infections due to their ability to infect and kill antibiotic resistant bacteria. Furthermore, phages can penetrate biofilms which are a common cause of chronic and difficult-to-treat infections.

Phage therapy has a long-standing history in Eastern Europe. In the West, since 2007 the group at the Queen Astrid Military Hospital (QAMH) in Belgium has been treating patients with phage therapy utilizing protocols based on the experiences of the George Eliava Institute of Bacteriophages. A retrospective analysis was performed on 100 consecutive cases of phage therapy which included an assessment of phage-resistant bacteria isolated from some of these cases.

METHODS: Our group at KU Leuven received 21 isolates from 5 patient cases with varied infections, but all caused by the pathogen *Pseudomonas aeruginosa*. Whole genome sequencing was performed on these isolates combining long-read (Illumina) and short read technology (Nanopore) in order to determine single-nucleotide polymorphisms (SNP) and other structural variations. Assessment of the phenotype of these strains analysis was also performed that included virulence and MIC (minimal inhibitory concentration) assays.

RESULTS: Analysis of sequencing results show changes in phage-resistant isolates recovered from patients correlates with changes in known phage receptors. For one patient, which was treated with three phages that target three different receptors, resistance to all three was observed. For this particular case, however, we see changes associated with resensitivity of pan-resistant bacteria to antibiotics which was demonstrated in patient antibiogram results as well as in MIC assays. A fitness cost on the bacteria was observed as well when resistors were isolated from patients that received phages that target multiple receptors.

DISCUSSION & CONCLUSIONS: This works adds to further evidence that combined phage and antibiotic therapy can be a successful strategy to treat patients with resistant or difficult-to-treat infections. A thorough understanding of phage receptor and the effects of resistance on the bacteria should be determined in order to develop a successful treatment strategy.

Debridement, antibiotics, irrigation, and implant retention in a preclinical model of FRI in sheep.

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INTRODUCTION: Fracture related infection (FRI) represents one of the major complications in orthopaedic and trauma surgery. Debridement, Antibiotics, Irrigation, and implant Retention (DAIR) is a surgical treatment protocol suitable for some patients with fracture related infection (FRI). However, DAIR is only indicated for a limited portion of patients due to poor results in challenging cases such as chronic infections, those with implant instability, or with poor soft tissue coverage. A better understanding of the reasons for failure of DAIR may allow for the development of tailored treatment approaches that increase success rates and expand the indications, with substantial benefits for patients.

The aim of this study was to establish a large animal model of FRI with plating osteosynthesis, including early (2 weeks) and delayed (5 weeks) DAIR. Moreover, DAIR with an additional intramedullary channel lavage (IML) approach was evaluated after 3 weeks with a deeper inoculation method to guarantee intramedullary canal (IMC) infection. Additionally, fracture healing overtime was monitored using the implant load sensor (fracture monitor), prior to and after DAIR procedure.

METHODS: Healthy, skeletally mature, female Swiss Alpine sheep (*n*=16) were included in this study. Briefly, a 2 mm osteotomy was created in the tibia and fixed with a 10-hole 5.5 mm steel plate on which the fracture monitor was fixed. Animals received $2.5x10^6$ CFU/animal of *Staphylococcus aureus* MSSA (ATCC 25923) pipetted on the osteotomy side in the early and delayed groups or inoculated using collagen sponges within the intramedullary channel (IMC) to establish a deep infection. Revision surgery with DAIR was performed at 2 (*n*=3), 5 (*n*=5) and 3 weeks (*n*=8). Four animals of the 3 weeks groups received DAIR plus an IMC lavage (flushing saline within the IMC). After revision surgery, the sheep were treated systemically for 2 weeks with flucloxacillin and for 4 weeks with rifampicin and cotrimoxazole. After 2 further weeks off antibiotics, the animals were euthanized. Bacteriological culture was performed at revision and at the end of the study. Clinical CT was performed at revision, at 8 weeks post initial surgery and at euthanasia.

RESULTS: Systemic antibiotic treatment failed to clear the infection in the early and delayed DAIR groups while the IML approach resulted in complete eradication of the bacterial burden. Additionally, the fracture monitor showed a correlation between fracture healing and bacterial burden.

DISCUSSION & CONCLUSIONS: The developed large animal model proved how conventional DAIR fails to treat the infection in a delayed FRI treated with DAIR or in deep bone infection cases and that only a proper and intense debridement of the IMC results in infection clearance. The continuous monitoring of bone healing could help to understand the relationship between FRI and mechanical stability in an unprecedented way. This model may allow to test different antimicrobial interventions with implant retention and to further improve early FRI diagnosis with the use of the fracture monitor, leading on the long term to an increase in health outcomes of patients.

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Real-Time Fluorescent Microscopy Assessment of PAD4-Mediated NETosis and NET Degradation by *S. aureus* Nuclease During Nidus Formation on Implants

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INTRODUCTION: Bone infection remains a catastrophic outcome of orthopaedic surgery,[1] and *Staphylococcus aureus* is the most common pathogen.[2] Studies on soft tissue infection have demonstrated roles for host protein arginine deiminase 4 (PAD4) in generating protective neutrophil extracellular traps (NETs) and *S. aureus Nuc* in the conversion of NETs into eDNA biofilm.[3] To assess their importance during nidus formation on metal implants, we developed a novel in vitro real-time fluorescent microscopy model with quantitative outcomes of bacteria and neutrophil swarming and volume.

METHODS: All animal studies were performed under protocols approved by the UCAR. 12-week-old Catchup mice with tdTomato expressing neutrophils were sacrificed to collect neutrophils from the tibia and femur and cultured with *S. aureus* (EGFP$^+$ USA 300) contaminated, etched titanium pins. Longitudinal scanning confocal microscopy (LSCM) with an environmental chamber was performed for 6 hours, and then SYTOX Blue was added to the co-culture to stain extracellular DNA. GSK484 (PAD4 inhibitor) and *S. aureus* strain *Nuc$^\Delta$* AH1680 were used to assess the role of NET formation and degradation respectively. The volume of bacteria and neutrophils was calculated to evaluate nidus formation with neutrophil swarming behaviour.

RESULTS: SEM demonstrated robust *S. aureus* growth within the etch on the pin surface, which served as the region of interest (ROI) for LSCM. SEM also confirmed NETosis following neutrophil addition to *S. aureus* contaminated pins. LSCM confirmed neutrophil swarming towards *S. aureus* on the pin, phagocytosis of bacteria, and NETosis. Cell tracking results demonstrate that swarming neutrophils in the USA300 or *Nuc$^\Delta$* AH1680 co-cultures show significantly higher displacement and velocity than co-cultures treated with the NETosis inhibitor (GSK484). Longitudinal assessment of nidus formation demonstrated that neutrophils first swarm into the ROI by 3 hours and their numbers increase out to 6 hours producing a modest nidus with low bacteria counts and a predominance of host cells. PAD4 inhibition resulted in dramatically increased *S. aureus* volume primarily comprised of bacteria in static biofilm, and reduction of neutrophils swarming. In contrast, *Nuc$^\Delta$ S. aureus* were efficiently cleared by large numbers of swarming phagocytic neutrophils by 6 hours, suggesting that degradation of NETs into eDNA is required for biofilm formation. SYTOX blue staining of these co-cultures confirmed the presence of NETs in and around the nidus, the absence of NETs in GSK484 treated cultures, and robust NETs in and around the nidus formed by Nuc$^\Delta$ AH1680. Quantitative analyses confirmed the increase in bacteria volume and lack of neutrophil swarming in GSK484 treated cultures.

DISCUSSION & CONCLUSIONS:
Here we describe the first in vitro real-time imaging model of *S. aureus* nidus formation on metal implants in the presence of phagocytic neutrophils with quantitative outcomes of host and bacteria cell volumes and neutrophil behaviour. We also validate the critical role of NETosis in preventing implant infections, and *S. aureus* nuclease in NET remodelling to generate eDNA for biofilm formation. Studies to demonstrate this using intravital imaging[4] are ongoing.

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Elucidating the Osteoimmunology of Bone Infection and Development of a Passive Immunization for *S. aureus* Osteomyelitis

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INTRODUCTION: Osteomyelitis is the bane of orthopaedic surgery, and most are caused by *Staphylococcus aureus*. Although the number of infections following elective orthopaedic surgery is low (1-5%), reinfection rates are very high (up to 30%) at a cost of $17,000-$150,000 per patient, which has led to the orthopaedic paradigm that *S. aureus* infection of bone is incurable. Additionally, these bone infections are known to be a non-random event that is largely determined by specific host-pathogen interactions, as infections are caused by only a few prevalent nosocomial strains (i.e. MRSA USA300), and implementation of the most rigorous surgical systems is incapable of reducing infection rates below 1%. To answer the major questions about host-pathogen specific factors that impact the incidence of osteomyelitis and clinical outcome, and develop diagnostics and therapies, we commenced the AOTrauma Clinical Priority Program on Bone Infection (AO-CPP), whose primary goal was establishing a biospecimen clinical registry of 300 patients undergoing surgery for *S. aureus* confirmed bone infection.

METHODS: To elucidate the immune proteome in these patients, we developed a custom multiplex-Luminex immunoassays to correlate the human immune proteome against 8 *S. aureus* antigens with clinical outcome of bone infection using the AO-CPP registry. We also developed a novel bioinformatic approach to diagnose bone infections and prognose treatment success, which concluded that there are "protective" and "susceptible" immune proteomes that predict infections control vs adverse outcomes (arthrodesis, reinfection, amputation, and septic death). We also developed monoclonal antibodies as candidates for passive immunization and demonstrated their safety and efficacy in mice and sheep.

RESULTS: Our preclinical research validated this information in murine models of MRSA implant-associated osteomyelitis by demonstrating that antibodies against glucosaminidase (Gmd) are prophylactic and therapeutic, while antibodies against the iron-regulated surface determinant proteins (IsdA, IsdB, IsdH) exacerbate surgical site infections by inducing Trojan horse leukocyte formation and *S. aureus* dissemination to internal organs. In murine models, we showed that anti-Gmd passive immunization is protective against implant-associated osteomyelitis via direct inhibition of binary fission and opsonophagocytosis of megaclusters, and anti-Gmd synergize with vancomycin to cure established MRSA osteomyelitis. We also demonstrated the feasibility of a single clinically relevant administration of anti-Gmd monoclonal antibody in sheep, which revealed no complications or adverse events, and the estimated circulating half-life was 23.7 day. Towards clinical development of a passive immunization, we humanized our lead anti-Gmd mAb and used it to quantify endogenous levels in osteomyelitis patients, which ranged from < 1 ng/mL to 300 microg/mL, with a mean concentration of 21.7 microg/mL. We also estimated the circulating half-life of endogenous anti-Gmd antibodies in sera of 12 patients with cured osteomyelitis to be 120.4 days. Of note is that this dose was 8-fold greater than the endogenous anti-Gmd levels observed in osteomyelitis patients and was predicted to have a half-life of > 3 weeks. By assessing 1-year clinical outcomes we showed that all patients had measurable humoral immunity against some *S. aureus* antigens, but only 20 (6.7%; $p < 0.0001$) had high levels of anti-Gmd antibodies (>10 ng/mL) in serum prior to surgery, and that these antibodies are non-neutralizing.

DISCUSSION & CONCLUSIONS: Passive immunization with anti-Gmd mAb is a rationale therapy for *S. aureus* osteomyelitis, and further clinical development is warranted.

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