Principles of management of infections of the long bones – Surgeon’s view.

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INTRODUCTION:

The management of infections of the long bones remain cost- and time-consuming and can be equally challenging for the patient and the treating team. Most long bone infections are secondary to open fractures despite all efforts to reduce the risk by adequate soft tissue and bone management with a staged approach.

Even applying optimal care to management of open fractures, the infection rate for III degree open fractures remains up to 24% (1,2,3).

Once an infection / osteomyelitis is confirmed by clinical signs, blood parameters, swabs or aspiration, basic principles for the treatment should be applied in a multidisciplinary approach involving at least surgical and infectious disease specialists. The strategy depends on various parameters. It is important to determine the reason for the infection (acute haematogenous infection, acute contiguous osteomyelitis, osteomyelitis related to open fracture or at the site of prosthetic material, or patient’s general condition and expectation) and obviously the general condition of the patient (4). From the surgical viewpoint the most important aspect of treatment is to debride necrotic material, provide stability and vascularity in the infected area. Those principles apply to almost all circumstances of long bone infection. An implant removal is preferred to reduce non vital material in the infected zone, but this should not compromise the stability of an unhealed fracture. In those cases either an External Fixator should be applied, or in some situations of periarticular fractures the implant must be kept so as not to create an unstable, infected non-union. In certain situations, when a stable implant bone fixation is present, the strategy can be also to suppress the infection with targeted antibiotics to gain time until the fracture is healed and the implant can be removed safely.

If an extensive soft tissue debridement is required, almost the same surgical principles apply in infection surgery as in tumour surgery. Only when the infection is eradicated can a reconstruction of the bone and soft tissue defect commence. This can include extensive bone grafting free vascularized bone transfer or bone lengthening procedures, as well as soft tissue coverage with vascularised muscle flaps.

Depending on the severity of the infection, the amount of required surgery and treatment time has an enormous impact on the patient’s general, mental as well as social status. Those aspects must also be addressed in a multidisciplinary team involving orthopaedic and plastic surgeons, general physicians, infectious disease specialists and psychologists.

Treatment cost estimations can vary between each case as well as country, but complex infection management of long bones with limb reconstruction can add up to $500K purely in medical in-hospital costs (1).

The keynote lecture will highlight parameters, which have to be considered, when treating infections of bong bones from the surgical viewpoint.
Surgical treatment for infected hip and knee replacement

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Surgical treatment options for infected hip and knee replacements are: surgical removal of dead, damaged and infected tissue (debridement) with prosthesis retention and long-term antibiotic treatment; 1-stage revision; 2-stage revision; excision; or amputation. Surgical debridement and retention is considered in early PJI with pathogens susceptible to antibiotics and in patients unfit for more extensive surgery. However this approach may require lifelong antibiotic treatment.

Surgical revision of an infected TJR involves prosthesis removal, debridement, antibiotic treatment and joint replacement. The prosthesis is replaced in the same operation (1-stage) or delayed for 3-6 months (2-stage). In a 2-stage revision a temporary “spacer” may be fitted, but the patient has no hip or knee joint until it is replaced in the second operation. In England and Wales in 2009, treatment was 1-stage (26%), 2-stage (70%) and excision (3%). The best treatment option is unclear.

There are no RCTs comparing 1- and 2-stage revision of infected TJR. Gallo and colleagues reviewed longitudinal studies and calculated overall re-infection rates after 1- and 2-stage hip PJI revision of 9.2% and 7.4% respectively. Wolf and colleagues also reported an increased re-infection rate after 1-stage (12.3%) compared with 2-stage revision (6.5%) of infected THR. However, in the studies they classified as 2-stage, more patients died. Furthermore, using preferences provided by a group of outpatients and surgeons and the probabilities of re-infection, death, repeat treatment and mechanical complications in a Markov model, the authors concluded that published studies favoured 1-stage revision.

To compare outcomes of 1- and 2-stage revision of infected hip replacements we systematically reviewed studies that included populations representative of patients in routine clinical practice. We did not include studies where patients were selected for a particular treatment by health status or infection. We also excluded studies with selected follow up, for example those only including patients with a successful second stage implantation.

We searched literature databases EMBASE and MEDLINE, reference lists and citations to March 2011 for appropriate longitudinal studies and clinical trials. With a search strategy based on hip replacement, infection and revision, and 1-stage and 2-stage surgery we found 313 articles of which 138 described outcomes after surgical treatment of infected hip prostheses. Two reviewers checked articles independently and identified 62 relevant studies including 4197 patients. To compare rates of re-infection within 2-years we created pooled random effects estimates using the Freeman Tukey arcsin transformation to stabilise the variances.

Irrespective of surgical treatment used, the overall 2-year rate of re-infection was 10.1% (95% CI 8.2, 12.0). In 11 studies with 1225 patients with infected hip prostheses receiving exclusively 1-stage revision, the rate of re-infection was 8.6% (95% CI 4.5, 13.9). In a fixed effects analysis the pooled incidence rate was 11.4% (95% CI 9.7, 13.3). The discrepancy with the random effects model was mainly explained by the large study of Buchholz and colleagues [45]. Excluding this study with the less specific outcome of need for second exchange and other re-infection, the pooled random effects estimate was 7.7% (95% CI 3.6, 13.4). After 2-stage revision exclusively in 28 studies with 1188 patients, the rate of re-infection was 10.2% (95% CI 7.7, 12.9).

It appears as if there is no strong evidence to show superiority of either strategy and there remains a clear need for a randomised controlled trial of one versus two stage revision in both hip and knee replacement.
FISH-based detection of bacteria in orthopedic implant-related infections

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INTRODUCTION: Correct diagnosis of infection and the identification of the responsible microorganisms are crucial for an adequate treatment of orthopedic implant-related infections. Besides blood tests and histologic analyses, these infections are routinely detected by culturing patient materials such as periprosthetic tissue, synovial fluid or sonication fluid1 (SF) from explanted biomaterials2,3. In the orthopedic field, infections can be difficult to diagnose due to a small amount of material, low infectious loads, or due to a fastidious organism4. As a direct consequence, patients may suffer from an undiagnosed and untreated implant-related infection.

Currently, no rapid and sensitive diagnostic method is available to detect these so-called low-grade infections. To solve this problem, we are searching for a sensitive method with a low chance on false-negative results due to contamination. For this purpose, the technique fluorescence in situ hybridization (FISH) was evaluated for its potential for detection and identification of bacteria in SF.

METHODS: SF was collected by sonicating retrieved implants from 62 patients. All samples were subjected to standard bacterial culture for clinical diagnostics. In addition, a commercially available FISH kit (miacom diagnostics, Germany) specifically designed for blood tests (hemoFISH Masterpanel) was used for detection of bacteria. The FISH kit contained 16S rRNA probes, non-sense probes, probes for Staphylococcus spp., Staphylococcus aureus, Streptococcus spp., Streptococcus pneumoniae, Streptococcus agalactiae, Enterococcus faecium, Enterococcus faecalis, Enterobacteriaceae, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Acinetobacter spp., and Stenotrophomonas maltophilia. All FISH analyses were performed according to the protocol provided with the kit. The results obtained with bacterial culture and FISH were compared, considering the culture results as the gold standard.

RESULTS: Bacterial culture resulted in 27 positive and 35 negative cultures. Comparing FISH results obtained with the positive control (16S rRNA) probe with bacterial culture, we found 24 samples which tested true positive and 33 samples true negative. Furthermore, 3 samples tested false-negative and 2 samples false-positive. The species cultured with the highest incidence were Propionibacterium acnes and Staphylococcus epidermidis, both from 8 sonication fluid samples. As no probe for Propionibacterium acnes was present in the FISH kit, these bacteria were only detected by the positive control probe. In addition, the latter samples all tested positive with the Staphylococcus spp. probe.

DISCUSSION & CONCLUSIONS: The present study shows that FISH holds promise to be used as a diagnostic tool for identifying orthopedic infections. The greatest advantage of using FISH for this application is saving time. Compared to bacterial culture, which takes days, FISH results are available within an hour after SF collection. Another advantage is that slow-growing and even dead bacteria can be detected. Interestingly, 2 samples tested positive using FISH that were culture negative. This result could indicate a higher sensitivity for detection of bacteria with FISH.

Before FISH can be used for diagnostic purposes, the technique needs to be optimized to prevent false negative results, for use on other patient materials and for detection of bacterial strains relevant for the orthopedic field, like Propionibacterium acnes.

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Antibiotic resistance of commensal *Staphylococcus aureus* and coagulase negative *Staphylococci* in an international cohort of surgeons

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**INTRODUCTION:** Nasal colonization with antibiotic resistant bacteria represents both a risk factor for the colonized individual and their immediate contacts. Despite the fact that healthcare workers such as orthopaedic surgeons are at a critical interface between the healthcare environment and an at-risk patient population, the prevalence of antibiotic resistant bacteria within the surgical profession remains unclear. This study offers a snapshot of the rate of nasal colonization of orthopedic surgeons with multi-resistant staphylococci including Methicillin-resistant *S. aureus* (MRSA) and Methicillin-resistant Coagulase Negative Staphylococci (MRCoNS).

**METHODS:** We performed a prospective, observational study obtained at a single time point in late 2013. The participants were active orthopaedic, spine and head & neck surgeons from 75 countries. The prevalence of nasal carriage of the different bacteria and the corresponding 95% confidence interval were calculated.

**RESULTS:** From a cohort of 1,166 surgeons, we found an average *S. aureus* nasal colonization rate of 28.0% (CI 25.4;30.6) and MRSA rate of 2.0% (CI 1.3;2.9), although significant regional variations were observed. The highest rates of MRSA colonization were found in Asia (6.1%), Africa (5.1%) and Central America (4.8%). There was no MRSA carriage detected within our population of 79 surgeons working in North America, and a low (0.6%) MRSA rate in 657 surgeons working in Europe. High rates of MRCoNS nasal carriage were also observed (21.4% overall), with a similar geographic distribution. Recent use of systemic antibiotics was associated with higher rates of carriage of resistant staphylococci.

**DISCUSSION & CONCLUSION:** In conclusion, orthopaedic surgeons are colonized by *S. aureus* and MRSA at broadly equivalent rates to the general population. Crucially, geographic differences were observed, which may be at least partially accounted for by varying antimicrobial stewardship practices between the regions. The elevated rates of resistance within the coagulase negative staphylococci are of concern, due to the increasing awareness of their importance in hospital acquired and device associated infection.
The value of pre-clinical testing of antimicrobial biomaterials
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Implant-associated infections remain a huge challenge in orthopaedic and orthopaedic trauma surgery and there is a tremendous need for antimicrobial biomaterials. Biofilm-building and intracellular invasion strategies are among the most relevant virulence factors of bacteria involved in implant infections. Biomaterials are of high interest as they enable the local delivery of antibiotics or antimicrobial agents to the wound without any significant systemic side effects. Ideal biomaterials for the use in implant-associated bone infections should be safe in its use without disturbance of wound healing and biodegradable without the need for removal of the material. In vivo testing is mandatory in the pre-clinical assessment of such materials. Beside safety testing on biocompatibility of the product, the antimicrobial effect should be targeted in a clinically relevant model in order to mimic the further clinical scenario for the product as close as possible. There are several clinically relevant infection models particularly for infection prevention that have recently shown e.g. their usefulness in the approval of antibiotic-coated or silver-coated implants. However, there is still a lack of in vivo models for the treatment of implant-associated infections, e.g. for two-stage revision surgery for chronically infected arthroplasty. We have recently started to develop such a model to mimic explantation surgical practice of chronically infected arthroplasty. After induction of the infection, the infected implant is explanted after 4 weeks together with the implantation of an antibiotic-loaded spacer in rabbits (stage 1) (Fig. 1 and 2). Stage 2 surgery consists of removal of the spacer and implantation of the new implant after 4 weeks). Based on this model, we hope to better evaluate new treatment strategies for chronically infected arthroplasties, e.g. of new spacers, duration of antibiotic treatment etc. In all in vivo models, the three R’s: Replace, Reduce, Refine, should always be respected.
INTRODUCTION: Biofilms have a major impact on human health being present on a wide range of interfaces in the human body, including device surfaces.

METHODS: We use Fluorescence in situ hybridization (FISH) to visualize, identify, and quantify amount, composition, and interactions of in vivo grown biofilms in clinical samples [1].

RESULTS:
(i) Pathogen identification: FISH with specific probes in sections of artificial heart valves can prove infection and identify the causing microorganisms in endocarditis patients. This is helpful in particular in culture-negative cases that pose a significant diagnostic problem [2].

(ii) Biofilm activity: Within prosthetic heart valve sections, we detected structured biofilms that were highly organized showing stratification with zones of elevated rRNA levels alternating with layers of only DAPI positive cells that were presumably dead. We also found FISH positive bacteria in culture-negative samples and samples from patients under antibiotic therapy (Fig. 1). The high signal intensity of FISH correlates to a high ribosomal content of the bacteria indicating metabolic activity at the time of surgery. To detect the activity of single bacterial cells more precisely we developed FISH probes for the 16S-23S intergenic spacer region that is only present in actively transcribing cells. Using this ‘spacer FISH’ we detected positive cells in heart valves of patients under adequate therapy.

(iii) Biofilm architecture: In implant samples from peri-implantitis patients we analysed the composition of multispecies biofilms comprising periodontal species and yet uncultured bacteria.

(iv) Biofilm quantification: FISH combined with the unspecific nucleic acid stain DAPI and analyzed by digital image analysis is a powerful tool to quantify biofilms in situ. We present biofilm quantification results for medical device applications, i.e. an in vivo catheter model with Staphylococcus epidermidis biofilms.

DISCUSSION & CONCLUSIONS: In summary, these findings show the versatility and impact of FISH for diagnosis of device-associated infections and analysis of biofilms in the clinical setting. Spacer-FISH allows visualizing the effect of antimicrobial therapy on in vivo grown biofilms and thus, translate laboratory research results into the clinical situation in the patient.

FISH provides unique in situ information, which can guide therapeutic decisions in biofilm-associated infections.
Macroscopic fluorescence imaging of biofilms
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INTRODUCTION: Whereas Fluorescence Microscopy and Confocal Laser Scanning Microscopy are used for determining the 2D- and 3D-distributions of bacteria including the extracellular polymeric substances in a biofilm by fluorescence staining techniques, exact enumeration of bacteria is cumbersome and restricted to small spots on a substrate. In phase contrast microscopy the number of adhering bacteria can be determined with great accuracy in real time during deposition, but the applicability of the technique is mainly restricted to flat and transparent substrates. The aim is to evaluate Macroscopic Fluorescence Imaging (MFI) as a novel methodology to partly fill in unmet functionality in either of these methods: enabling the enumeration of bacteria on non-transparent substrata like Titanium, but also having full potential in enumerating bacteria in 3D-structures like biofilms.

METHODS: Fluorescently engineered Staphylococcus aureus ATCC 12600 pMV158 GFP bacteria were grown under flow in different concentrations of TSB nutrient medium to form a biofilm, after deposition of inoculating bacteria from a flowing bacterial suspension in PBS in a parallel plate flow chamber (PPFC) on a glass substrate. The number of bacteria as a function of time was obtained from the fluorescence radiance data averaged over the entire surface of the flow chamber with three separately grown cultures. Bacterial numbers presented were inferred from single bacterial fluorescence [1,2]. For fluorescence imaging, a flow chamber was placed on the sample stage inside a bio-optical imaging system (IVIS Lumina II, Caliper LifeScience, Hopkinton, MA, USA). The excitation wavelength was set at 465 nm, while a broad band-pass emission filter (515-575 nm) was used to measure fluorescence emission.

RESULTS: Numbers of S. aureus ATCC 12600 pMV158 GFP bacteria appeared to be related to the distance from the entrance of the flow chamber. At the start of the experiment there is a positive correlation with distance, whereas during time nutrient depletion caused a decreasing biofilm thickness as a function of distance from the entrance.

DISCUSSION & CONCLUSIONS: We demonstrated that MFI could be applied to enumerate bacterial adhesion and growth and acquire quantitative information even from bacteria residing and accumulating in three dimensional structures like biofilms, allowing the study of multi-species microbial adhesion and biofilm growth.

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Preventing implant associated infections by silver coating

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INTRODUCTION: The burden of implant-associated infections (IAI) has grown and has become an immense economic and health care issue. Here we examine a pre-clinical silver-coated alloy against biofilm-forming Staphylococcus (S.) aureus and S. epidermidis in vitro and in vivo for its activity to prevent IAI and its possible toxicity against eukaryotic cells.

METHODS: Silver coordination polymers were coated on titanium aluminium niobium (TiAlNb) alloys. We measured their bactericidal activity against S. aureus SA113 and S. epidermidis SE1457 in vitro in an agar inhibition assay. To examine the additive effect of antibiotics we exposed 24 h old biofilms to silver (AgNO3) and vancomycin (50 mg/l) or daptomycin (30 mg/l) in vitro and measured the residual biofilm by crystal violet staining and counted the adherent bacteria by plating. Silver-coated cages were assessed in vivo in a previously published subcutaneous tissue cage mouse model. Bacteria were injected into the silver coated tissue cages either perioperatively or postoperatively 14 days after implantation. In infections with methicillin resistant S. aureus, we additionally administered vancomycin (200 mg/kg i.p.) or daptomycin (50 mg/kg i.p.) perioperatively. After infection, the tissue cage fluid (TCF) was aspirated repetitively to assess planktonic bacteria, silver concentration and leucocyte counts. To assess the infection prevention rate, tissue cages were explanted either on day 9 or 14 and examined for regrowth by incubation in tryptic soy broth.

RESULTS: In the agar inhibition assay, silver coated TiAlNb alloys produced an inoculum dependent inhibition zone of 30.1 mm for 10^4 CFU/ml to 17.7 mm for 10^7 CFU/ml of S. epidermidis and of 22.6 mm for 10^4 CFU/ml to 13.3 mm for 10^7 CFU/ml of S. aureus, respectively. In vivo, the effect of silver coated TiAlNb cages against S. epidermidis depended on the inoculum and the modality of infection. Perioperative injected S. epidermidis at an inoculum of 10^5 CFU/cage was completely cleared whereas 10^7 CFU/cage resulted in a 20% prevention rate and 10^8 CFU/cage could not be cleared. In the postoperative setting, 10^6 CFU/cage of S. epidermidis resulted in a 27% prevention rate. The lower prevention rate postoperatively is explained by the decreasing silver concentration in the TCF (from 82 mg/l on day 2 to 11 mg/l on day 14). Infection with S. aureus at 10^3 CFU/cage did not result in a clearance neither peri- nor postoperatively. We therefore assessed the additive effect of silver to vancomycin or daptomycin. Addition of silver to the antibiotics decreased the biofilm by 33% and the adherent bacteria by two log_10 in vitro. In vivo, the perioperative administration of daptomycin to silver coated cages resulted in a 100% and vancomycin in a 33% prevention rate in a perioperative infection with methicillin resistant S. aureus (see Figure). The leukocytes in the TCF showed only a modest decrease in viability (minimal 90%) at 2 days and a rapid recovery after 6 days (100% viability) indicating a low cytotoxic effect of silver on these cells.

Figure: Perioperative infection of uncoated and silver coated tissue cages with methicillin resistant S. aureus 43300 (300 CFU/cage), addition of saline, daptomycin (DAP) or vancomycin (VAN)

CONCLUSIONS: TiAlNb alloy is a good candidate for silver coating. The coated silver has good antistaphylococcal properties. In combination with preoperative antibiotic prophylaxis it can prevent implant infections and is probably safe to use in humans.
Novel anti-infective implant substrates: Controlled release of antibiofilm compounds from mesoporous silica-containing macroporous titanium

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INTRODUCTION: Bone implants with open porosity enable fast osseointegration, but also present an increased risk of biofilm-associated infections. Since an implant, as a biocompatible surface, presents a favorable support for microbial adherence and because the local immune system is temporarily repressed at the implant/tissue interface due to the occurrence of a foreign body response, the implantation site is inherently at risk for microbial contamination [1,2]. To reduce microbial biofilm formation on titanium substrates, we designed a novel implant material enabling controlled release of antibiofilm molecules.

METHODS: The novel implant material consisted of a mesoporous SiO₂ diffusion barrier with controlled drug release functionality integrated in a macroporous Ti load-bearing structure. Using an in-house made in vitro tool consisting of Ti/SiO₂ disks in an insert set-up (Fig 1), through which molecules can diffuse from feed side to release side, a continuous release without initial burst effect of various broad-spectrum antibiofilm compounds was sustained for at least 9 days. We used the fungal pathogen Candida albicans and the bacterial pathogen Streptococcus mutans as a model to assess anti-infective properties of the new titanium substrates.

RESULTS: We found that the C. albicans and S. mutans biofilm growth on the compound-release side was significantly inhibited, establishing a proof-of-concept for the drug delivery functionality of mesoporous SiO₂ incorporated into a high-strength macroporous Ti-carrier.

Fig. 1. In vitro test tool based on a 12-well plate system. (a) The permeable membrane of polystyrene cell culture inserts is replaced by the experimental porous Ti/SiO₂ disks, (b) effectively separating the well (dark blue feed compartment) from the cup (light blue release compartment) by a mesoporous diffusion barrier (gray Ti/SiO₂) for controlled release of the compound through its pores (as indicated by the arrows).

DISCUSSION & CONCLUSIONS: Next-generation implants made of this composite material and equipped with an internal reservoir (feed side) can yield long-term controlled release of antibiofilm compounds, effectively treating infections on the implant surface (release side) over a prolonged time.

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Novel antibiotic release coatings for cementless arthroplasty fixations


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INTRODUCTION: Over the last 5 years there has been a steady increase in the ratio of cementless to cemented hip replacements [1]. Given that, for cementless fixation, antibiotic delivery is necessarily systemic there is a potential risk of impaired local infection control in the patient and possible selection of antibiotic resistance in the pathogen. In the revision situation, both cemented and uncemented fixations receive systemic prophylaxis. However, while this is routinely augmented by local antibiotic delivery via antibiotic-loaded bone cement, there is currently no local antibiotic delivery for uncemented reconstructions. Given that more uncemented revisions are conducted than cemented, and that the surgery time for a revision is longer than that of a primary procedure, the potential infection rate associated with a revision is greater than that of primary surgery [2]. There is therefore an urgent clinical need to develop a therapeutic coating system that is able to release antibiotic(s) within a surgical site to eliminate pathogenic microorganisms, while at the same time allowing the regeneration of local bone tissue.

METHODS: Sol gel coatings were produced through a conventional hydrolysis-condensation route using Silane based precursors [3]. Antibiotics were incorporated into the coatings at loadings equivalent to that conventionally used in bone cement, notably 1.25 Wt %. In vitro, eluted antibiotic was measured by LC-MS-MS. Antimicrobial efficacy was conducted using biofilms of Staphylococcus aureus and S. epidermidis grown on 96 pin Nunc-TSP plates then submerged in wells containing antimicrobial sol-gel coatings. Biofilms at 0, 24, 48, 72 h were re-suspended and plated on agar. In vivo studies involved hydroxyapatite (control) and hydroxyapatite + gentamicin coated Titanium rods 2mm x 1mm Ø implanted into rat femora for 28 days, N=6. Micro-computer tomography image analysis was conducted on explant rods to assess osseointegration. Histology slides were used to assess cell behaviour.

RESULTS: Figure 1 presents the elution characteristics of the therapeutic coating compared to bone cement, having the same antibiotic loading. The minimum inhibitory concentration (1 μg/ml) is exceeded by the sol gel system within the first two hours. Figure 2 shows the kill efficacy of the coating against 4 bacterial strains, showing a minimum, log-4 kill within 24 hours.

![Fig. 1: Elution properties of sol gel vs. bone cement.](image1)

![Fig. 2: Kill efficacy of sol gel coating.](image2)

Figure 3 presents a cross-section of an explanted sol gel coated Ti rod taken from a rat femur.

![Fig. 3: Cross section of femur showing bone bridging to implant.](image3)

DISCUSSION & CONCLUSIONS: A novel sol gel coating has been developed that delivers, by controlled release, therapeutic agents to the implant site without adversely affecting osteoblast differentiation and osteointegration. Kill efficacy has been proven against four major bacterial strains, including an MRSA clinical isolate. Results from an in vivo healing model show the novel coating does not adversely affect healing and bone integration is noted within 4 weeks.

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Biofilms cause recalcitrance of staphylococcal joint infection to antibiotic treatment

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INTRODUCTION: The pathogenesis of joint infections is not well understood. In particular, we do not know why these infections respond poorly to antibiotic treatment.

METHODS: We used an ex-vivo approach to investigate the bacterial factors contributing to joint infection by isolating uninfected synovial fluid (SF) from patients undergoing joint surgery, emulating the situation patients encounter when developing joint infections. A transposon bank was used to identify genes impacting joint infection. Biofilm formation and bacterial aggregation were monitored using microtiter plate assays, confocal laser scanning microscopy, and evaluation using a cellometer.

RESULTS: We show that even with antibiotic concentrations that far exceed the expected bactericidal levels, Staphylococcus aureus bacteria added to the synovial fluid samples are not eradicated and are able to colonize model implant surfaces, i.e., titanium pins². We show that this phenotype is due to biofilm formation, which significantly lowers the efficiency of antibiotic treatment¹. Screening a transposon bank identified bacterial fibronectin- and fibrinogen-binding proteins as important for the formation of macroscopic clumps in SF, suggesting an important role of fibrin-containing clots in the formation of bacterial aggregates during joint infection. Pre-treatment of synovial fluid with plasmin led to a strongly reduced formation of aggregates and increased susceptibility to antibiotics.

We found while specific S. aureus surface proteins are a prerequisite for agglomeration in synovial fluid, that low activity of the Agr regulatory system and subsequent low production of the phenol-soluble modulin (PSM) surfactant peptides cause agglomerates to grow to exceptional dimensions³. Our results indicate that PSMs function by disrupting interactions of biofilm matrix molecules, such as the polysaccharide intercellular adhesin (PIA), with the bacterial cell surface. Together, our findings support a two-step model of staphylococcal prosthetic joint infection: Interaction of S. aureus surface proteins with host matrix proteins such as fibrin initiates agglomeration; thereafter, the bacterial agglomerates grow to extremely large sizes owing to the lack of PSM expression under the specific conditions present in joints.

DISCUSSION & CONCLUSIONS: Our findings provide a mechanistic explanation for the extreme resistance of joint infection to antibiotic treatment, lend support to the notions that fibrin- and fibronectin-binding proteins, Agr functionality and PSM production play a major role in defining different forms of S. aureus infection, and have important implications for anti-staphylococcal therapeutic strategies. Specifically, we suggest that current prophylactic antibiotic choices, despite high penetration into the synovial fluid, may need to be reexamined.

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Infection strategies of *Staphylococcus aureus* to cause chronic osteomyelitis

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INTRODUCTION: *S. aureus* is the most frequent pathogen to cause osteomyelitis that often develops into a chronic course and becomes extremely difficult to eradicate. *S. aureus* is endowed with a multitude of virulence factors, such as adhesins and toxins that are regulated by complex bacterial regulatory networks. Chronic infections have been highly associated with altered bacterial phenotypes, i.e., the small colony variants (SCVs) that dynamically appear after bacterial host cell invasion and are highly adapted for intracellular long-term persistence. In our work we analyse the impact and the mechanism of bacterial phenotype switching and the involvement of bacterial virulence and regulatory factors.

METHODS: We performed different types of in-vitro and in-vivo long-term infection models with *S. aureus* and analysed the bacteria at different stages of the infection process. For this, we used multiple methods such as real-time PCR, proteomics, functional assays and imaging methods to analyse and visualize the bacteria within the host. Further on, we generated and tested a large set of bacterial mutants to investigate the impact of defined virulence and regulatory factors in the bacterial adaptation process.

RESULTS: We show that viable bacteria can persist within host cells (including osteoblasts) or host tissue (predominantly in bone tissue) for several weeks. Persistence induced bacterial phenotypic diversity, in particular the development of SCVs. The SCVs revealed typical changes in virulence factor expression, such as increased expression of adhesins and decreased expression of toxins. Yet, the SCV phenotypes were highly dynamic and rapidly reverted back to the fully virulent wild-type form when leaving the intracellular location and infecting new cells. Using bacterial mutants in the chronic infection experiments revealed that during acute infection bacteria express toxins and destroy host cells, whereas during chronic adaptation they downregulate their virulence. Bacterial mutants in SigB were not able to persist within host cells and did not form SCV phenotypes.

Fig. 1: Schemata of bacterial adaptation during chronic infection

DISCUSSION & CONCLUSIONS: Our results demonstrate that bacterial phenotype switching is an integral part of the infection process that enable the bacteria to hide inside host cells and escape from the host immune response and antimicrobial treatments. The dynamic bacterial switching and adaptation phenomenon is mediated by the tight crosstalk of *S. aureus* regulators, in particular by the stress factor SigB that is crucial to mediate bacterial persistence and SCV formation.

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Staphylococcus aureus protein A (SpA) binding to osteoblasts hinders bone remodelling in osteomyelitis infections resulting in a net bone loss

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INTRODUCTION: Osteomyelitis (OM) is a progressive inflammatory microbial infection of any bone of the skeleton. It is predominately caused by Staphylococcus aureus, a gram positive bacterium which is a commensal of humans. When S. aureus gains access to sterile bone, inflammation coupled with bone loss and destruction occurs. Chronic infection is diagnosed by x-ray radiology via the presence of pathological fractures resulting in sequestra (avascularised necrotic bone), often forming the foci of infection. Here we report a pathogenic mechanism where S. aureus cell wall Protein A (SpA) binds to osteoblasts resulting in an inhibition of osteogenesis [alkaline phosphatase (ALP), osteogenic genes (collagen type-1, osteopontin and osteocalcin) and subsequent mineralisation]. Moreover stimulation of osteoclastogenesis occurs [Receptor activator of nuclear factor kappa-B ligand (RANKL) release, osteoclast recruitment and increased Tartrate resistant acid phosphatase (TRAP) levels] when S. aureus binds to osteoblasts. These events may collectively result in bone loss and destruction in OM patients.

METHODS: S. aureus Newman wild-type, Newman mutants ∆SpA and pCU1 (SpA+), were cultured overnight in Brain Heart Infusion broth for >18hrs. S. aureus strains were then fixed in 4.8% formaldehyde and washed before diluting to 1x10⁹ cells/ml. Bacteria were allowed to adhere for 2 hrs on 6-well plates before seeding MC3T3-E1 pre-osteoblasts (5x10⁵ cells/well) in vitro. Osteoblastic cells were cultured in α-MEM medium containing L-glutamine, foetal bovine serum and penicillin/streptomycin with osteogenic supplements (dexamethasone, ascorbic acid and β-glycerolpentahydrate). At 4 hours, day 7, 14 and 21 osteoblast cells were assed for proliferation (haemocytometer and trypan blue) and pro-osteogenic markers: ALP levels (p-nitrophenol), osteogenic genes (RT-PCR) and mineralisation (Alizarin red and Von Kossa staining). RANKL was measured from osteoblasts, while TRAP was measured from RAW 264.7 osteoclastic cells (2x10⁴ cells/well) using ELISA kits. Conditioned 4 day media from osteoblasts ± S. aureus (± SpA) was used with osteoclast cell studies for TRAP activity and migration studies, using 8µm pore hanging cell culture inserts.

RESULTS: S. aureus Newman wild-type binding to osteoblasts resulted in an inhibition of proliferation (P<0.0001), ALP levels (P<0.05), osteogenic markers (collagen type-1, osteopontin and osteocalcin) and subsequent mineralisation (P<0.001).1,2 Osteoblasts exposed to S. aureus released soluble RANKL (P<0.01), which in turn recruited osteoclastic cells and increased their TRAP levels (P<0.05).2 Removal of SpA from S. aureus cell wall resulted in a recovery of proliferation, expression of osteogenic markers followed by mineralisation and with no pro-osteoclastogenic events (P=NS). Over expressing SpA in S. aureus resulted in an increase of adverse events on osteogenic markers and increased osteoclastogenic activity in vitro.1,3

DISCUSSION & CONCLUSIONS: Previously we have identified S. aureus SpA binding to tumor necrosis factor receptor I(TNFR1) on osteoblasts.1 Here we identify the effect of SpA binding to osteoblasts.2 In addition to inflammation,3 S. aureus mimics an osteoporotic disease-like characteristic by hindering bone remodelling through increased bone resorption and lack of pro-osteogenic events resulting in a net bone loss. SpA is expressed in >90% of S. aureus strains and can contribute to bone loss and destruction in OM patients, leading to chronic infection with sequestra development and pathological fractures.

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The interaction of bone marrow mesenchymal stem cells with S. aureus is mediated by fibronectin binding protein

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INTRODUCTION: Most studies on the pathogenesis of osteomyelitis have focussed on the interaction between bacterial virulence factors and osteoblasts. However, there is another player in this scene and this is the mesenchymal stem cell (MSC), the precursor cells of osteoblasts. To this end, we wanted to identify the role of bone marrow MSCs in osteomyelitis through investigating their molecular interaction with S. aureus.

METHODS: Confluent monolayer of human MSCs (n=3) in 96 well plate were infected with 3 bacterial strains of S.aureus: wild type (WT), a mutant lacking the expression of fibronectin binding protein (ΔFnBPA/B) and a mutant supplemented with plasmid expressing intact FnBP type A (ΔFnBPp4A). Cells were infected at MOI 100 for 90 minutes and adhesion and invasion were quantified after 3 hours using an immunofluorescence assay and viable bacterial colony count.

RESULTS: S. aureus WT was able to adhere to MSCs (Figure 1). This adhesion was greatly reduced in the absence of FnBP (strain ΔFnBPA/B) and restored with the use of strain ΔFnBPp4A. The difference in internalisation between FnBP deficient and FnBP positive groups was statistically significant (Figure 2).

DISCUSSION & CONCLUSIONS: To date, there are no studies to define the molecular interaction between S. aureus and bone marrow MSCs. Indeed, very few studies have addressed the interaction between MSCs and S. aureus. Our results demonstrated the capacity of S. aureus to adhere to and internalize bone marrow MSCs. We identified FnBP as a critical player in this interaction. The results of this study shed light on a new paradigm in the pathogenesis of osteomyelitis, namely, the interaction with the bone niche precursor cells. This may explain the persistent nature of the disease and open the door for novel interventions in the disease.

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Staphylococcus epidermidis, commensal to pathogenic?

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INTRODUCTION: The commensal Staphylococcus epidermidis is a leading cause of nosocomial biomedical implant-associated infections. They successfully evade the host’s immune system due to its ability to adhere and form a biofilm on surfaces. In the past it has been hypothesised that S. epidermidis populations colonising the skin and mucus membranes and those causing infections differ significantly in the prevalence of key factors involved in adhesion and biofilm accumulation. Here we took a combined phenotyping and genomics approach to investigate (i) if pathogenic S. epidermidis form biofilms better than commensals, and (ii) if this is related to genomic elements such as those involved in the production of cell-wall and intercellular adhesins involved in adhesion and biofilm formation.

METHODS: Genomic DNA from the 117 S. epidermidis isolates (70 pathogenic and 47 commensals) were sequenced using an Illumina HiSeq 2500 machine, de novo assembled and resulting data was archived in the Staphylococcal Bacterial Isolate Genome Sequence database (BIGSdb). Phylogenetic trees were constructed from 53 rMLST genes and implemented in CLONALFRAME. For biofilm phenotype analysis, 98 S. epidermidis isolates were cultured in 8-well chamber slides, stained with SYTO9, and visualised with a Zeiss Confocal Laser Scanning Microscope (CLSM). Biofilm thickness, biovolume and roughness coefficient (Ra) were determined from the CLSM z-stacks using COMSTAT software. The presence/absence of genes involved in adhesion and biofilm formation was also examined.

RESULTS: Genotypic classification randomly distributed the isolates into three separate clades (Fig. 1), with the isolates in the main clade, Clade A, being diverse in biofilm thickness, biovolume, and Ra. All S. epidermidis isolates formed aggregates and biofilms (Fig. 2), but the magnitude of adherent cell aggregates and the thickness of the biofilm formed differed depending on the isolate. Statistically, only biofilm thickness had a significant association with clade distribution.

Fig. 1: Population structure of A) 117 S. epidermidis isolates, and B) an enlargement of the 98 isolates in Clade A. The scale (0.1) is in coalescent units and represents the number of substitutions per site.

Fig. 2: Example of CLSM biofilm images: A) orthogonal view of Z-stacks; B) 3D image of the Z-stacks; C) 3D cross-section of Z-stacks. Scale of images is μm.

The only significant association between the presence/absence of key adhesion and biofilm formation genes and phenotype was between aap, sesE, and biofilm thickness.

DISCUSSION & CONCLUSIONS: The study confirmed S. epidermidis isolates are from diverse lineages, and that these divergences lead to the bacteria forming different biofilm structures due to variations in the presence or absence of key genes involved in adhesion and biofilm formation.
Immune tolerance mechanisms in tissues during chronic inflammatory diseases

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Substantial progress in understanding mechanisms of immune regulation in allergy, asthma, autoimmune diseases, tumors, organ transplantation and chronic infections has led to a variety of targeted therapeutic approaches. Allergen-specific immunotherapy (AIT) has been used for 100 years as a desensitizing therapy for allergic diseases and represents the potentially curative and specific way of treatment. The mechanisms by which allergen-AIT has its mechanisms of action include the very early desensitization effects, modulation of T- and B-cell responses and related antibody isotypes as well as inhibition of migration of eosinophils, basophils and mast cells to tissues and release of their mediators. Regulatory T cells (Treg) have been identified as key regulators of immunological processes in peripheral tolerance to allergens. Skewing of allergen-specific effector T cells to a regulatory phenotype appears as a key event in the development of healthy immune response to allergens and successful outcome in AIT. Naturally occurring FoxP3+ CD4+CD25+ Treg cells and inducible type 1 Treg (Tr1) cells contribute to the control of allergen-specific immune responses in several major ways, which can be summarized as suppression of dendritic cells that support the generation of effector T cells; suppression of effector Th1, Th2 and Th17 cells; suppression of allergen-specific IgE, and induction of IgG4; suppression of mast cells, basophils and eosinophils and suppression of effector T cell migration to tissues. In addition to Treg cells Breg cells also play an important role in allergen tolerance and specific IgG4 production. Similar mechanisms are valid in immune tolerance-related disease, such as autoimmunity, tumor, organ transplantation and chronic infections. New strategies for immune intervention will likely include targeting of the molecular mechanisms of immune tolerance and reciprocal regulation of effector and regulatory T and B cell subsets.
On the humoral immune response evoked by *Staphylococcus aureus* bacteraemia and osteomyelitis.

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**INTRODUCTION:** *Staphylococcus aureus* (*S. aureus*) is the most frequent (up to 67%)¹ cause of human osteomyelitis, *S. aureus* osteomyelitis often occurs after open bone fractures, orthopaedic treatment (bone reconstruction, joint replacements), and diabetic foot ulcerations. As a consequence of continuous population ageing, the incidence of *S. aureus* osteomyelitis is steadily increasing². Unfortunately, *S. aureus* osteomyelitis is often difficult to treat and cure. Once an osteomyelitis developed towards the chronic stage, it results in life-long recurrent exacerbations with increased chance of further complications like bacteraemia, endocarditis, metastatic infection of other body sites³ and malignant transformation⁴. Despite the fact that *S. aureus* pathogenesis has been studied for decades *in vitro* and in animal models, we still have little insight in the mode of action of the individual virulence factors involved in osteomyelitis in humans. In order to shed more light on this subject we studied the humoral response in longitudinally collected sera from 10 patients with chronic osteomyelitis and compared them with sera from 10 bacteraemia patients and 20 age-matched healthy volunteers. Furthermore we generated biofilms on both polystyrene (PS) and human bone to determine the local production of virulence factors.

**METHODS:** We characterized IgG responses in sera of 10 patients with chronic osteomyelitis against 50 primarily virulence factors of *S. aureus* using a Luminex®-based assay⁵. Furthermore the presence of the same proteins was determined in biofilms of the osteomyelitis causing *S. aureus* strains grown on polystyrene (PS) and human bone using a competitive Luminex®-based assay⁶. For conformation of *in vitro* expression of these proteins we used transcriptomic approaches. Finally we confirm *in vitro* biofilm formation on human bone using scanning electron microscopy.

**RESULTS:** We observe significantly increased IgG levels in osteomyelitis patients compared to 20 age-matched healthy controls for 15 antigens while no differences are observed compared to IgG levels of the bacteraemia patients. The presence for 7 of these proteins was confined in biofilms on PS and/or bone. Interestingly 7 other proteins were found to be expressed by *in vitro* grown biofilms yet none of them developed a significant elevated IgG level in any of the osteomyelitis patients.

**DISCUSSION & CONCLUSIONS:** Our data provide further insights in the development of the humoral response in patients with *S. aureus* infections like osteomyelitis. In the future studies like these might lead to new therapy targets to fight *S. aureus* osteomyelitis.
A new paradigm for the diagnosis of periprosthetic joint infection

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INTRODUCTION: Total joint arthroplasty continues to gain acceptance as the standard of care for the treatment of severe degenerative joint disease, and is considered one of the most successful surgical interventions in the history of medicine. However, infection of these implants, called Periprosthetic Joint Infection (PJI), remains one of the biggest challenges facing orthopaedics today. PJI can lead to additional surgeries, revision, fusion and amputation.

DIAGNOSIS OF PJI: It is important to accurately diagnose PJI because its management differs from that of other causes of arthroplasty failure. The most common symptom of PJI is pain. In acute infection, the local signs and symptoms (e.g., severe pain, swelling, erythema, and warmth at the infected joint) of inflammation are generally present. On the other hand, chronic infection usually has a more subtle presentation, with pain alone, and is often accompanied by loosening of the prosthesis at the bone-implant interface. The diagnosis of PJI has proven quite challenging, as both acute and chronic infections can be difficult to differentiate from other forms of inflammation.

The reported literature on the diagnosis of PJI has focused on evaluated laboratory tests that were never developed specifically for the diagnosis of PJI. These include the erythrocyte sedimentation rate (ESR), the serum C-reactive protein (CRP), the synovial fluid white blood cell count and the leukocyte differential. Because these tests were not made for the purpose of diagnosing PJI, it has been the responsibility of the orthopaedic community to evaluate and recommend their interpretation. This has resulted in significant confusion regarding the appropriate thresholds and optimal combination of these tests. These difficulties were the motivation for the development of a specific test for the detection of PJI.

THE SYNOVASURE® TEST FOR PERIPROSTHETIC JOINT INFECTION (PJI): The promising diagnostic capabilities of synovial fluid biomarkers for PJI have already been reported in the literature1,2. These biomarkers include inflammatory proteins, cytokines, and microbicidal peptides/proteins that are known to be involved in the host response to infection.

Studies have demonstrated that the alpha-defensin microbicidal peptide present in human neutrophils is an ideal biomarker for PJI due to the distinct separation it achieves between positive and negative results3,4,5. A specific test allowing to measure the concentration of the alpha-defensin in the synovial fluid has been developed4,5. The specificity and the sensitivity of this test for the detection of a PJI are respectively 96% and 97%4,5 and an independent study by the Mayo Clinic6 (Phoenix, AZ, USA) confirmed these results. This test has been proven to have also a high reproducibility, its results not being influenced by antibiotics.

DISCUSSION: A lateral flow version of this test (Synovasure PJI, distributed exclusively in Europe by Zimmer GmbH) has been recently developed. It allows reading the results in 10 minutes and it doesn’t require any laboratories for its interpretation. Currently, this test device is in clinical evaluation in more than 200 European hospitals.

CONCLUSIONS: In case that the clinical evaluation of this test device is positive, this method will be a new paradigm for the diagnosis of periprosthetic joint infection.
Local prophylaxis of implant-related infections using a hydrogel as carrier

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**INTRODUCTION:** Currently, no clinical options are available to prevent infections on uncemented orthopedic implants. Therefore we investigated the efficacy of DAC hydrogel (disposable antibacterial coating1, Novagenit, Italy) as carrier for various agents to prevent infections in an in vivo implant model.

**METHODS:** Titanium rods were implanted in the left tibiae in 48 New Zealand White rabbits. Prior to implantation, the implant bed was contaminated with 10^5 colony forming units of S. aureus. In the experimental groups, DAC hydrogel was loaded prior to be coated on the rods with: 2% w/v vancomycin (Van2 group, N=6), 5% w/v vancomycin (Van5 group, N=6), 10% w/v bioactive glass, (BonAlive, Finland) (BAG group, N=6) which is antibacterial2 and osteoconductive3, or 0.5% w/v N-acetyl cysteine (NAC group, N=6), which inhibits bacterial growth and decreases biofilm formation4. Two control groups were included: no hydrogel (No gel group, N=6) or hydrogel-only coating on the rod (Gel group, N=12).

Blood parameters (erythrocyte sedimentation rate (ESR) and neutrophil counts) were measured weekly. Following explantation on day 28, in accordance, the Van2 and Van5 groups showed lower scores for infection and inflammation compared with the Gel group. Further, the Van2 group demonstrated more bone-implant contact than the Gel group. These results suggest a less severe or absence of infection in these animals from the Van2 and Van5 groups. In contrast, blood values, histological scores, and bone-implant contact of the BAG and NAC groups were comparable with the Gel group. These results suggest that the infection was not prevented in the BAG and NAC groups.

**DISCUSSION & CONCLUSIONS:** Local application of vancomycin-loaded DAC hydrogel successfully reduced implant-related infections. Loading of the DAC hydrogel with BAG or NAC did not prevent infection. It is possible that BAG in powder form, as used in the present study, dissolved before the antibacterial effect could take place. Instead, BAG granules may be a viable alternative. Next, it is possible that the NAC concentration was too low to prevent infections in an in vivo environment, although this concentration was proven effective in vitro for its antibacterial properties1.

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Dendritic cell induced T-cell responses to biomaterials in presence of staphylococcal infection

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INTRODUCTION: Staphylococcal bacteria are major pathogens in medical device (biomaterial)-associated infection. The combination of a biomaterial and bacteria can provoke inflammatory reactions of non-expected nature, resulting in increased susceptibility to infection. A major cell type orchestrating immune responses towards either immune activation or immune tolerance are dendritic cells (DCs), which activate and instruct T-cells. Activated (matured) DCs present antigens to T-cells and together with co-stimulatory molecules and secreted cytokines can activate and polarize naïve T-cells into different effector T helper (Th) cells (figure 1). We aimed to obtain insight in the activation of DCs and subsequently in their potency to activate and polarize T-cells in response to biomaterials, staphylococci, and to their combination. We studied poly(trimethylene-carbonate) (PTMC), a high interest experimental material for resorbable implants in a variety of surgical indications.

METHODS: Human monocyte-derived DCs were cultured with or without PTMC discs in the presence or absence of Staphylococcus aureus or Staphylococcus epidermidis bacteria. The activation state of DCs was assessed by measuring their maturation markers HLA-DR, CD83 and CD86 by flow cytometry and by measuring secreted cytokines TNF-α, IL-10, IL-6, IL-23, IL-12p70 and IL-1β by ELISA. PTMC and/or staphylococci-activated DCs were subsequently co-cultured with CFSE-labelled autologous naïve CD4+ T-cells, and T-cell proliferation was assessed by flow cytometry. For T-cell polarization, DCs were co-cultured with autologous naïve CD4+ T-cells with or without PTMC discs in presence or absence of S. aureus or S. epidermidis. The resulting intracellular IFN-γ and IL-4 levels of the T cells were analyzed by flow cytometry, as markers for Th1 or Th2 polarization, respectively. Experiments were performed with cells of seven different donors.

RESULTS: DC culture on PTMC discs did not induce DC activation, nor did the cultured DCs induce T-cell proliferation and polarization. DC culture with staphylococci or with the combination of PTMC and staphylococci induced DC activation as well as the capacity of the DCs to induce proliferation and polarization of naïve T-cells into Th1 cells. There was no difference between the level of DC activation, nor between the numbers of proliferating T-cells and the percentage of Th1 or Th2 polarized T-cells after incubation with PTMC combined with staphylococci, or with the bacteria alone.

DISCUSSION & CONCLUSIONS: PTMC alone did not measurably influence DC activation or subsequent T-cell proliferation and polarization. S. aureus activated DCs and induced a DC type which caused strong T-cell proliferation and predominantly Th1 polarization. As far as S. epidermidis induced DC activation, this also led to T-cell proliferation and Th1 polarization, but with lower numbers of T-cells proliferated and polarized. The response to the bacteria was not different in presence or absence of PTMC, but strongly predominated over the response to the biomaterial itself. So, in a bacterial species-dependent way the presence of infection may alter the immune response as provoked by the biomaterial alone.

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Pathogenesis of staphylococcal biomaterial-associated infection

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INTRODUCTION: Infection of inserted or implanted medical devices (biomaterials”) can have disastrous consequences, including removal of the device. Implantation of a biomaterial provokes an inflammatory response known as the “foreign body response”. Staphylococcus epidermidis and Staphylococcus aureus are the major causes of biomaterial associated infection (BAI), which in absence of a foreign body hardly ever cause infection. Infections may arise early, from contamination during the implantation or insertion procedure by bacteria from the skin of the patients or from the surgical environment, or late by bacteria reaching the implant from other sites of the body by hematogenous spreading. Bacteria entering during surgery may adhere to the material to form a biofilm, and may enter the surrounding tissue and become internalized within host cells.1 Thus, the tissue surrounding a foreign body must be considered a niche for bacteria that cause BAI. In order to design strategies to prevent BAI, it is vital to understand the in vivo course of bacterial infection. The aim of the present study therefore was to compare the pathogenesis of early (i.e. infection during surgery) and delayed S. aureus experimental BAI (i.e. infection 2 days after implantation).

METHODS: Titanium implants were placed subcutaneously in the back of mice and an inoculum of 106 CFU of S. aureus ATCC 49230 in 25 µl saline was injected either along the implant immediately after implantation (i.e. early infection), or 2 days later (i.e. delayed infection). Mice were sacrificed at 1 or 4 days after implantation, and biopsies were collected to assess the bacterial colonization of both the biomaterial and the surrounding tissue. Each group consisted of 9 mice, each with 2 implants (n = 18 implants).

RESULTS: After 1 and 4 days, significantly more bacteria were cultured from tissue of mice with an early infection, than of mice with a delayed infection (p<0.05) (Fig. 1). All tissue biopsies of mice with early or delayed infection were culture positive after 1 day. The delayed group appeared to be less susceptible to infection, since after 4 days, the number of culture positive tissue samples (39%) was significantly lower than in the early infection group (83%) (p<0.05). Moreover, after 1 day the implants had significantly lower levels of bacterial colonization in the delayed infection group, both in numbers of bacteria (p<0.001) and the numbers of culture positive implants (p<0.01). After 4 days, numbers of bacteria and infected implants were very low in both groups, with no difference between the early and delayed infection groups.

DISCUSSION & CONCLUSIONS: Mice were more susceptible to early than to delayed S. aureus infection. In delayed infection, less bacteria colonized the implant surface. However, even in delayed infection bacteria were cultured from the tissue, implying that in order to protect against early as well as delayed infection, antibacterial strategies should be directed against bacteria both in biofilms and in peri-implant tissue.

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Recent developments for *Staphylococcus aureus* vaccines: clinical and basic science challenges


Bacterial vaccines have made dramatic impacts upon the morbidity and mortality of a number of common pathogens, but a vaccine to prevent *Staphylococcus aureus* infections has proven to be illusive. With successful bacterial vaccine, the organisms are all part of the transient flora, whereas, *S. aureus* is part of the normal human flora. This means that *S. aureus* is much better adapted to the host. The failure of several staphylococcal antigens to protect humans from infection clinical trials using active or passive immunization has stimulated a re-examination of the fundamental assumptions about staphylococcal immunity in humans vs. animals, especially rodents. This has spurred an active debate about the appropriate models for vaccine development and an examination of our current understanding of the protective immunity in humans. A major factor in the development of previous bacterial vaccines was a biomarker that predicted human protection, e.g., antibodies to tetanus toxin or to pneumococcal polysaccharide. While antibodies against a number of staphylococcal antigens have proven to be an excellent biomarker for protection in rodents, these have not been translated to human infections. Thus, while much work remains, there is a growing consensus that T cell immunity plays an important role in protecting humans. Moreover, the presence of anti-staphylococcal toxin antibodies correlates with reduced disease severity in humans. Some of the most important recent advances concerning potential biomarkers, adjuvants, novel antigens, and the role of pre-existing immune status of vaccinees in vaccine-associated mortality will be discussed.
Osteoimmunology of implant-associated osteomyelitis and development of a passive immunization for MRSA
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INTRODUCTION: In Orthopaedics there is great concern over the very poor outcomes of two-stage revision total joint replacement (TJR) surgery from Methicillin-resistant Staphylococcus aureus (MRSA) osteomyelitis. Thus, non-traditional interventions like passive immunization are warranted, particularly for immunocompromised patients and the elderly who are typically poor responders to active vaccines. Recent studies on the osteoimmunology of MRSA osteomyelitis in a murine model, have demonstrated the roles of Staphylococcal abscess communities (SACs), NETosis, and biofilm formation, which has lead us towards the development of a passive immunization to prevent and treat this condition. Additional studies have elucidated the immune proteome against S. aureus in mice and humans, to further our understanding of “protective” and “non-protective” humoral immunity against this pathogen, and have revealed that the autolysin proteins, amidase (Amd) and glucosaminidase (Gmd), are immuno-dominant, protective antigens in a murine model of implant-associated osteomyelitis, and our development of anti-Amd and anti-Gmd monoclonal antibodies as a potential passive immunization therapy for two-stage revision of S. aureus-infected TJR.

METHODS: To assess the efficacy of anti-Gmd and anti-Amd passive immunization, alone and in combination therapy, mice (n = 5) were passively immunized with PBS (Placebo), anti-Gmd, anti-Amd or a combination of these antibodies (40 mg/kg, i.p.) 24 hr prior to infection with USA300 LAC::lux, and the effects on SAC formation were determined by histology as previously described. Representative infected tibiae on day 14 post-infection are shown from histology sections that were stained with modified Gram stain to identify SACs (Fig. 1A-H). Note that Placebo-treated tibiae show a central nidus of bacteria surrounded by an eosinophilic pseudocapsule within the abscess area (SACs) (A,B), which are absent in mice treated with anti-autolysin mAbs: Anti-Gmd (C,D), Anti-Amd (E,F) and combination Anti-Amd + Anti-Gmd(G,H).

DISCUSSION & CONCLUSIONS: Implant-associated osteomyelitis from MRSA remains a major clinical problem without definitive solutions. Using a murine model we have shown the potential of passive immunization with anti-Gmd and anti-Amd mAb. Future studies to further develop these mAb into a human therapy are warranted.

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Redox active compound improves infection control in biofilm-associated infected femur model

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INTRODUCTION: Implant-associated infections affect more than 50,000 orthopedic cases a year and Staphylococcus aureus is one of the most common pathogens. Its ability to persist locally is related to its ability to form and maintain biofilm structures. Fixation devices and total joint components provide a surface for bacterial adherence and foster formation of biofilms. Reports that bacteria actively modulate their redox environment in a biofilm suggested a novel treatment for biofilms. We postulated that an oxidant scavenger would interfere with the maintenance of the biofilm structure and improve clinical outcomes. We tested a redox-active metalloporphyrin compound (MnTE-2-PyP or MnTE) in an in vitro biofilm model and an infected fracture model.

METHODS: In vitro: A known biofilm-forming subtype of S. aureus (ATCC 29213) was used. Biofilm assessment was done using crystal violet assay for extracellular polymeric structure (EPS) and NBT assay for reactive oxygen species. S. aureus was diluted and plated on sterile 96-well PVC plates. Cultures were grown over 24 hours, treated with varying concentrations of drug (30 μM) or PBS after 12 hours of growth. Absorbance for each assay was read using a microplate reader.

Animal Model: Male C57BL6 mice (20-25 grams) were used. A midshaft femur fracture was created through a lateral incision, treated with IM fixation using an 8 mm section of 23 gauge needle and infected with S. aureus (sensitive to cephalaxin). Three treatment groups (1) no active drug treatment, (2) cephalaxin 250 mg/mL in drinking water, (3) MnTE-2-PyP and cephalaxin) with five to 10 mice per group were tested and four independent replicates were done. Femurs were harvested at the end of two weeks. Bone was weighed, homogenized, sonicated then plated for quantitative cultures.

RESULTS: MnTE decreased the EPS structure in S. aureus biofilms in a dose response fashion that corresponded with changes in redox state as measured by NBT assay (Fig 1). In an animal model MnTE acted synergistically with cephalaxin and showed significantly lower CFU/g bone after two weeks of treatment.

![Figure 1](image1.png)

**Fig. 1:** Panel A. Crystal violet Assay for EPS in S. aureus biofilm treated with varying doses of MnTE. Panel B. NBT assay for reactive oxygen species produced within S. aureus biofilm treated with same dosing levels of MnTE.

DISCUSSION & CONCLUSIONS: A redox-active metalloporphyrin decreased the EPS structure of a staph biofilm in vitro and also showed synergistic effect with antibiotic to reduce bone bacterial counts in a mouse infected fracture model.

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405 nm light technology shows potential for patient safe decontamination during orthopaedic surgery

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INTRODUCTION: Infection rates following orthopaedic arthroplasty surgery are as high as 4%, while the infection rates are even higher after revision surgery [1]. The duration of routine arthroplasty surgeries is typically between 1 and 2 hours. 405nm High-Intensity Narrow-Spectrum Light (HINS-light) has bactericidal activity against Healthcare-Associated Infection (HAI) related bacterial pathogens including MRSA [2] and hence may aid in reducing the incidence of infections that arise from environmental contamination during arthroplasty surgery.

METHODS: Immortalised rat osteoblast (OST 5) cells were exposed to 405 nm light in Dulbecco’s Phosphate Buffered Saline (DPBS) at different dose rates (18 - 45 J/cm²) at 37°C and 5% CO₂. Unexposed samples were treated the same way. After 72 h post treatment at 37°C, cell viability (MTT reduction), cell function (ALP assay) and cell proliferation rate (BrdU assay) were measured. Bacteria including S. aureus, E. coli, P. aeruginosa, K. pneumoniae, S. epidermidis and A. baumannii were exposed to 405 nm light in Phosphate Buffered Saline (PBS) at doses ranging from 4.5 – 36 J/cm² at room temperature. After 24 h post incubation at 37°C, viable bacteria were enumerated as % surviving CFU/plate as compared to unexposed samples. Statistical analysis was performed for both mammalian cells and bacteria using unpaired Student’s t-test and differences considered significant when p<0.05

RESULTS: After 72 h post light treatment, no significant difference was observed between the unexposed and 405 nm light exposed samples for up to a dose rate of 36 J/cm² in cell viability, cell function and proliferation rate (fig 1-a). However, at higher doses (45 J/cm²) there was a decrease in all the cell response parameters measured. Significant inactivation of all bacterial organisms was achieved by exposure to 18 J/cm² and up to a 100 % inactivation of all species was shown after administering a dose of 36 J/cm² of 405 nm light (fig 1-b).

Fig. 1: Effect of 405 nm light treatment on (a) OST 5 cell response parameters after 72 h incubation (n=8 ± SEM), (b) Survival of a range of clinically relevant bacterial pathogens after 24 h incubation. (n=4 ± SEM)

DISCUSSION & CONCLUSIONS: Exposure of mammalian cells for up to a dose of 36 J/cm² does not cause any detectable effect on osteoblast viability. However, this dose of light is still bactericidal to a variety of gram positive and gram negative bacteria tested in this study. These results suggest that exposure to a dose of 36 J/cm² may be suitable for use for continuous decontamination during orthopaedic surgery whilst being safe for tissue exposure.

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Synthetic antimicrobial and antibiofilm peptides from controlled release coatings to prevent implant-associated infection and derangement of immune responses

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INTRODUCTION: Infection occurs in 0.5 – 10% of patients receiving an indwelling biomedical device (“biomaterial”). Bacteria may form biofilms on the biomaterial surface and colonize the surrounding tissue. In order to prevent biomaterial-associated infection, it is necessary to develop antimicrobials not subject to resistance development which do not only kill the microorganisms, but also neutralize the bacterial compounds that cause excessive immune dysregulation. In the EU project BALI we have therefore developed novel Synthetic Antimicrobial and Antibiofilm Peptides (SAAPs) based on the primary structure of the human antimicrobial proteins LL-37 and thrombocidin-1.

RESULTS: The peptides kill a broad spectrum of Gram-positive and -negative (antibiotic resistant) bacteria at concentrations ranging from 0.8 - 8 µM in phosphate-buffered saline. In presence of 50% human plasma the bactericidal concentrations are 2 – 32-fold higher, depending on the peptide and tested strain. The SAAPs prevented biofilm formation of Staphylococcus aureus at concentrations of 3.2 – 12.8 µM. They also neutralized bacterial pro-inflammatory molecules, e.g. they inhibited the induction by lipopolysaccharide (LPS) or by UV-killed S. aureus bacteria of the production of IL-12 and IL-8 by leukocytes in whole blood .

Studies on membrane model systems mimicking bacterial and mammalian plasma membranes revealed that the SAAPs preferentially interact with anionic lipids characteristic for bacterial plasma membranes. The high permeabilization of those membranes as compared to mammalian membranes reflects the high antimicrobial activity and low cytotoxicity of the SAAPs.

The different SAAPs were eluted from innovative Polymer-Lipid Encapsulation matriX (PLEX) coatings composed of pharmaceutically approved polymers and lipids. The final PLEX was tailored to accommodate an initial high rate, short term release in the first two days followed by zero-order kinetic release over approximately 30 days. In the mouse subcutaneous biomaterial–associated infection model, SAAP-containing coatings applied on titanium implants reduced the numbers of S. aureus colonizing the implants at one day after challenge. In a rabbit humerus intramedullary nail infection model the LL-37 –derived peptide OP-145, previously named P60.4Ac¹, reduced numbers of culture-positive implants and bones after 4 weeks by 67%. Moreover, the percentage of culture-positive soft tissues was reduced by 80%. Coatings with other SAAPs are presently under investigation.

DISCUSSION & CONCLUSIONS: SAAPs combined with PLEX controlled release coatings can prevent metalimplant-associated infections, and thus may contribute to patient uneventful recovery and to reduction of healthcare costs.

ACKNOWLEDGEMENTS: This work was supported by FP7-HEALTH-2011 grant 278890, BALI – Biofilm Alliance.
Mechanisms by which *Staphylococcus* and *Acinetobacter* inhibit osseointegration

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**INTRODUCTION:** Implant infections are among the most difficult problems in orthopaedics. Moreover, prolonged infection and progressive inflammation result in osteolysis around the orthopaedic implants and impair osseointegration. *S. aureus* is the most common orthopaedic pathogen and *Acinetobacter* is becoming increasingly common in both military and civilian hospitals. The goals of this project were to determine how *S. aureus* and *Acinetobacter* affect osseointegration.

**METHODS:** The implant infection model was based on our previously described murine model of osseointegration that utilized rigorously cleaned Ti-6Al-4V screw shaped implants (0.8 mm diameter, 3.5 mm length) [1]. Implants without or with adherent bacteria (0.5-2x10^6 CFUs of *S. aureus* per implant or 1-3x10^7 CFUs of *Acinetobacter* per implant) were screwed into pilot holes in the mid-diaphysis of the femur of 6-8 week old mice matched for age, gender, and genetic background. Macrophage recruitment was measured in MAFIA mice, in which the c-fms promoter drives expression of GFP, by fluorescence imaging after exposing the femurs, implants, and surrounding soft tissues. Osseointegration failures were defined as implants that were not fixed in the femurs at the time of euthanasia. Osseointegration was also measured by biomechanical pull-out testing as we described previously [1]. Bacterial bioburden was assessed by longitudinal bioluminescent imaging and by determination, on the implants and in femur homogenates, of the number of CFUs and copies of the *S. aureus*-Xen36 luxA gene and the *Acinetobacter adeR* gene. TNFα, IL1β, IL6, and MCP-1 were also measured by ELISA in the femur homogenates.

**RESULTS:** All three methods for measuring bacterial bioburden demonstrated that our protocol successfully created chronic localized infections. Both *S. aureus* and *Acinetobacter* inhibited osseointegration by recruiting macrophages, inducing production of pro-inflammatory cytokines, and causing local osteolysis. To determine mechanisms by which *Acinetobacter* initiates this inflammatory cascade, we studied mutant strains that have impaired biofilm formation and/or impaired quorum sensing. Surprisingly, impaired biofilm formation had no detectable effect while impaired quorum sensing significantly reduced the effects of *Acinetobacter* on osseointegration and IL1β levels without detectably affecting the levels of TNFα, IL6, or MCP1. Consistent with an important role for IL1β, *Acinetobacter* had less effect on pro-inflammatory cytokines and osseointegration in IL1R−/− mice. Comparison of wild-type and TLR2−/−;TLR4−/− double knockout mice showed that the effects of *S. aureus* are mostly independent of TLR2 and TLR4, while the effects of *Acinetobacter* are partially dependent on the TLRs. Finally, the immunomodulatory peptide IDR-1018 [2] significantly increased macrophage recruitment and reduced the *S. aureus* bioburden as well as the effects of *S. aureus* on implant osseointegration and TNFα levels without detectably affecting the levels of IL1β, IL6, or MCP1.

**DISCUSSION & CONCLUSIONS:** Our murine model demonstrated that both *S. aureus* and *Acinetobacter* impair osseointegration of orthopaedic implants. The discrepancy between our results and previous reports that *Acinetobacter* does not cause osteolysis [3,4] may be due to the absence in those studies of implants that osseointegrate or to the use of *Acinetobacter* strains with impaired quorum sensing. This murine model will also allow testing of novel approaches, such as IDR-1018, to treat implant infections and thereby preserve osseointegration. Our results suggest that agents that block quorum sensing may be especially useful for *Acinetobacter* infections.
Staphylococcus aureus infection causes hyper mineralisation by osteoblasts in a 3D extra-cellular matrix environment

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INTRODUCTION: Osteomyelitis is an inflammatory bone disease most commonly caused by Staphylococcus aureus¹. Previous research conducted by our host infection group revealed the interaction between S. aureus surface protein, protein A, and osteoblast integrin, TNFR1, resulting in bone destruction and bone loss². As is typical for most studies of this type, this was conducted in standard 2D tissue culture. However, infection takes place in the 3D extra-cellular matrix and this 2D environment may not adequately represent the physiological microenvironment³. Therefore the aim of this study is to develop an in vitro 3D model of bone infection using collagen-based scaffolds that have been developed for bone regeneration, and to use it to elucidate the pathogenesis of osteomyelitis specifically the protein A interaction between osteoblasts and bacteria following infection.

METHODS: S. aureus Newman wildtype and SpA knock out were grown statically for 18hours in brain heart infusion broth, fixed and set to an OD1. MC3T3 – E1 pre-osteoblastic mouse cell line was used in this study. The collagen glycosaminoglycan (CG) scaffolds were fabricated by a freeze-drying process in which chondroitin 6-sulfate is mixed with acetic acid and fibrillar type I collagen then crosslinked using 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide to generate a scaffold with ~99% porosity to support osteoblast culture and bone regeneration, a technique developed by our tissue engineering research group. MC3T3s were seeded onto the CG scaffold at a density of 500,000 cells and after 7 days were infected with S. aureus Newman and ΔSpA. 7, 14 and 21 days post infection, osteoblast proliferation, mineralisation and alkaline phosphatase activity were measured using PicoGreen, StanBio and Sensolyte assays respectively. Apoptosis was measured using FACs analysis and cell/ bacteria/scaffold interaction was visualised using confocal microscopy using DAPI and anti-staphylococcal antibody/ FITC staining.

RESULTS: The CG scaffolds supported cell and bacterial infiltration after 7 days post-seeding. However, following infection osteoblast proliferation was significantly reduced at 14 and 21 days compared to uninfected constructs.

S. aureus induced osteoblast apoptosis specifically at day 14, however not at day 7 or 21. However, and very interestingly, alkaline phosphatase expression and matrix mineralization was significantly higher following infection compared to scaffolds with uninfected cells. None of these effects demonstrated by the wildtype S. aureus Newman are seen in a mutant strain of S. aureus lacking expression of SpA.

DISCUSSION & CONCLUSIONS: This study demonstrates, in a 3D extra-cellular matrix, new findings on the pathogenesis of bone infection above that previously documented in 2D culture. S. aureus infection using this scaffold based model results in inhibition of osteoblast proliferation and cell death. However, the remaining infected osteoblasts produce more mineral, which has not been previously shown. Additionally, all effects caused by S. aureus wildtype are not seen when SpA is absent demonstrating the importance of this virulence protein in the progression of osteomyelitis. This study also clearly demonstrates the importance of 3D models to fully understand the pathogenesis of disease and provide more physiologically relevant conditions.

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Modelling the multidisciplinary nature of biomaterial associated infection
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INTRODUCTION: The study of biomaterial associated infection is increasingly becoming a field involving a broad range of scientific disciplines. Until recently the role of the simultaneous interplay between material, host and pathogen has been largely negated. However, the recent paradigm shift towards considering biological interactions associated with infection has generated a requirement for increasingly complex models. These new multidisciplinary models could open the door to novel methods for fighting infection beyond antibiotics.

METHODS: Traditionally biomaterial associated infection research has largely consisted of simple in vitro bacterial adhesion and biofilm growth assays or in vivo measures of infection rates. However, more recently host responses have also been included as important variables in studies. For example, parallel plate flow chambers have been used to combine bacterial contamination with osteoblast adhesion and immune cells interactions in vitro.[1] Meanwhile increasingly complex in vivo assays are being developed. These models include the application of an implant with bacterial contamination and the monitoring of host responses at terminal time-points.[2-3] However, due to the terminal nature of the measurements these studies typically require a large quantity of animals to attain results. Furthermore, ex vivo observations of cellular interactions from such methods are in fixed tissue, limiting the amount of information about dynamic processes which may be occurring. An alternative is to use in vivo imaging systems (IVIS). By labelling bacteria or host cells with fluorescent markers it is possible to image the development of infection through the tissue of a live animal, truly in vivo.[4] This permits the reuse of animals for multiple time-points, thus reducing the size of the study. However, the detail attained by IVIS is relatively low resolution and requires additional ex vivo measurements to assess cellular level biological interactions.

Therefore, there is a requirement for investigating more appropriate multidisciplinary in vitro and in vivo models of biomaterial associated infection. To this end we are developing new models of biomaterial associated infection. The first aspect of this is to develop novel, detailed and accurate methods of investigating biological interactions in vivo. Meanwhile, new in vitro multidisciplinary methods will be developed by reverse engineering in vivo observations.

RESULTS: We have developed novel methods of quantifying host-pathogen-biomaterial interactions in highly detailed and biologically relevant models both in vitro and in vivo. In vitro it was observed that different methods of cellular signalling between immune cells and healing cells is important in the development of host responses. Meanwhile, new in vivo models permit biological interactions associated with biomaterial associated infection to be observed on a cellular level in live animals in a non-terminal manner.

DISCUSSION & CONCLUSIONS: Multidisciplinary approaches are driving a new wave of research and possibilities for combatting biomaterial associated infection. Furthermore, intravital imaging of host-pathogen-material interactions is now possible on a cellular level in vivo. This combined with improved multidisciplinary in vitro assays permits highly relevant research, marking an important shift in research. Ultimately these techniques will lead to novel anti-infective measures and a reduction in the number of animals used in future research.

ACKNOWLEDGEMENTS: The intravital imaging portion of this work was made possible by an AO start-up grant: Project no S-14-35R, AO Foundation, Switzerland.
The role of biomechanical stability on Staphylococcus epidermidis osteomyelitis in a murine fracture model

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INTRODUCTION: Infection associated with orthopedic devices is one of the main causes for implant failure. Implant instability is considered a risk factor for infection; however, little experimental data is available confirming this belief, or describing the underlying mechanisms. In this study, a murine femur osteotomy model was used to investigate infection progression when fractures were fixed with rigid or non-rigid (i.e. flexible) constructs [1-2]. Implant-associated osteomyelitis was induced by inoculating \(S.\) \(epidermidis\), one of the leading etiologic agents for device-associated infections [3], in the osteotomy site.

METHODS: Rigid and flexible MouseFix\(^\text{TM}\) titanium plates were used to fix osteotomized femora in C57Bl/6 mice (female, 20-28 weeks old). Infection was induced by inoculating a clinical isolate of \(S.\) \(epidermidis\) (\(10^4\) CFU) into the osteotomy site. Mice were sacrificed at 3, 7, 14, 30 and 40 days after surgery (n=6-9 per group). Viable bacteria were quantified from the implant, bone and soft-tissue. Bone cells were cultured and stimulated to collect supernatants for cytokine quantification. Lymph node cells and bone cells were characterized by flow cytometry.

RESULTS: The rigid plate resulted in a stable fixation, which lead to healing by intramembranous ossification. The flexible plate resulted in an unstable fixation, leading to a more endochondral ossification, with stabilizing callus formation. When infected, bacterial contamination led to a mild infection based on clinical scores and spleen and lymph node cell counts. Mice started to clear bacteria from day 7. At the early time-points (7, 14, 30 days), mice with a rigid implant cleared the infection in 12/21 mice, compared with only 5/22 mice receiving a flexible implant. At the latest time point however, when fractures had completely healed in both implant groups, the infection rate increased and was similar between groups. Quantitative bacteriology indicated that the bacterial count in the soft tissues decreased, whilst the number of bacteria colonizing the implant and bone remained steady. All infected animals displayed high levels of secreted TNF-alpha, IL-10, KC, MCP-1 and G-CSF at day 7, and were consistently higher in animals with flexible fixation. In the animals that cleared the infection, IL-17 producing-cells were generally elevated in local lymph node (day 7, 14).

Figure 1 Histology sections (Giemsa&Eosin staining) of C57Bl/6 mouse femur with rigid plate (left) or flexible plate (right) at day 14.

DISCUSSION & CONCLUSIONS: Our model results in an increased infection rate in flexible constructs. Intriguingly, at the final time-point, the infection rate increased and the differences between the groups were no longer observed. When studying associated immune response, cytokines typical of innate immunity were present at day 7, with highest values seen in the flexible group. However, and despite the presence of bacteria in tissues, cytokine levels were reduced to similar baseline levels from day 14 on. This suggests that after an initial inflammatory phase, regulatory mechanisms lead to a non-inflammatory status that may interfere in bacteria clearance and may lead to late reseeding of infection once the healing phase is complete. Finally, the data from the lymph node cells suggest that a Th17 type response is important for the clearance of infection.
Implantation of mesenchymal stroma cells for stimulation of long bone healing aggravates *Staphylococcus aureus* induced osteomyelitis.

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**INTRODUCTION:** Large bone defects show a considerable rate of infections due to the need for long term osteosynthetic stabilization and repeated surgeries. Mesenchymal stroma cells (MSCs) have been successfully used to stimulate regeneration of such bone defects. But next to their osteogenic potential, MSCs are known to modulate the host immune response. Thus, their application might contribute to the risk for osteomyelitis. Aim of this study was to unravel a possible impact of MSCs on osteomyelitis when implanted into a *Staphylococcus aureus* (SA) contaminated and orthopaedic implant-stabilized bone defect in rats.

**METHODS:** In a pilot study (n=6), a hydrogel-supported osteomyelitis ostectomy model was developed and the minimal SA dose inducing a local bone infection was evaluated. In the main study, half of the SA-infected animals were treated with 1.6 million MSCs (n=5 per group and time point, 3 and 4 weeks). Non-infected animals served as controls. Osteomyelitis was assessed by blood count, osteomyelitis score, µCT analysis and histology. Additionally, gene expression levels of immunomediators were quantified in human MSCs after SA exposure in vitro. For statistical comparison a Mann-Whitney-U test with post-hoc Bonferroni correction was performed.

**RESULTS:** All SA-infected animals developed a local bone infection with periost reactions, osteolysis, instability and deformity, which was aggravated in the MSC-treated animals (Fig. 1) according to significantly higher osteomyelitis scores. Increased blood neutrophils and positive SA re-isolation confirmed a local bone infection. µCT analysis revealed a significantly enhanced distal bone loss with spongy alteration of cortical bone in the MSC-group after 3 weeks, whereas in the MSC-free group this occurred not before 4 weeks. Histology confirmed changes in bone structure, increased attraction of (M1) macrophages and osteoclasts, abscess formation and SA accumulations with worst infection seen in the MSC group at 4 weeks. In vitro, human MSCs up-regulated pro- and anti-inflammatory mediators after SA exposure.

**DISCUSSION & CONCLUSIONS:** Our study for the first time showed that MSC implantation into an orthopaedic implant-stabilized bone defect contaminated with only a few bacteria aggravated osteomyelitis. This argues in favour of a local immunosuppressive action of implanted MSCs in an infectious bone environment. Studies on antibiotic carrier augmentation or antibiotic treatment are warranted to decide whether MSC implantation is a safe and promising therapy for orthopaedic implant-stabilized bone defects at high risk for bone infection.

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Trauma, osteomyelitis and reconstruction of long bones
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INTRODUCTION: Osteomyelitis of long bones after trauma is a complication with serious impact on patient recovery and often associated with further limitations. Infection after closed fractures occur in 1.5% of the cases and after open fractures up to 40%. Infect eradication and reconstruction of long bones after osteomyelitis is a time consuming process depending on the defect size, bone quality and associated soft tissue defects.

The following study gives an overview to incidence of posttraumatic musculoskeletal infections, bacterial spectrum, surgical strategies and options for bone reconstruction.

METHODS: Data from 934 patients treated for implant associated infections were collected from 2004 – 2014 and bacterial growth analyzed. Clinical problems and surgical concepts for the reconstruction of long bone defects are presented.

RESULTS: In 75% the lower extremity was affected. Table 1 shows the first results of microbiological differentiation. In 68% of the patients the bacteria changed during the treatment.

<table>
<thead>
<tr>
<th>Percentage</th>
<th>Bacteria</th>
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<tbody>
<tr>
<td>38 %</td>
<td>Coag. neg. staphylococci</td>
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<tr>
<td>26 %</td>
<td>Staph. aureus</td>
</tr>
<tr>
<td>8 %</td>
<td>Enterococcus supp.</td>
</tr>
<tr>
<td>4 %</td>
<td>Pseudomonas</td>
</tr>
<tr>
<td>24 %</td>
<td>Others</td>
</tr>
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To estimate the required time for bone reconstruction a rough rule is “each centimeter bone defect extends the treatment one month”.

DISCUSSION & CONCLUSIONS: Patients risk factors, patient individual decisions, surgical options, clinical course of the infection etc. influence the steps during treatment of posttraumatic osteomyelitis. Treatment of osteomyelitis after trauma and reconstruction of long bone defects is a costly and time consuming process. Interdisciplinary team work is the essential clue to succeed and reach an optimal result for each individual patient.

Fig. 1: Images of a 23y female with a 10 cm bone defect after open tibial fracture. An interim PMMA gentamycin spacer was used for dead space management during the eradication of the osteomyelitis.

Fig. 2: After 3 years and 14 operations the bone reconstruction is consolidated after distraction osteogenesis. The moderate varus deviation of the mechanical axis was accepted.
Strategies for reducing infection in open fractures
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Open fractures are plagued by high complication rates, among which infection is the most common. These complications lead to higher morbidity and poor patient outcomes. Despite meticulous surgical care and use of adjunctive therapies, infection rates remain at or above 20%, due to the limitations of conventional therapies. Understanding why open fractures pose such a challenge will help with the development of effective therapies.

The open wounds are often grossly contaminated at the time of injury, and surgical debridement of the wound and implementation of antibiotics are often delayed for hours. The soft tissue envelope is damaged and perfusion of the wound is impaired which can reduce availability of systemic antibiotics. Because of the extent of the soft tissue injury and often the poor health of the patient, the most severe wounds cannot be closed for days to weeks which increase the chance of contamination with nosocomial pathogens. The bone defect often requires grafting to promote healing but this graft can act as a foreign body and be a nidus for infection. Implanted hardware is required for stabilization, which provides another foreign body within the wound for the bacteria.

The bacteria have many survival strategies to thwart the immune response and antimicrobial effects, and biofilm is likely the most significant challenge. Bacteria are often thought of as being free-floating organisms, which is their planktonic state. The fact is, however, that the vast majority of bacteria are found within their biofilm state. Bacteria adhere to the surface of the wound or orthopaedic implant and quickly produce a glycocalyx. A mature biofilm can form in 5-10 hours which reduces immune cell effectiveness and drug penetration. A small number of the cells within this biofilm are known as persisters. These cells have extremely slow metabolism which make them less susceptible to the majority of antibiotics. Therefore, successful anti-infection strategies must address the challenges the biofilm presents.

A single approach will likely not be completely effective; therefore, improvements need to be made at each level of care or procedure with a number of recent advances. Immediate administration of systemic antibiotics reduces infection by approximately four-fold, which underscores the effectiveness of antimicrobials when bacteria are within their planktonic stage. Unfortunately, immediate administration of antibiotics is often not possible. Negative pressure wound therapy is commonly used to help manage the soft tissue injury, but NPWT reduces the effectiveness of local antibiotic depot. Ring-fixators have been shown to have low infection rates; it is believed that this is because they do not introduce a foreign body into the wound bed which occurs with intramedullary nails and plates. Local antibiotics, usually delivered by PMMA beads or spaces, are also a mainstay because they allow for high levels of drug within the wound bed. There are some emerging therapies that may also be useful. Vancomycin powder has been used for spine surgery for years and sprinkling 2 grams of powder into open fractures is starting to be explored. Local antibiotic delivery devices that can work in conjunction with negative pressure wound therapy have been developed. Orthopaedic implant infection is a significant issue and various approaches to protect the devices from bacterial colonization are being investigated. Bone grafts that promote healing while reducing infection by eluting antibiotics have been shown to grow bone in contaminated defects. Several different biofilm dispersal agents have been discovered, which prevents biofilms from forming or disperses the bacteria that are within biofilms. Biofilms dispersal agents hold promise but there is uncertainty over their effectiveness and mechanisms of action. There is a renewed interest in antibiotics that do not require bacteria replication to be effective, such as rifampin. These antibiotics are much more effective against biofilm in vitro; more rigorous preclinical models and clinical studies are needed. Many of these antibiotics are harmful to host cells, so their effects on fracture healing must be examined as well.

Open fractures pose some of the biggest difficult wounds for surgeons to manage and the role of the biofilm increases the risk for infection. Fortunately, there are emerging therapies that may further reduce infection rates by addressing the biofilm challenge.
Efficacy of a lipid-and-polymer-based drug delivery coating containing doxycycline for the prevention of implant-related osteomyelitis.

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INTRODUCTION: One of the most challenging complications in trauma surgery is the development of implant-associated bone infections, in particular those caused by antibiotic resistant pathogens such as methicillin-resistant Staphylococcus aureus (MRSA). Prophylactic administration of antibiotics is mandated for open fractures, however penetration of systemically applied prophylactic antibiotics to the fracture site may be compromised in cases with significant vascular damage. In the present study, we aimed to characterize and determine the efficacy of a biodegradable Polymer-Lipid Encapsulation Matrix technology (PLEX) loaded with doxycycline for the local delivery of antibiotic directly to the fracture site via implant coating.

METHODS: Coated intramedullary nails implants - Pre-clinical evaluation: Titanium aluminium niobium (TAN) implants were coated with a PLEX-doxycycline coating. Antibiotic elution from the coating and antibacterial activity were monitored in vitro. A rabbit humeral intramedullary (IM) nail model of infection was used to evaluate the prophylactic efficacy of the coated nail for the prevention of osteomyelitis caused by a doxycycline sensitive methicillin sensitive S. aureus (doxy^S MSSA) and a doxycycline resistant methicillin resistant S. aureus (doxy^R MRSA) strain.

Coated bone filler – Clinical evaluation: β tricalcium phosphate (TCP) granules bone graft substitute was coated by PLEX-doxycycline coating (BonyPid^SM.1000). Each 10 g vial contains 56 mg of doxycycline. Eighteen Gustilo type III open tibia fractures and one open femur fracture patients (12 Gustilo type IIIA and 7 type IIIB) were implanted with BonyPid-1000 during the first surgical intervention and stabilized by external fixation. Patients had periodic laboratory, bacteriology and radiology follow-up.

RESULTS: In vitro elution studies revealed that 25% of the doxycycline was released from the PLEX-coating during the first day, following by a 3% release per day for 27 days. Comparison of antibacterial activities in the elute from coated versus uncoated discs indicated that the released doxycycline is highly effective against doxy^S MSSA for at least 14 days. In the rabbit IM nail study, all rabbits receiving a PLEX-doxycycline coated nail were completely culture negative in the doxy^S MSSA-group besides the surrounding bone that displayed a normal physiological appearance in histological sections and radiographs. In the doxy^R MRSA-group, a statistically significant reduction in the number of culture positive samples was observed for the PLEX-coated group, although the reduction in bacterial burden did not reach statistical significance. In the clinical evaluation, according to principal investigators, no death and serious adverse events as well as adverse events related to BonyPid-1000 were reported during 6-12 months follow up. No events of amputation occurred, no infection of the target bone was reported in any of the patients during 6-12 months following implantation of BonyPid-1000. In contrast, in the historical control group (n=51) with a similar severity of open fracture (24 Gustilo type IIIA and 27 type IIIB) at the same hospital, receiving only Standard of care treatment, a 26% bone infection rate was reported. Callus, which represents the initiation of bone healing process, was seen in 15 patients (79%) within 8-12 weeks post BonyPid-1000 implantation. Immediate wound closure was done in 9 out of 19 patients following BonyPid-1000 implantation. In the remaining 10 patients, 4 needed soleus muscle transfer- and 6 required delayed primary closure and skin grafting.

DISCUSSION & CONCLUSIONS: The doxycycline-PLEX coating provided adequate prophylaxis against implant-related infection in preclinical and clinical studies.
Anti-bacterial hydrogel coating of osteosynthesis implants. Early clinical results from a multi-center prospective trial.

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INTRODUCTION: Infection after osteosynthesis remains a feared complication in trauma surgery. Various antibacterial coatings for implanted biomaterials are under study, aimed at reducing bacterial colonization and biofilm formation; however, only few technologies are currently available in the clinical setting. In vitro and in vivo studies have demonstrated the safety and efficacy of a fast resorbable (<96 h) antibacterial-loaded hydrogel coating (DAC®, Novagenit Srl, Italy), that can be applied intra-operatively and has recently received the CE mark. Here we present the first clinical results of a multi-center prospective trial, performed on vancomycin-loaded DAC in trauma patients, under the European 7th Framework Programme (collaborative research project number 277988).

METHODS: In this prospective, controlled, single blind study, 98 patients (37 in Italy, 32 in Greece and 19 in Austria), undergoing internal osteosynthesis for closed fractures, were randomly assigned to receive vancomycin-loaded DAC coating or to a control group, without coating. The study was approved by local Ethical Committee and all patients gave their written informed consent. Pre- and post-operative assessment of laboratory tests, wound healing (ASEPSIS score), clinical score (SF-12 score) and x-rays was performed at fixed time intervals, by a blind investigator. Statistical analysis was performed with Fisher exact test or Student's t test as appropriate. Significance level was set at p<0.05.

RESULTS: On average, wound healing, clinical scores, laboratory tests and radiographic findings did not show any significant difference between the two-groups at a mean 6 months follow-up (min: 3, max: 15 months). Two early infections of the surgical site, treated with antibiotic therapy in one case and with early hardware removal and external fixation in the other were observed in the control group. No local or systemic side effects, that could be related to DAC hydrogel coating, were noted. In particular, no radiological signs of interference with bone healing were noted.

DISCUSSION & CONCLUSIONS: This study shows that a novel, fast-resorbable anti-bacterial hydrogel coating can be safely used in patients undergoing internal osteosynthesis for closed fractures. Further studies are needed to confirm in the clinical setting the efficacy of the coating to effectively prevent implant-related infections, as previously successfully tested in animal models.

ACKNOWLEDGEMENTS: The financial support was provided by the European 7th Framework Programme (collaborative research project number 277988).
Are infections still a problem in orthopedics?

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The most commonly used arthroplasties in Europe are the hip and knee arthroplasties which represent over a 1000000 interventions per year. The most devastating complication occurring after the implantation of an arthroplasty is infection. This can occur early, in the days and weeks that follow the operation, or late, many years after the implantation. The currently reported rates of infection are between 0.5 and 2% but depending upon the circumstances this can rise up to 10%\(^1\). Infection is devastating because it leads to pain, destruction of bone stock, implant loosening and failure and if left insufficiently or un-treated it can lead to major disability and death. This is a terrifying outcome for an elective operation aimed at improving the patient’s function. The infecting organism is most commonly staphylococcus aureus (74%) the other types of bacteria playing a much smaller role. Methicillin resistant staphylococcus aureus (MRSA) has emerged in the last decade as one of the most dangerous purveyors of infection and this organism has colonized many European hospital where it has become endemic\(^2\). Infection is determined by many different factors\(^3\). Patient related such as advancing age, female gender, ASA (Anaesthesia score, I to IV, the higher the riskier the procedure for the patient) score, BMI or body weight (very high or very low), previous trauma to the hip causing the arthritis, duration of operation and length of preoperative stay. Obesity plays an important role: In the last 5 years there have been 3 infections in our institution and all three patients had a BMI of over 35 which seems to be a cut-off point. Duration of surgery is an important factor and hip or knee operations lasting more than 2 hours place the patient at heightened risk for infection. Surgical volume of a given surgeon or a given institution plays a role. Large volume surgeons and hospitals tend to have fewer infections than surgeons and hospitals with smaller volumes. Finally in the context of infection clean care is good care but great care depends on team care.
Translational research challenges for a drug releasing fracture fixation device

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INTRODUCTION: Implant related infection remains a significant clinical problem associated with open reduction and internal fixation of fractures. Despite the use of systemic antibiotics, implant surfaces can provide a substrate for bacterial colonization, biofilm formation and infection. The consequences can include delayed healing, osteomyelitis, secondary surgeries and amputation. A combination medical device that combines an intramedullary tibia nail with gentamicin as a prophylactic for bacterial colonization was developed. Translational research for combination medical devices is particularly challenging. Characterization of the combination device with respect to fabrication, in vitro and in vivo antibiotic delivery, and preclinical efficacy studies were essential.

METHODS: A suspension of particulate gentamicin in a solution of PDDL is used to dip-coat implants. After the polymer solvent is evaporated the device is packaged behind a moisture barrier and then terminally sterilized by gamma irradiation.

The amount and the chemical integrity of gentamicin coated on the nail were assessed using LCMS. The anti-colonization activity was determined in a variety of rat and sheep models.

RESULTS: The dip coating process results in a durable, uniform coating that contains 0.19 mg/cm² (±0.05 mg/cm²) of gentamicin. The major chemical species found in gentamicin were identified in LC chromatographs and the chemical identity of all the major components and inert co-isolates were identified and tracked throughout the manufacturing process. There were no chemical transformations detected.

The release of the antibiotic begins upon exposure of the implant to aqueous media and follows zero order kinetics. A majority of the antibiotic is released in the first 24-48 hours. The coating degrades without any significant inflammatory reactions or toxic by-products, and no adverse reactions are expected.

Studies in rats and sheep revealed that the gentamicin coating significantly or entirely eliminates colonization of bacteria on the implant surface.

DISCUSSION & CONCLUSIONS: The key translational research challenge was the development of a validated coating process that generated a durable coating of the implant. In addition, the chemical integrity of the antibiotic with respect to both major active species and inert co-isolates had to be painstakingly established. The data requirements to support translation of the product into the clinic involved a substantial investment in process validations including significant shelf life studies and analytics for the uniformity and chemical stability of the coating and the impregnated gentamicin. These validation studies were augmented with robust preclinical bench studies and animal testing to confirm antibiotic activity and in vivo anti-colonization effects.
Current concepts in diagnosis and treatment of periprosthetic joint infection

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INTRODUCTION: Periprosthetic joint infection (PJI) is a rare but serious complication of joint replacement. In 2013, clinical guidelines for the diagnosis and management of PJI have been published by the Infectious Diseases Society of America (Clin Infect Dis 2013;56:e1-25, 2013). Thus, despite missing data from controlled studies, a consensus from international experts, based on published observational studies and clinical expertise, is now available.

DIAGNOSIS: If PJI is suspected, case history, clinical examination, plain radiograph and serum CRP should be performed as first step. If this screening does not exclude PJI, arthrocentesis for cell counts, differential and culture in synovial fluid should be performed. If synovial cell counts are above the defined threshold, diagnostic surgery (inspection, 3-6 tissue biopsies for histopathology and culture, debridement and sonication/culture of modular parts) should be performed. If cultures remain negative, molecular diagnosis on sonicate fluid should be perfomed. This is especially important in patients pretreated with antibiotics.

TREATMENT: PJIs can be classified in acute hematogenous (<3 weeks duration of symptoms), early postinterventional (<1 month), and chronic PJI (all others). Implants are highly susceptible to infection. They are not only at risk for infection during the perioperative period, but remain susceptible to hematogenous seeding during their entire life-time. Traditionally, implant-associated infections were treated by removal of the biofilm-coated device. During the last 2 decades, novel concepts have been evaluated. In patients with acute infection the implant can be retained, provided that a biofilm-active antibiotic is used. Patients qualifying for implant retention are those with acute hematogenous PJI (<3 weeks of duration of symptoms) and those with early postinterventional PJI (during the 1st postoperative month). In contrast, patients with chronic PJI (>3 weeks of duration of symptoms) have a low chance to be cured with implant retention. Cure by the first treatment attempt is crucial, because with each treatment failure, tissue damage and functional integrity is worse. Therefore, early referral to specialized centers is advised. Cure requires a combination of both, an appropriate surgical procedure and long-term antimicrobial therapy. We propose a rational treatment algorithm which allows choosing the optimal surgical strategy for each patient. Each surgical treatment option should be combined with a prolonged antibiotic treatment, preferably with an agent acting on slow-growing and adhering microorganisms. This requirement is fulfilled by rifampin in staphylococcal, and a fluoroquinolone in gramnegative infections. The excellent activity of rifampin on implant-adhering microorganisms has been shown in vitro, in animal models, and in several clinical studies. In order to avoid emergence of resistance, rifampin must always be combined with another agent. Traditionally, fluoroquinolones are excellent combination partners. Following the novel treatment concepts, using rifampin-combinations against staphylococci or a fluoroquinolone against gramnegative bacilli and choosing the optimal surgical procedure, the chance for eradication of orthopaedic implant-associated infection is 80-90%.

DISCUSSION & CONCLUSIONS: Given the limited efficacy of traditional antibiotics in implant-associated infections, novel strategies such as coating of the device, vaccination against biofilms, and quorum-sensing inhibitors are promising future options for prevention and treatment.
Efficacy of DOXICOLL - a novel drug delivery systems for infected bone tissue

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INTRODUCTION: A major challenge in bone healing remains the presence of bone infection caused by pathogenic microorganisms resulting in a severe prolonged inflammatory disease, followed by bone destruction (osteomyelitis) [1,2]. Local antibiotic release is commonly used to draw back poor bone penetration of drug administered systemically (intravenously or orally) [1]. The aim of our study is to develop new drug delivery system as membrane based on collagen and doxycycline, with antimicrobial properties, controlled biodegradation and release of antibiotic, with biocompatible and osteogenetic properties, able to treat the infected soft and hard tissue.

METHODS: Doxicoll – a drug delivery system based on collagen and doxycycline (5:1) was prepared in form of membrane as we previously described [3]. The membrane was characterized by scanning electron microscopy, swelling ability, enzymatic degradation and microbiological tests for sterility validation. In vitro doxycycline release from collagen membrane was performed using a sandwich device adapted to a dissolution equipment. The amount of drug released at different intervals of time was spectrophotometrically evaluated. For the assessment of the doxycycline release mechanism from the tested film, the Power law model (eq. 1) was applied to the kinetic data:

\[
\frac{m_t}{m_\infty} = k \cdot t^n
\]

where \( m_t/m_\infty \) is the fraction of drug released at time \( t \), \( k \) is the kinetic constant and \( n \) is the release exponent that can be related to the drug transport mechanism. Surgical defects were created in 12 animals that were divided in: Control group (defect contaminated by Porphyromonas gingivalis (PG)); and Doxicoll group (defect contaminated by PG, filled with bone graft and covered by Doxicoll). Animals were sacrificed at 14th and 28th days post-surgery. The tibiae of 5 animals were cut sagitally (14th day) and used to determine the bacterial load of CFUs/g bone and the tibiae of 25 animals were embedded in paraffin, serially cut, and stained with hematoxylin and eosin for analysis under light microscopy. The formation of new bone in the cortical area of the defect was histopathologically evaluated. 10 human subjects were treated with Doxicoll for periodontal inflammation, after extraction and to coat the bone defects.

RESULTS: The SEM images showed compact membrane with pore size lower than 50 µm, 17% swelling capacity, about 40% degradation in first 10 days because doxycycline inhibit collagenase. The sterility doze was determined to be 25 kGy. The doxycycline release from collagen film showed a non-Fickian drug diffusion transport mechanism. An initial drug burst release was noticed in the first hours of experiments ensuring a rapid diminishing of bacteria on an infected wound, followed by a slower and gradual drug release during the next days, ensuring the protective effect against infections over a required period to favour long-term healing. The osteogenesis degree of Doxicoll group (1.80±0.45) was found significantly higher. Least bacterial count was observed in the Doxicoll group compared to other groups. The clinical trials showed bone reconstruction after 1 to 3 months treatment with Doxicoll.

DISCUSSION & CONCLUSIONS: Collagen assures biocompatibility with cells and fast regeneration of soft and hard tissue. The doxycycline is slowly released to keep antimicrobial site. Doxicoll could be a favorable material to prevent bacterial infection and to enhance bone formation.

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Antimicrobial hydrogels based on collagen and zinc oxide

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INTRODUCTION: Natural polymer such as collagen is widely used in regenerative medicine because of its similarity with extracellular matrix [1]. Zinc oxide is a mineral that exhibit antibacterial, antifungal and anti-inflammatory properties [2]. Recently, natural antimicrobial agents for infection were proved to be plant essential oils. The aim of this study was to develop, characterize and evaluate antibacterial activity of composite hydrogels based on collagen and zinc oxide with or without essential oils and mimic the composition of bone.

METHODS: Hydrogels based on type I fibrillar collagen gels with different concentrations of zinc oxide (0; 25; 50 and 75%) were obtained and named G1, G2, G3 and G4. The rheological performance of the collagen hydrogels was assessed using flow rheometry (MultiVasc-Rheometer Fungilab equipped with a standard spindle TR 9). The flow patterns were recorded at 37°C (Ultrathermostat ThermoHaake P5) and the rheological descriptors specific to Power law model (eq. 1) were determined.

\[
\eta = m \cdot \gamma^{-n}
\]

where, m and n are parameters correlated with the designed hydrogels composition and determined through the linearization of eq. (1) by double logarithmic method. It can be assessed that m value matches the viscosity obtained for the shear rate of 1·s⁻¹ (the logarithm for the shear rate equal to one is zero). Collagen-zinc oxide with and without Eucalyptus camadulensis Dehnh. Oil, 0.5% (v/v) [3] were antimicrobial evaluate against S. aureus and E. coli.

RESULTS: The rheological profiles presented in Fig. 1 showed that the designed hydrogels presented a non-newtonian pseudoplastic behaviour with shear thinning which facilitates the hydrogels flow, allowing their good manipulation. The fitting parameters of the Power law rheological model are given in Table 1.

DISCUSSION & CONCLUSIONS: The rheological analysis shows that the adding of ZnO determines an obvious increase of viscosity and more than that, the formulation containing a concentration of ZnO 75% reported to collagen dry substance leads to the highest hydrogel viscosity (Table 1). The presence of eucalyptus oil does not significantly modify the rheological parameters of collagen-zinc oxide hydrogels but have a high influence against S. aureus and E. coli, being promising composite in bone reconstruction.

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Table 1. The fitting parameters of the Power law rheological model for collagen-zinc oxide hydrogels.

<table>
<thead>
<tr>
<th>Hydrogel</th>
<th>m</th>
<th>n</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>10.921</td>
<td>0.732</td>
<td>0.9914</td>
</tr>
<tr>
<td>G2</td>
<td>30.825</td>
<td>0.854</td>
<td>0.9996</td>
</tr>
<tr>
<td>G3</td>
<td>47.678</td>
<td>0.802</td>
<td>0.9975</td>
</tr>
<tr>
<td>G4</td>
<td>50.930</td>
<td>0.859</td>
<td>0.9996</td>
</tr>
</tbody>
</table>

Fig. 1: Plots of viscosity as a function of shear rate for the collagen hydrogels G1-G4 evaluated at 37°C.
The effects of polymethylmethacrylate with gentamicin on the healing of infected segmental bone defects in rabbits.

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INTRODUCTION: Large bone defects resulting from high-energy trauma are often associated with soft-tissue injury and infection.1,2 Masquelet et al. described a reconstruction method by placing a polymethylmethacrylate (PMMA) spacer in the bone defect along with debridement and soft-tissue reconstruction.1,2 The primary aim of this study was to evaluate the effects of a PMMA induced membrane on the healing capacity of infected and non-infected bone defects in rabbit ulnas. The secondary target was to investigate the role of infection and periosteal resection in bone regeneration.

METHODS: A 15-mm bone defect was created in the distal third of the ulna in 37 rabbits allocated in five experimental groups: group 1, bone defect filled with spacer; group 2, bone defect with no spacer; group 3, infected bone defect filled with spacer; group 4, infected osteoperiosteal defect filled with spacer and group 5, infected bone defect with no spacer. Infection was induced with an inoculum of 5x10^8 colony-forming units (CFU) of Staphylococcus aureus inside the medullary canal of a devitalized bone segment, and debridement was performed after 2 weeks. The bone defect was filled up with the spacer of PMMA with gentamicin in groups 1, 3 and 4 and all infected animals received systemic gentamicin for 4 weeks. The spacer was left in place for 4 weeks and then surgically removed. The animals were followed radiologically for up to 12 weeks, at which point the regenerated area was submitted to histological analysis.

RESULTS: All inoculated animals developed clinical S. aureus infection. Seven animals died during the study. Overall, the rabbits exhibited excellent recovery of infection and soft tissues healing.

The size of the bone defect increased by 8%, from 15 mm to 16.2 mm in the infected animals (p = 0.00) after debridement. The spacer caused differences in the regenerative pattern over time (p = 0.00) especially because it blocked new bone formation inside the defect. During the first four weeks all new bone formation was restricted to the periphery around the spacer. In particular, bone regeneration was initially more pronounced in the groups without a spacer, but over time this effect was lost, and increased regeneration was associated with spacer use. However, this effect was not statistically significant at twelve weeks. Infection had a negative impact on this regeneration (p = 0.00) (Figure 1), although the worst results, associated with periosteal resection, exhibited no statistical significance.

Fig. 1: Bone regeneration after spacer use. A-Non-infected bone defect. B-Infected bone defect. HE; 2.5x/methylene blue/blue II/pararosaniline; 2.5x).

DISCUSSION & CONCLUSIONS: The recent discovery that PMMA induces the local formation of a biological membrane with regenerative bone properties make the use of such spacers desirable in cases of bone defects. In this study the spacer acted as a barrier to bone formation inside the defect during the first few weeks. The spacer encapsulation by new bone resulted in a well delimited cavity, but after the spacer removal, bone regeneration was greater than in the untreated bone defects groups. Infection seriously hinders regeneration and that this effect persists even after the infection is cured.

ACKNOWLEDGEMENTS: State of São Paulo Research Funding Institution (FAPESP) supported this study.
Potential for a novel acrylic bone cement formulation against staphylococcus bone infection

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INTRODUCTION: Acrylic bone cement (BC) as a carrier material for antibiotic delivery remains a key strategy to control bone associated infections \cite{1,2}. Two major drawbacks still limit its use, the inadequate drug delivery and the inflammatory reaction around the implant \cite{3}. In this context, the concept proposed in the present work consists in including relevant additives (chitosan and diclofenac) to address the referred drawbacks. Chitosan nanoparticles (CS) were used as a porogen to increase antibiotic release and diclofenac, a well known anti-inflammatory drug, aims to suppress inflammation around the implant. The selected model antibiotic was levofloxacin, a fluoroquinolone with high anti-staphylococcal activity and adequate penetration into osteoarticular tissues \cite{2}. The modified BC matrices were evaluated concerning solid-state characterization, drug release profiles and cell-material interaction.

METHODS: Commercial BC (CMW\textsuperscript{1}® , DePuy Synthes) was used to prepare different matrices. BC matrices were characterized regarding surface structure (FEG-SEM), contact angle (Wilhelmy plate) and \textit{in vitro} drug release (HPLC). Preliminary biocompatibility screening was conducted based on direct contact assays on mouse fibroblast (L929) and osteoblast-like cells (MG63). Studies were conducted by phase-contrast and fluorescent microscopy.

RESULTS: FEG-SEM and contact angle analysis revealed no significant change in the outer or inner structure of the material due to the incorporation of the nanoparticles or the drugs. BC matrix loaded with 7.5\% (w/w\textsubscript{BC}) of CS nanoparticles presented the best properties as significant increase in antibiotic release was observed, comparing to control, while diclofenac liberation was sustained (Table 1).

Regarding direct contact of BC with the cells, phase-contrast micrographs were taken to the interface of the cell layer with outer contact areas of the composites. As illustrated in Fig. 1, only the cells in contact with filter paper containing SDS (positive control) did not grow, all the others presented a monolayer of viable cells. Also fluorescence microscopy showed that cells were well spread out on the surface of the composites, indicating a good cell adhesion and proliferation.

<table>
<thead>
<tr>
<th>Matrices</th>
<th>Lev (%)</th>
<th>Diclo (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lev[BC]</td>
<td>6.6±0.6</td>
<td>-</td>
</tr>
<tr>
<td>Diclo[BC]</td>
<td>-</td>
<td>8.9±0.6</td>
</tr>
<tr>
<td>Lev_Diclo[BC].CS</td>
<td>14.2±0.9</td>
<td>8.4±1.1</td>
</tr>
</tbody>
</table>

Fig. 1: Phase-contrast micrographs

DISCUSSION & CONCLUSIONS: \textit{In vitro} approaches are an important first step in the development of novel antibiotic-loaded acrylic BC aiming to select optimal matrices in terms of drug release and biocompatibility to be further evaluated. In the present work, the selected formulation released an average of about 14 \% of the total incorporated levofloxacin during 3-week test while maintaining structural properties and presenting good \textit{in vitro} interaction with fibroblast and osteoblast cells. Further work is underway to gain insights on the antimicrobial and anti-inflammatory properties of the novel BC composite.

ACKNOWLEDGEMENTS: Portuguese government (Fundação para a Ciência e Tecnologia), project EXCL/CTM-NAN/0166/2012 and COST TD1305 (iPROMEDAI).
Antimicrobial efficacy and cytotoxicity of a new antimicrobial peptide

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INTRODUCTION: Infections of the bone are difficult to treat, especially if they are biomaterial associated infections and a biofilm has been formed. The development of new antimicrobial active substances might improve the therapy. Antimicrobial peptides (AMPs) are naturally occurring peptides of the innate immune system. It is supposed that they have a broad range of action against Gram+/− bacteria, viruses and fungi, they also target bacteria within a biofilm and induce no or rarely resistance.

In this study a new AMP derived from Bactencin was used and the antimicrobial effect on different bacteria, the efficacy on biofilms and the effect on osteoblast-like cells was analysed.

METHODS: The cationic AMP was synthesised with SPOT-synthesis1. Peptide concentration was determined with NanoDrop.

MIC/MBC: E. coli, P. aeruginosa, S. epidermidis, S. aureus und E. faecalis and a serial dilution of the AMP starting with 267 µg/ml (n=8).

Biofilm: Fluorescence in-situ-hybridisation (FISH) combined with DAPI was used to investigate the effect of the AMP on S. epidermidis biofilm.

Cytotoxicity: Primary human osteoblast-like cells (hPOB) were cultured under standard conditions or in differentiation medium with the AMP. Cell vitality, osteogenic differentiation and gene expression (qRT-PCR) was analyzed.

RESULTS: The AMP inhibited the growth of all investigated bacteria at concentrations of 4 up to 33 µg/ml. Bacteria were killed at concentrations of 8 up to 133 µg/ml.

After AMP-exposure biofilm thickness as well as amount of FISH positive cells was reduced considerably. Also the biofilm structure was altered.

The highest investigated concentration (267 µg/ml) had no negative effect on the metabolic activity, alkaline phosphatase (ALP) activity and mineralisation of the osteoblast-like cells (Fig. 2).

DISCUSSION & CONCLUSIONS: This newly developed AMP showed an antimicrobial activity against clinically relevant Gram positive and negative bacteria and was also effective against bacteria that already formed a biofilm. All tested concentrations showed no negative effect on the growth and differentiation of human primary osteoblast-like cells. Taken these results together, this AMP has promising properties for the use in the treatment of bone infections or the prophylaxis of implant associated infections.

ACKNOWLEDGEMENTS: This study was supported by the BMBF (BCRT FKZ 1315848A.)

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Animal models of dose-related methicillin-resistant *Staphylococcus epidermidis* infected non-union after osteosynthesis

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**INTRODUCTION:** *S. epidermidis* is commonly associated with biofilm formation on implanted medical devices [1]. Methicillin-resistant *S. epidermidis* (MRSE) is known to be one of the leading causes of fracture healing delay [2], even though *in vivo* models have not been developed yet in order to investigate the impact of MRSE infections on bone repair and non-union establishment.

This study presents the first animal model of dose-dependent MRSE-induced non-union in rat femoral fractures. This model aims to evaluate the role of low-grade – difficult to detect – MRSE infections in delaying bone healing and to develop a useful tool to assess novel diagnostic and prophylaxis strategies to treat infected non-unions.

**METHODS:** Wistar male rats were divided into four groups consisting in a sham control group and three groups locally infected with increasing colony forming units of MRSE. In all groups, a femoral osteotomy was performed and synthesized with stainless-steel implantable devices. After surgery, rats were weekly checked for clinical signs of infection, pain and suffering. The bone responses to healing and infection were evaluated through an integrated approach based on imaging, histological and microbiological analyses 8 weeks after surgery. Evidence of biofilm deposition was observed by scanning electron microscopy.

**RESULTS:** During the clinical follow-up, the body weight measured a loss that indicated a dose-dependent trend in the infected rats over time. After 8 weeks, the control group showed a complete bone healing without signs of infection. About 67% of rats belonging to the 10³ MRSE group showed both a reduced bone healing and a microbiological positivity for coccal colonies, without evident clinical signs of infection. Conversely, severe signs of acute infection were found in the 83% of animals inoculated with 10⁵ CFU of MRSE that represented a medical case of osteomyelitis and non-union development together with slime formation. The 10⁸ MRSE group showed a greater amount of biofilm on the metal implant surface, which determined the establishment of a chronic infection and the failure of fracture healing in all the rats.

**DISCUSSION & CONCLUSIONS:** Given the growing impact of orthopaedic infections, in this study, we propose a preclinical animal model of infected non-union induced by a common nosocomial bacterium, the *S. epidermidis*. Thanks to the combination of several diagnostic techniques, our results demonstrated a direct correlation between altered bone healing and bacterial load. We create the first preclinical model of subclinical, subacute and chronic MRSE-related non-unions.

Our models might constitute relevant tools to test and validate diagnostic and prophylaxis strategies to detect and prevent non-union caused by low-grade contaminations. Moreover, our study prepares the scientific ground for further investigations on bacterial changes, adaptation and biofilm formation related to orthopaedics.

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The role of physico-chemical properties on the activity of antimicrobial GL13K-peptide coatings

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INTRODUCTION: Infections are the most prevalent cause of failure for dental implants and orthopedic prostheses. The antimicrobial-peptide GL13K, derived from the Parotid Secretory Protein (PSP), showed bactericidal and bacteriostatic properties against putative pathogens associated with oral infections. We fabricated biofunctionalized Ti-surfaces by anchoring the antimicrobial GL13K-peptide using a simple and reliable chemical route. We have previously demonstrated that the GL13K-antimicrobial coating was effective in vitro against P. gingivalis and S. gordonii, pathogen and primary colonizer during dental peri-implant infections; has strong resistance to degradation; and long-sustained antimicrobial activity, even after being sterilized. Here, we investigated the role of the physico-chemical properties of the GL13K peptides (charge, amphiphilicity, and secondary structure) and obtained coatings on the effectiveness of the peptide coatings to prevent biofilm growth.

METHODS: Ti discs were activated by etching in NaOH (eTi), silanized with CPTES (eTi/CPTES), and coated with GL13K (NH3-GKIILKASLKL-C(O)NH2) in a pH=10.5 Na2CO3 solution (eTi/CPTES/GL13K). Coatings of non-antimicrobial GK7 (NH3-GQIINLKC(O)NH2) (eTi/CPTES/GK7) and a scrambled version of GL13K (eTi/CPTES/GL13KR1) peptides were also tested as controls. GK7 is a more hydrophilic and less positively-charged peptide than GL13K and GL13KR1. XPS and visualization of fluorescent-labelled peptides were conducted to confirm immobilization of the peptide coatings. Water contact angles (WCA), surface charge (Zeta potential-ZP) and secondary peptide-structure (Circular dichroism-CD) of the GL13K and control coatings were performed. Peptide coatings for CD experiments were formed on CPTES-silanized glass slides. CD tests were also performed in buffer solutions of the different peptides from physiological pH to pH=10.5. ATP-activity, Colony-Forming Units, live-dead cell staining, and SEM of S. gordonii biofilms were assessed on the coatings at different incubation periods.

RESULTS: All bacterial tests showed that only the GL13K-coatings were strongly bacteriostatic and bactericidal and prevented biofilm formation. GL13K and GL13KR1 coatings had no significant differences in ZP and WCA>120°; i.e., they were highly positive-charged and highly hydrophobic. The two peptides in a buffer at pH=7.4 showed dominant random coil conformations. However, GL13K and GL13KR1 peptides gradually changed their secondary structure with a notable but different contribution of β-sheet and α-helix conformation, respectively, up to pH=10.5. The peptides in the coatings maintained their preferential specific conformations and stabilised them after long periods of incubation in PBS.

DISCUSSION & CONCLUSIONS: Both GL13K and GL13KR1 peptides were rearranged on the coatings to expose their most hydrophobic residues at the solid-water interface. This feature of the coatings is likely responsible for their previously-proved strong resistance to hydrophilic and enzymatic degradation. Notably, the basic solution (pH=10.5) used for tethering the peptides on the surfaces triggered folding of peptides from unordered to the predicted ordered conformations by effective neutralization of their abundant cationic side chains, which in turn defined the specific antimicrobial activity of the obtained coatings. We demonstrated that high local concentrations of GL13K on bacteria membrane models induced the adoption of β-sheet structures and thus, disruption of the membranes. These and previous findings demonstrate that the GL13K-coatings are highly effective in their sustained antimicrobial activity because of high peptide concentration, appropriate physico-chemical properties, and stabilized β-sheet structures.
Sonodynamic antimicrobial effect of curcumin in mouse model of post-traumatic *Staphylococcus aureus*-infected femoral osteomyelitis

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INTRODUCTION: Osteomyelitis is most commonly caused by *Staphylococcus aureus* (*S. aureus*), leading to non-union [1]. Sonodynamic therapy (SDT) is an alternative non-antibiotic antimicrobial treatment *in vitro* [2]. We hypothesized that SDT with curcumin as sonosensitizer eradicated *S. aureus* in mouse model of femoral post-traumatic osteomyelitis.

METHODS: Post-traumatic osteomyelitis model was developed in 33-40g male ICR mice by right femur open osteotomy with bone cutter and 25G needle as intramedullary fixation, as modified previously [3]. *S. aureus* Xen29 (4x10⁸ CFU) was inoculated near fracture site, followed by SDT with percutaneous injection of 100μM curcumin and ultrasound insonation at 1.1MHz central frequency and 1.5W/cm² intensity for 10s once on day 2 post-osteotomy. Efficacy of SDT was assessed with Xen29 bioluminescence intensity (BLI) *in vivo*. The fracture site was harvested on day 7 post-operation for histological analysis with Gram stain/iodine solution.

RESULTS: SDT with curcumin reduced the bioluminescent signal of Xen29 *in vivo* (Fig. 1). Bacterial amount in SDT group in terms of net BLI decreased by 35.7% (*p*<0.05) as compared with control group. Histology of harvested femurs showed SDT reduced *S. aureus* burden, with less and smaller bacterial colonies found.

DISCUSSION & CONCLUSIONS: SDT using curcumin as sensitizer demonstrated its anti-bacterial effect in osteomyelitis animal model. Curcumin was initiated by ultrasound energy to generate reactive oxygen species (ROS) [4]. ROS might induce oxidative damage to lipid, proteins and DNA/RNA and finally eradicate bacteria. Moreover, SDT provided instant anti-bacterial effect on *S. aureus* [2]. Such advantage allows multiple SDT until complete elimination of bacteria. To conclude, SDT with curcumin reduced *S. aureus* in post-traumatic femoral osteomyelitis mouse model.

ACKNOWLEDGEMENTS: This project is supported by AO Research Fund (Project no.: S-12-07L).

Fig. 1: Overlaying photo of in vivo bioluminescent signal of Xen29 before (A) and after (B) SDT. (C) Relative net BLI was calculated by dividing net BLI after treatment over that before treatment.

Fig. 2: Histological section of Gram stained femurs, representative of control (A), SDT (B), curcumin only (C), ultrasound only (D) groups. Scale bar 100μm. Asterisks and arrows indicate bone and bacterial burden respectively.
Dhvar5 Antimicrobial Peptide Immobilization for the development of an antibacterial coating

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INTRODUCTION: The implant-induced local immune depression in association with the biofilm virulence factors and the rising of antibiotic resistance, urges the need for new more efficient and long-lasting therapeutic approaches. Antimicrobial peptides (AMPs) are a new class of promising agents highly selective, with a broad spectrum of activity, and with a low tendency to induce resistance. Dhvar5 is such a peptide, derived from Histatin5. However, the use of AMPs is hampered by the peptide low bioavailability due to fast degradation or to aggregation, as the use of higher peptide titers results on higher toxicity. AMP immobilization onto a biomaterial surface could be the pathway to overcome these difficulties. The aim of this work was to study the immobilization of Dhvar5, testing different peptide orientations (C and N-terminal) and the inclusion of a spacer, in order to create an antimicrobial surface.

METHODS: Dhvar5 derived-peptides with different spacers and with a cysteine (Cys) at the end (Table 1), were assembled by Fmoc/tBu solid-phase synthesis.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Dhvar5 (D)</td>
<td>Nt-LLLFLKRRKKRKY-Ct</td>
</tr>
<tr>
<td>Cys-AB-D</td>
<td>Nt-C-aminobutanoicacid-Dhvar5-Ct</td>
</tr>
<tr>
<td>Cys-GG-D</td>
<td>Nt-C-GlyGly-Dhvar5-Ct</td>
</tr>
<tr>
<td>Cys-AH-D</td>
<td>Nt-C-aminohexanoicacid-Dhvar5-Ct</td>
</tr>
<tr>
<td>D-AH-Cys</td>
<td>Nt-aminohexanoicacid-Dhvar5-C-Ct</td>
</tr>
</tbody>
</table>

Chitosan ultrathin films were obtained through spin-coating on gold substrates. Then the chitosan films were modified with N-acetyl cysteine (NAC), through aqueous carbodiimide chemistry, followed by disulfide bridge Dhvar5 immobilization using oxidative conditions (DMSO). The success of the reaction was assessed by Infrared reflection absorption spectroscopy (IRRAS), Ellipsometry, Water Contact Angle and quantified through phenanthrenequinone (PHQ) chromogenic reaction. Bacterial adhesion studies were performed with S. aureus ATCC33591 strain and the viability of the attached bacteria was evaluated using LIVE/DEAD® Bacterial Viability Kit (Baclight). Gold substrates were used as controls. Statistical analysis performed with One-Way ANOVA, with Tukey Post-test.

RESULTS: All surface characterization techniques corroborated the successfulness of the immobilization reaction, as the variations observed were congruent with the applied chemistry. The quantification assay showed a tethered concentration of around 2ng/mm². Bacterial adhesion assays showed a reduction on the number of adhered bacteria onto modified surfaces, comparing to gold control surfaces (p<0.05). This reduction was strikingly high (81-86%) on the Nt immobilization using a longer spacer (AH and GlyGly) (p<0.05). Between these two spacers no significant difference was observed, meaning that length is more important than flexibility.

DISCUSSION & CONCLUSIONS: Dhvar5 covalently immobilized onto a chitosan thin coating by the N-terminus (exposing the cationic end), improves the antimicrobial effect of the coating by decreasing S. aureus colonization. This effect was enhanced when longer spacers were used independently of its flexibility. In opposition, Dhvar5 covalent immobilization by the C-terminus did not change bacterial adhesion to chitosan and even induced bacterial adhesion to chitosan coatings.

ACKNOWLEDGEMENTS: This work was financed by FEDER funds through the Program COMPETE and by Portuguese funds through FCT in the framework of the projects: PTDC/CTM/101484/2008;PEst-C/SAU/LA0002/2013; Pest-C/QUI/UI0081/2013. Fabiola Costa acknowledges FCT for the PhD grant SFRH/BD/72471/2010.
Effects of sterilization techniques on glass and resin-based materials
C. Farrugia, J Camilleri

INTRODUCTION: Tooth tissue loss through caries or traumatic injuries leads to infection of the dental pulp and eventual tooth loss. Thus teeth are restored by replacing dentine with glass-based materials and enamel by resin composites. Hybrids of these material types also exist. These materials are placed in an environment full of microbes thus their antimicrobial activity is important. Antimicrobial testing procedures require disinfection or sterilization treatment.[1] The aim of this study was to assess the changes sustained by the materials after sterilization treatment.

METHODS: The materials investigated included a glass-ionomer cement (Chemfil Superior), a composite resin (SDR) and two hybrids (Ionoseal and Dyract Extra). The test materials were sterilized using alcohol, steam, ultraviolet light (UV) and ethylene oxide and any changes to these materials were then assessed by SEM, Fourier transform infrared (FT-IR) spectroscopy and microhardness testing.

RESULTS: The steam sterilization caused changes to the surface of Dyract with a number of bubbles present on the material surface. Ethylene oxide affected the microstructure of the glass ionomer and the hybrids with deposition of chlorine and calcium respectively in Chemfil and Dyract and flattening of the Si–O stretching vibrations. UV sterilization resulted in changes in surface microhardness (P < 0.05).

DISCUSSION & CONCLUSIONS: The different sterilization techniques affected the microstructure of the materials under investigation particularly ethylene oxide. These results highlight the need for standardization of methodologies used for assessment of antimicrobial activity of materials used in medical devices as well as further assessment of which sterilization methods used on the materials prior to their use. Changes in the material also lead to results of antimicrobial testing being non-representative of what will occur in the biological environment and possibly different behaviour of the material during its use.
**Staphylococcus epidermidis** biofilms on reinforced PLLA with Mg and MgZn

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**INTRODUCTION:** To improve the mechanical behaviour of biodegradable polymers, a present research line is oriented to the reinforcement of the polymer matrix with metal particles. Magnesium and its alloys are promising candidates because of their biodegradability. The aim of this study is evaluate the formation of biofilms of *Staphylococcus epidermidis*, an opportunistic pathogen primarily responsible for surgical failure, on hybrid systems of a biodegradable and resorbable PLLA matrix (1) reinforced with Mg and MgZn particles.

**METHODS:** The hybrid systems tested were PLDA (P), PLDA+10%wt Mg (P-Mg) and PLDA+10%wt MgZn (P-MgZn), and for biofilm assays the strain selected was *S. epidermidis* ATCC 35983, routinely maintained in Tripticase Soy Broth. Bacterial cultures in exponential phase, at a bacterial concentration spectrophotometrically adjusted, were incubated with the discs at 37°C for 24h on sterile polystyrene culture plates. The bacterial biofilms were quantified with the BacTiter-Glo™ Microbial Cell Viability Assay. The light emission reaction was measured by a luminometer in Relative Light Unit (URL). Each assay was performed in duplicate and repeated three or more times. Results are expressed as means ± standard deviation. Significance differences were determined by the one-way analysis of variance (ANOVA).

Morphology of biofilms grown on substrates was observed by scanning electron microscope (SEM). To this purpose, samples after biofilm grown experiments were fixed and dehydrated. Subsequently, were vacuum dried, sputter-coated with Au, and observed using a SEM (Quanta 3D FEG; FEI Company).

**RESULTS:** In respect to the PLDA control matrix, both reinforced polymers reduce the bacterial biofilm viability after 24h of incubation (Fig 1). The reductions reach up to 28% for P-Mg (p=0.03) and 32% for P-MgZn (p=0.001) with respect to the control.

**DISCUSSION & CONCLUSIONS:** The inclusion of metal particles of Mg and MgZn in the PLLA matrix provides to the polymer with a high anti-biofilm surface, despite biofilm morphology are similar. The combination of the degradation results of Mg and MgZn and the modifications induced in the surrounding media near the surface layer is the most probably reason behind the biofilm reduction observed.

**ACKNOWLEDGEMENTS:** Authors thank to the Ministerio de Ciencia e Innovación (Spain) and FEDER (MAT2012-37736-C05-01-03-04), Junta Extremadura (GR10031 and GR10149) and Research Support Unit of University of Extremadura (SAIUEx).

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Evaluation of the antimicrobial and cytotoxic activity of metal ions in different eukaryotic cell culture media

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INTRODUCTION: Bacterial infections especially biofilm triggered infections cause tremendous health problems in humans. More than 45% of new infections in hospitals can be traced back to microbial biofilm contaminated medical devices (Bixler and Bhushan, 2012). Therefore, it is highly desired to develop “antimicrobial” implants, catheters, and other medical surfaces. Some metal ions, e.g. silver and copper, have been shown to possess antibacterial properties. These new developments have to prove both, the cytocompatibility and the antimicrobial activity. For the evaluation of materials with such functionality reliable antimicrobial assays mimicking in vivo conditions are necessary. The in vivo environments differ dramatically in tissue cell types, biological fluids and micro environments such as the oral cavity, urinary tract or tissues surrounding bone implants. Specific bacterial species cause particular infections within these environments. For bone implants for example skin bacteria Staphylococcus aureus, S. epidermidis and Pseudomonas aeruginosa are the most common cause for the infections.

METHODS: In our study different staphylococci and P. aeruginosa species have been tested for their ability to grow planktonically in typical media for eukaryotic cell culture, Dulbecco’s Modified Eagle Medium (DMEM) or Roswell Park Memorial Institute medium (RPMI), both + 10% fetal calf serum (FCS). The growth was compared to commonly used bacterial media Brain Heart Infusion (BHI) and Tryptic Soy Broth (TSB). Different concentrations of metal ions (AgNO₃, CuCl₂, ZnCl₂ and Ga(NO₃)₃) were applied to the bacterial cells grown in DMEM and RPMI. The antimicrobial activities of the metal ions were analysed by measuring bacteria proliferation at optical density (OD 600). Cytotoxicity of the metal ions was tested on normal human dermal fibroblasts (NHDF) in DMEM + 10% FCS and THP-1 macrophages in RPMI + 10% FCS. After exposure to the metal ions for 24 h, cell metabolic activity was evaluated using MTT assay.

RESULTS: The minimal inhibitory concentration (MIC) of AgNO₃ is 8 µM for P. aeruginosa having an initial cell number of 6.7 x 10⁶ CFU/ml in DMEM + 10% FCS after 24 hours of incubation (Fig. 1 A). Lethal dose 70 or cytotoxic limit (LD70) of AgNO₃ was found to be 22.2 µM for NHDF (Fig 1B). Under the same conditions much higher concentrations of copper ions were needed to reach MIC and LD70 (Table 1).

DISCUSSION & CONCLUSIONS: It was found that a concentration of 8 µM of silver ions is enough to inhibit the growth of P. aeruginosa and not to outreach the LD70 of NHDF. Furthermore, MIC of copper ions exceeded the cytotoxicity limit of NHDF. Thus, copper ions may have limitations to be used as antimicrobial agents in implant or other medical devices.

ACKNOWLEDGEMENTS: This study was support by Commission for Technology and Innovation CTI (No. 16302.2 PFNM-NM).
Increased release time of antibiotics from bone allografts through a novel biodegradable coating

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Introduction
Use of local antibiotics in connection with bone substitutes has 3 indications: prevention of primary infection, implantation into a contaminated area or the prevention of recurrent infection. These cases all have their requirements in terms of choice of antibiotics and length of release time, so there is no universal coating technique for all cases. We aimed to develop a sustained-release coating for bone allografts, which can achieve long-term release characteristics for several antibiotics.

Materials and Methods
Human bone allografts (50±5 mg each) were coated with 10 mg/ml amoxicillin, ciprofloxacin, vancomycin or gentamycin. The simple coating methods were soaking (method A) or freeze drying (method B). The controlled release film coating was prepared in single or double layers. When using one layer, the antibiotics were mixed with 2% chitosan and the coating was freeze dried (method C). The preparation of the two layer controlled release coating involved a freeze drying step with the antibiotic followed by a water insoluble film preparation step by using 4% Na-alginate, which was converted with 10% CaCl₂ solution to Ca-alginate (method D). Preparations were incubated in 2 ml H₂O and antibiotic release was measured in the supernatant by spectrophotometry. For the antibiotic that we found best, the MIC (minimal inhibitory concentration) was also assessed.

Results
The antibiotic coating experiments showed that the best concentration was 10 mg/ml using all the antibacterial agents and with method B, we were able to enhance the coating compared to the conventional soaking method with every antibiotic. All the antibiotic content was released within 48 hours, so method A, B and C were only suitable for short-term antibiotic release. With method D we were able to prepare a coating that released amoxicillin over a 7 day period; ciprofloxacin was released over a 28 day period and vancomycin and gentamycin release exceeded 50 days well over the MIC concentration.

Discussion and Conclusions
Simply adding antibiotics or freeze-drying them onto the allograft only reaches a 2-day release profile, which is even shorter than the closure time of the surgical wound. Insoluble alginate coating achieved a sustained release of each selected antibiotic to various lengths, depending on the physico-chemical characteristics of the drugs. Amoxicillin may be applicable for primary prevention while ciprofloxacin or vancomycin and gentamicin is suitable for more challenging cases.

Acknowledgments
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Disclosures
Dr. Lacza is founder and CEO and Dr. Hornyák is an employee of Lacerta Technologies GmbH, which holds a pending patent of the technology.
3D printed bioceramics for dual antibiotic delivery to treat implant-associated bone infection

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INTRODUCTION: Implant-associated infections are challenging to treat due to biofilm, where the bacteria may evade the immune response and antibiotics [1]. Treating chronic bone infections typically involves local antibiotic delivery via poly(methyl methacrylate) (PMMA) bone cement to augment the systemic antibiotics, which include rifampin since it may be effective against biofilm-forming species. PMMA cannot deliver rifampin, as this antibiotic impairs polymerization. Local rifampin therapy for implant-associated bone infections had yet to be investigated in vivo. In this study, rifampin- and vancomycin-laden calcium phosphate scaffolds (CPS) were fabricated by 3D printing to treat an implant-associated Staph. aureus bone infection in a murine model.

METHODS: The CPS were fabricated using a modified 3D printer [2]. The vancomycin dose was kept constant at 5 wt% of the CPS, but the rifampin dose was varied to include 0% (Vanco-CPS), 0.05% (Lo-Rif+Vanco-CPS) or 0.5% (Hi-Rif+Vanco-CPS). Sustained release was achieved by coating Hi-Rif+Vanco-CPS with poly(lactic-co-glycolide) (Hi-Rif+Vanco-CPS/PLGA). Bone cement spacers mixed with 5 wt% vancomycin were formed in a custom mold (Vanco-PMMA).

Animal studies were approved by the University Committee on Animal Research and surgeries were performed according to a recently described murine model of implant-related bone infection [3]. A Ti-coated PEEK fixation plate was installed on the femur of each BALB/cJ mouse. A 0.7 mm osteotomy was cut in the mid-diaphysis and a collagen sheet, inoculated with 8x10^4 CFU of bioluminescent Staph. aureus (Xen36), was placed into the defect. The infection established for 7 days before the revision surgery, at which point the tissue was debrided and the osteotomy was widened to 3 mm to place a CPS or PMMA spacer (n≥8/group). The infection was monitored for 21 days by bioluminescent imaging (BLI). Micro-CT scans were performed after the revision surgery and at the end of the study to measure volumetric bone changes. The tissues and implants were separated for bacterial CFU counts.

RESULTS: The bacterial burden, based on BLI, was significantly reduced at early time points after revision by all Rif+Vanco-CPS compared with Vanco-PMMA. These reduced BLI values on days 8-10 were correlated with reductions in bone resorption (r=0.44, p=0.0002) and total CFU counts (r=0.49, p<0.0001). Bacterial CFU counts (Fig. 1) and volumetric bone resorption were significantly reduced by local rifampin therapy compared to Vanco-PMMA. Vanco-CPS (without rifampin) was not significantly different from Vanco-PMMA. Bacteria persisted on the biofilm-ridden fixation hardware, but the mean bacterial load was reduced by local rifampin therapy.

DISCUSSION & CONCLUSIONS: Localized co-delivery of rifampin and vancomycin from 3D printed CPS, which is not possible with PMMA, significantly improved the outcomes of implant-associated osteomyelitis. As Vanco-CPS was not different from Vanco-PMMA, this study supports the importance of supplemental rifampin against S. aureus. Bacterial persistence on the fixation plates highlights the challenge of treating biofilms and reaffirms the importance of implant exchange.

ACKNOWLEDGEMENTS: This study was supported by the AOTrauma Research CPP on Bone Infection.

Fig. 1: Bacterial colonization of the bone and soft tissues was significantly reduced by local rifampin therapy. Note that culture negative bones were only achieved with groups receiving rifampin.
Surfactin or iodined treatments to remove staphylococci biofilm

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INTRODUCTION: S. aureus, the pathogenic bacteria, as well as the opportunistic S. epidermidis bacteria have the ability to adhere to abiotic surfaces and to form biofilms. This is one of the major causes of the medical implant-related infections. At present, no medical treatment is available to eradicate bacterial biofilm. It is crucial to determine effective long-term treatment for the prevention of bacterial adhesion involved in these infections or for the biofilm removal.

METHODS: We have constructed a representative library of Staphylococcus strains involved in human infections. It consists out of 120 clinical isolates collected from hospitals in France. Almost all strains were isolated from infections of indwelling medical devices. To identify bacterial strains, a phylogenetic tree based on partial 16S rDNA and rpoB gene sequences was constructed by the neighbor-joining method using the program MEGA5.0. Biofilm formation was studied with a safranin or MTT biofilm assay and LIVE/DEAD BacLight staining and confocal microscopy.

We have examined 1) the effect of biosurfactant (surfactin) and 2) the oxidation of iodine potassium to I₂ by a fungal enzyme, laccase, on Staphylococci biofilm.

RESULTS: The most frequently isolated species were Staphylococcus epidermidis and CoN Staphylococcus aureus, followed by Staphylococcus lugdunensis, Staphylococcus capitis and Staphylococcus warneri.

Surfactin, a lipopeptide from Bacillus sp. was tested to hydrolyze biofilm after a 24-hour incubation or to inhibit bacterial adhesion in vitro. In the former case, no biofilm detachment is observed in our conditions. In the latter case, decreasing in biofilm formation is only observed in few strains, S. epidermidis or S. lugdunensis but in a minor extent.

We also investigated the possible effect of combined laccase and iodine treatments on biofilm degradation. Two biofilm producer strains are used in this study, Staphylococcus hominis and S. aureus. Mortality increased significantly although the biofilm remained still present.

DISCUSSION & CONCLUSIONS:

Unlike surfactant, we have shown that the iodinated treatment partially induced the removal of staphylococci biofilm. The iodine oxidation triggered by enzyme has induced a slight but significant decreasing in S. aureus biofilm while bacterial mortality was very high. Nevertheless the biofilm degradation is not sufficient to eradicate bacterial infection.
Modifying titanium implant surfaces with antibacterial copper: Is this feasible?

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INTRODUCTION: Implant associated infection is a burden for patients. It may appear not only directly after surgery but also after a long postsurgery period in some cases. Furnishing implant surfaces with infection inhibiting properties would therefore be a very welcomed approach. Here, we introduce the approach for modifying the surface of titanium implants with antibacterial copper. Based on the results of a number of studies [1-6], it is concluded that copper-modifying anodized implant surfaces is a feasible approach and has the potential for industrial up-scaling.

METHODS: Discs of cpTi grade 4 (⌀ 12 or 14 mm, 2 mm thick) were anodized and Cu-modified using the spark-assisted anodizing method run in a combined anodizing/deposition process using proprietary electrolyte and proprietary process parameters (KKS TioCelTM) [1,2] (Figure 1).

RESULTS: The anodized discs show a rough fine-porous outermost layer and an underlying conversion layer (Figure 2 left). The R_a-value of the outermost layer is ca. 0.6 µm. Using appropriate electrochemical process parameters, Cu can be deposited onto and/or into the titanium oxide layer as detected by XPS depth profile analysis using Ar^+ sputtering [3] (Figure 2 right).

DISCUSSION & CONCLUSIONS: The physicochemical and biological data obtained so far do strongly indicate that modifying the surface with copper is a feasible approach to give titanium implants an antibacterial property. The electrochemical technique is related to practice applications and has the potential to scale-up.

* Corresponding author.
INTRODUCTION: More than 2 million people/year suffer a bone fracture in the UK\(^1\). Reconstruction of bone defects represents a major clinical challenge and is addressed using a number of medical devices. Although medical device compositions and applications may differ widely, all attract microorganisms and represent niches for medical device associated infections. For open fractures, the risk of infection can be 55%\(^2\). These infections are often resistant to many of the currently available antibiotics and represent a huge and growing financial and healthcare burden. The aim of this study was a fundamental understanding of how the presence of host defence peptides (HDPs)\(^3\) and/or RGD can influence the outcome of cell vs. bacterial viability and proliferation.

METHODS: The antimicrobial activity of four HDPs; K5, K6, E6 and 1018, alone or in combination with RGD, was tested against three bacterial strains; *Staphylococcus (S.) aureus*, *S. epidermidis* and *Pseudomonas aeruginosa*, and their biofilms under both static and dynamic flow conditions using a commercially available microfluidic system, the BioFlux. Their performance was compared to Vancomycin, Polymyxin and Nisin, using a live/dead assay and luminescence.

The cytocompatibility of the above HDPs, alone or in combination with RGD was tested using breast cancer cell lines 231 and 468, human G-292 osteosarcoma cells, human antipose-derived stem cells (ADSCs, Lonza) and a MTT assay.

The performance of HDPs and/or RGD was also tested in *S. epidermidis* – G-292 co-culture systems using the BioFlux. *S. epidermidis* was introduced after an overnight G-292 culture.

RESULTS: The HDPs were effective against both Gram-positive and Gram-negative bacterial strains in the presence or not of RGD, while the RGD did not affect at all the bacteria viability. The E6 performed better against bacterial suspensions while the other three HDPs performed better against biofilms that were established using the BioFlux system. The antibiotics were more effective against bacterial suspensions than the HDPs at low concentrations such as 8 µg/ml; however the HDPs, in contrast to the antibiotics, were effective against both Gram-positive and negative strains and their biofilms, and performed better than Nisin.

The presence of HDPs at concentrations higher than 8 µg/ml significantly reduced the cell viability with the increase in HDPs concentration. The ADSCs were the most susceptible. The presence though of RGD at low concentrations in parallel to HDPs significantly increased the cell viability in comparison to HDPs alone.

In the case of *S. epidermidis* – G-292 co-culture systems in the BioFlux, the presence of HDPs significantly enhanced the cell viability, in comparison to the co-culture system without treatment, while the bacterial viability was reduced. The parallel presence of HDPs/RGD further supported cell viability and proliferation without compromising the antimicrobial and antibiofilm performance of the HDPs.

DISCUSSION & CONCLUSIONS: These results show promising signs for the use of RGD in combination with HDPs towards the preparation of antimicrobial materials that allow tissue integration. Orthopaedic implants would therefore be a great application for this kind of combined HDPs/RGD systems.

ACKNOWLEDGEMENTS: Dr S. Farmer, Dr E. Haney, Dr A. Hilchie (UBC) are acknowledged for their support with the antimicrobial and cytocompatibility assessment of HDPs and Dr S. Patel (Fluxion) for his help with the BioFlux. This work and MGK secondement at UBC were funded through WELMEC under grant number WT 088908/Z/09/Z and iMBE. This template was modified with kind permission from eCM Journal.
Fluorescence in situ hybridization (FISH) analysis of biofilms on device surfaces

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INTRODUCTION: Device-associated infections pose a significant risk for patients and increase health care costs. Causative agents may be difficult to diagnose in case of culture-negative samples. Here, we tested the impact of fluorescence in situ hybridization (FISH) in combination with digital image analysis for visualization, and quantification of in vivo grown biofilms on device surfaces.

METHODS: A rat central venous catheter infection model was investigated by FISH to gain further insights into the effect of antibiotics on biofilms. Biofilms were treated with daptomycin and vancomycin; PBS was used in the control group. Daptomycin and vancomycin were infused systemically in a high dosage regimen (100 mg/kg body weight/day and 160 mg/kg body weight every 12 hours, respectively; equivalent exposures in patients of 10 mg/kg/day and 1 g/person every 12 hours, respectively). We evaluated catheter-related bacteremia, metastatic infections and catheter culture in comparison to FISH results.

RESULTS: FISH visualized ribosome-containing, metabolically active bacterial cells and total biofilm mass, thus providing insight into the in situ effects of the antibiotics [1]. Local antibiotic treatment of the biofilms reduced the FISH-positive biofilm fraction from 36% (controls) to 28% for both daptomycin and vancomycin. Upon systemic treatment we observed significantly lower fractions of FISH-positive cells in biofilms that were treated with high dosage daptomycin (4%) as compared to vancomycin (19%) and controls (36%) (Fig. 1). We found ribosome-containing FISH-positive bacteria in biofilms that were culture negative when plated on agar, indicating cells that are viable but not cultivable and hence being responsible for recurrent infection.

DISCUSSION & CONCLUSIONS: In summary, these findings show the versatility and impact of FISH for analysis of device-associated infections in an in vivo setting. FISH might also be a valuable tool for further investigations of biofilm antibiotic recalcitrance.

ACKNOWLEDGEMENTS: Part of this study was supported by Novartis.
Epidemiology of musculoskeletal complications after intramedullary nailing of long bone fractures.

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INTRODUCTION: Intramedullary nailing (IMN) is the preferred treatment technique for shaft fractures of long bones [1-3]. The advantages of intramedullary fixation include early stable fixation, early mobilization of the adjacent joints, and preservation of soft tissue at the fracture site. Although previous studies published good results with this technique, reliable population-based incidence data on musculoskeletal complications are lacking. Therefore a retrospective study was performed to analyse the epidemiology and outcome after IMN of shaft fractures of long bones.

METHODS: Between January 1998 and January 2013, 1289 consecutive patients with shaft fractures were enrolled. Inclusion criteria were skeletal maturity and shaft fractures treated with IMN. Exclusion criteria were skeletal immaturity, primary treatment by plate osteosynthesis, primary treatment outside the University Hospitals Leuven, presence of metaphyseal fractures and presence of pathological fractures. Negative outcome measures such as infection and compromised fracture healing were retrospectively assessed.

RESULTS: During the study period, 907 patients with 931 fractures met the inclusion criteria. Of these, 39 patients were lost to follow-up, 13 died from trauma-related causes within the first 30 days after the accident, and 20 died from other causes (cardiovascular disease and cancer) leaving 835 patients with 859 fractures for a minimum follow-up period of 12 months.

The number of included patients and fractures in the different long bone groups, in combination with their epidemiological differences, is shown in table 1. Polytrauma patients are more prevalent in the lower extremity fracture group.

In the study population, 27 patients (3.0%) were diagnosed with an infection. Of these, 8 patients (0.9%) were diagnosed with a deep implant-related infection: 1 patient (0.1%) in the humeral shaft fracture group and 7 patients (0.8%) in the tibia shaft fracture group. Furthermore in the tibia shaft fracture group there were 21 patients (2.3%) with superficial infections.

In total, 138 patients (15.2%) with 140 fractures (15.0%) were diagnosed with compromised fracture healing: 6 patients (0.7%) in the humeral shaft fracture group, 27 patients (3.0%) in the femoral shaft fracture group and 105 patients (11.6%) in the tibia shaft fracture group.

DISCUSSION & CONCLUSIONS: IMN is a valid therapeutic option for shaft fractures of long bones. With respect to musculoskeletal complications there is a difference in the incidence between humeral, femoral and tibial shaft fractures. Compromised fracture healing is more prevalent in fractures of the lower extremity. Tibial shaft fractures have a higher incidence of compromised fracture healing, superficial and deep infection compared to humeral and femoral shaft fractures. Future strategies should focus more on preventive strategies in these high-risk populations.

Table 1. Population-based differences between patients treated with intramedullary nailing for shaft fractures of the long bones.

<table>
<thead>
<tr>
<th>Long bone fractures</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Humeral shaft fractures (125/13)</td>
<td>119 (94.4)</td>
</tr>
<tr>
<td>Femoral shaft fractures (197/140)</td>
<td>18 (9.4)</td>
</tr>
<tr>
<td>Tibial shaft fractures (198/210)</td>
<td>203 (96.1)</td>
</tr>
<tr>
<td>Male</td>
<td>140 (91)</td>
</tr>
<tr>
<td>Polytrauma patients</td>
<td>14 (10.5)</td>
</tr>
<tr>
<td>Active smokers</td>
<td>17 (17.4)</td>
</tr>
<tr>
<td>Others</td>
<td>24 (10.3)</td>
</tr>
<tr>
<td>Open fractures</td>
<td>3 (2.3)</td>
</tr>
<tr>
<td>Sealed fractures</td>
<td>9 (7.3)</td>
</tr>
<tr>
<td>Primary external fixation</td>
<td>3 (2.4)</td>
</tr>
</tbody>
</table>

N (number of patients/number of fractures)
Competition in dynamic flow adhesion between *S. mutans* and *S. sanguinis* on a titanium alloy surface

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INTRODUCTION: Hundreds of bacterial species colonize and co-exist in the mouth forming complex associations of microorganisms known as dental plaque or oral biofilm [1]. The oral biofilm usually comprises commensal microorganisms that are beneficial to the host, as they exclude colonization from pathogenic microorganisms that may induce disease [2]. The current concept of the role of oral bacteria in dental caries development involves an environment shift in the microbial composition. The oral flora tends to be increased in cariogenic species that competes more efficiently with the commensal flora for the tooth surface, eventually leading to disease [3].

Oral streptococci account for 20% of all supragingival microorganisms present in the oral biofilm, but constitute about 80% of the initial colonizers during early biofilm development. Certain streptococci have been associated with the onset and progression of carious lesions. *Streptococcus mutans* is an important player in caries pathogenesis due to its cariogenic traits. However, *Streptococcus sanguinis* is a normal colonizer of the dental enamel and its presence is associated with healthy biofilms and with the absence of caries. Studies in rats have shown that *S. sanguinis* may compete for tooth colonization and inhibit *S. mutans* during oral biofilm growth [4]. Results seem to support the idea of an antagonistic relation between both bacterial strains.

Due to the use of several biomaterials for the oral cavity repair, this work focuses on the bacterial attachment and co-adhesion on a metallic surface, as it can be also support for oral biofilm. This knowledge could help to lead toward strategies to control or influence the pattern of oral biofilm formation in oral prosthesis.

METHODS: Disks of Ti6Al4V of 25 mm in diameter were used. Experiments were carried out at 37 °C in a parallel-flow chamber by using a metallographic microscope (Olympus BX41, Barcelona, Spain). Once the system was filled with PBS solution, a *S. mutans* suspension of 3x10⁸ cells m⁻¹ in PBS was allowed to flow through the system at a flow rate of 2 mL min⁻¹ for 30 min. On a second stage, the flow was stopped and a newflow of *S. sanguinis* suspension of 3x10⁸ cells m⁻¹ in PBS was slowly opened at a flow rate of 2 mL min⁻¹ for 30 min. Live adhesion was followed as time passed by recording the images with a video camera connected to a computer and the initial adhesion rates (j₀) was calculated. Once the adhesion process was finished, with a final number of bacteria (n_f) on the Ti alloy surface, was exposed to the passing of three liquid-air interfaces (n_l-3). The experiment was repeated by changing the order of the bacterial strains.

RESULTS: No statistically significant differences were found neither j₀ nor n_f between Streptococcus over the first 30 min i.e. when only flows the first bacterial strain. For the second stage, both j₀ and n_f parameters were greater when *S. sanguinis* was flowed first.

DISCUSSION & CONCLUSIONS: When *S. mutans* is first adhered to the surface, the subsequent adhesion of *S. sanguinis* is obstructed and virtually all bacteria are detached with the third liquid-air interface. However, this obstruction is not observed when *S. sanguinis* flows first.

ACKNOWLEDGEMENTS: Ministerio de Ciencia e Innovación” (MAT2012-37736-C05-01;03) and “Junta of Extremadura-FEDER: European Regional Development Fund” (GR10149).
**Antimicrobial activity of bone cements with embedded organic nanoparticles**

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¹ *Cardiff Polymer Therapeutic Labs, School of Pharmacy and Pharmaceutical Sciences, Cardiff University, Wales.* ² *Casali Institute, Hebrew University of Jerusalem, Israel.*

**INTRODUCTION:** Infections after orthopaedic surgery are a very unwelcome outcome; despite the widespread use of antibiotics their incidence can be as high as in 10% of cases. This likely to increase as antibiotics are gradually losing efficacy as results of bacteria developing resistance, therefore novel antimicrobial approaches are required. Parabens are a class of compounds whose antimicrobial activity is employed in many cosmetic and pharmaceutical products. We developed propylparaben nanoparticles; the hydrophilicity of the nanoparticles results in superior antimicrobial activity than pure propylparaben that is hydrophobic. The nanoparticles were embedded in polymethylmethacrylate (PMMA) bone cement and the antimicrobial activity was determined against common causes of post-orthopaedic surgery infections such as: *Staphylococcus aureus*, MRSA, *S. epidermidis* and *Acinetobacter baumannii*.

**METHODS:** Organic nanoparticles were prepared according to Margulis-Goshen et al.[1]. The antimicrobial of PMMA bone cement samples containing paraben nanoparticles was determined through the estimation of the bacteria surviving on the sample surface, using the indirect method proposed by Berchet et al. [2] employing the time to detection principle (TTD). Cytotoxicity against osteoblast cells cultured on the bone cement containing paraben nanoparticles was assessed through the MTT assay.

**RESULTS:** The MIC of the paraben nanoparticles against all four bacteria tested was lower than pure paraben in virtue of the higher hydrophilicity of the nanoparticles. Bone cements containing increasing concentrations of antimicrobial compounds exhibited generally lower bacterial colonisation (longer time to detection) as can be seen in Table 1. TDD about 10 hours longer than the control (0% w/w) are compatible with a reduction of 6 log10 assuming a doubling time of 30 min.

Despite providing antimicrobial activity, in order to be a viable option, the organic nanoparticles must not induce negative effects on the other bone cements properties; for this reason, the cytotoxicity and compression strength of bone cements containing 7 % w/w of nanoparticles were determined. The results demonstrated that the nanoparticles did not have a detrimental effect on these two essential characteristics.

**DISCUSSION & CONCLUSIONS:** Gentamicin and tobramycin are the most common antibiotic used in PMMA bone cement in virtue of their thermal stability and broad spectrum. However, *S. epidermidis* strains such as: RP62a and various *A. baumannii*, both tested in this work, are resistant to these drugs rendering their use ineffective when such strains are involved. Our results demonstrated that organic nanoparticles made of propylparaben are effective against a wide spectrum of bacteria, including antibiotic resistant strains found in orthopedic infections; hence the use of these organic nanoparticles could offer not only a possible alternative to antibiotics but also solve some of the problems already associated with antibiotic resistance. Moreover, the amount of paraben nanoparticles required to provide effective antimicrobial activity is similar to amount of gentamicin or tobramycin used in commercial bone cements (2-4 % w/w) and is significantly lower than other antimicrobial agents such as chitosan derivative that require 20-30 w/w.

**ACKNOWLEDGEMENTS:** PP would like to acknowledge Arthritis Research UK (ARUK:18461) for funding this study.

---

**Table 1. Antimicrobial activity of bone cement containing paraben nanoparticles.**

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Nanoparticles conc. (% w/w)</th>
<th>TTD (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em></td>
<td>0</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>&gt; 24</td>
</tr>
<tr>
<td>MRSA</td>
<td>0</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>&gt; 24</td>
</tr>
<tr>
<td><em>A. baumannii</em></td>
<td>0</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>&gt; 24</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>0</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>&gt; 24</td>
</tr>
</tbody>
</table>

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Nanocarrier controlled delivery of antibiotics for acrylic bone cement applications

S Perni1, P Prokopovich1

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INTRODUCTION: Bone cement is routinely used in orthopaedic applications and antibiotics are added in order to prevent infections offset. Uneven distribution and uncontrolled release of the antibiotic are drawbacks associated with the mixing of such drug in the bone cement dough. Particularly, the antibiotic is released in the first weeks after implantation and, therefore, does not offer protection against infection that can occur months after the surgery. In order to control the antibiotic release and consequently extend the antimicrobial activity, silica nanocarriers containing gentamicin have been prepared through various synthetic routes.

METHODS: The carriers were synthesised using the Stöber protocol (hydrolysis of tetra ethyl silane in the presence of alcohol and alkaloids) and amino functionalised using (3-amino-propyl)triethoxy-silane. The antibiotic has been conjugated both directly to amino functionalised particles (Suberic acid bis(N-hydroxysuccinimide ester) or to succinylated amino functionalised particles and through direct entrapment of gentamicin in the nanoparticles during synthesis; moreover adsorptions on: amino functionalised, succinylated and un-functionalised have been performed. Alternatively, the antibiotic (naturally positively charged) has been deposited on the silica nanoparticles using the Layer-by-layer technique, sandwiching the drug between alginate layers. The obtained particles have been characterised in terms of porosity (BET), antibiotic content (TGA) and surface charge (zeta potential); whilst gentamicin release from all prepared nanocarriers has been quantified as function of time and pH of solution using the ortho-phthalaldehyde assay.

RESULTS: The direct entrapment provided the highest antibiotic load (12% w/w) but the release was completed after 4 hours, whilst the adsorption on unfunctionalised silica particles returned the lowest amount of antibiotic load (<1% w/w). For all other conjugated and adsorbed nanocarriers, gentamicin load was about (4% w/w) and the release was observed over a period of at least 2 days in all cases without significant differences among the synthetic route and pH. Using the LbL self assembly approach the released was sustained for at least 2 weeks; the use of multiple double layers of gentamicin and alginate did not improve the release profile or extended it (Fig. 1).

![Graph](attachment:image.png)

Fig. 1: Release profile of gentamicin from silica nanocarriers prepared through LbL (● double layer; ○ 4 double layers).

Once encapsulated in PMMA bone cement, the silica nanoparticles did not negatively affect the cytotoxicity and mechanical properties of the material.

DISCUSSION & CONCLUSIONS: Our results therefore, show that silica nanocarriers of antibiotics can be embedded in bone cement and extend the antimicrobial activity. Depending on the preparation route, the release can be controlled; for prolonged (many weeks), the coating of silica nanoparticles with antibiotic through LbL appear the most promising technique.

ACKNOWLEDGEMENTS: This work was supported by the Life Science Research Network Wales, an initiative funded through the Welsh Government’s Ser Cymru program.

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Possibilities of two-stage revision knee replacement for treatment of the deep periprosthetic infection in haemophilia patients.

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INTRODUCTION: The need of knee replacement for patients with a severe haemophilic arthropathy in the conditions of high risk of a periprosthetic infection demands development of approaches to treatment of these complications.

METHODS: Two-stage revision knee replacement was applied in 11 cases of a deep periprosthetic infection at 9 patients with a severe haemophilia (including one patient with FVIII inhibitor). The surgery was performed on both knees in 2 haemophilic patients. Two-stage revision surgery was indicated in cases of deep periprosthetic infection with formation of phlegmon and/or osteomyelitis. The first stage of the surgery consisted in implants and bone cement removing and sanitation of a joint cavity with antiseptic solutions (Lavasept 0.1-0.2%, Povidone-iodine 1%, Chlorhexidine 1%). The surgical sanitation consisted in total excision of a fibrous capsule with granulations, economy resections of the bone ends and removal of the infected cancellous bone. The bone resection was performed within an intact bone with the maximum preservation of a bone tissue. After surgical sanitation repeatedly carried out washing of a cavity of a wound with antiseptic solutions. The space between resected bones was filled with bone cement (PMMA) with addition of an antibiotic. The powder of Vancomycin 2 g was mixed with powder of bone cement 40 g before mixing with liquid. The insert of bone cement spacer was established so that as much as possible to fill postresection defect and to restore a tension of soft tissue. In case of the big defect a cement spacer reinforced with metal nail. Within 7 - 14 days after surgery wound cavity was daily washed by drainage system with antiseptic solutions (Lavasept 0.1%, 250 - 500 ml 1 - 2 time per day). The antibiotic therapy was admitted within 14 days beginning in 30 minutes prior surgery. The individual selection of drugs was performed in view of sensitivity of pathogenic microflora. The replacement haemostatic therapy for haemophilic patients was carried out according to standard surgical protocols, depending level of deficient factor. Time after surgery and weight of the patient. The second stage of the revision surgery was carried out within 4-12 months. After removal of a cement spacer and economy bone resection revision implants were implanted. Hinged and semi-hinged systems were used: -MATI-CITO (Russia), - GMRS (Stryker), - RT-PLUS (Smith&Nephew). In need of replacement of big bone defects used modular oncological systems. Antibacterial and replacement haemostatic therapy was carried out in the same volume, as well as after the first stage of treatment.

RESULTS: In 10 cases application of two-stage revision knee replacement at a deep periprosthetic infection in haemophilia patients led to knocking over of infectious process and restoration of function of an extremity. In one case repeated deep periprosthetic infection and implants instability were indications for joint fusion by individual locking construction. The standard replacement haemostatic therapy for haemophilia patients prevented bleeding complications in all cases.

Thus, two-stage revision knee replacement may be used for treatment of a deep periprosthetic infection in patients with haemophilia.

ACKNOWLEDGEMENTS: This template was modified with kind permission from eCM Journal.
**Characterisation of nasal Methicillin-resistant Staphylococcus aureus from orthopaedic surgeons**

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**INTRODUCTION:** *Staphylococcus aureus* is amongst the most frequent causative agent of hospital-acquired (HA) infections and device-associated (DA) infections causing 30-35% of all orthopaedic implant related infections. Methicillin-resistant *S. aureus* (MRSA) have emerged as a significant threat in both the hospital and community environment. Community-acquired (CA)-MRSA can be unpredictable, invasive and more deadly than HA-MRSA [1]. The spread of MRSA poses significant challenges. The hospital environment, the patient's endogenous microflora, and health care workers (HCWs) may all play a role [1]. Also surgeons themselves are at an elevated risk of nasal carriage of MRSA in comparison with non-surgical physicians [2]. The objective of this study was to perform a phenotypic and genotypic characterization of MRSA isolates from a recently undertaken prospective study where 1140 orthopedic, spine, cranio-maxillofacial (CMF) and Vet surgeons from all over the world were screened for staphylococcal colonization.

**METHODS:** Twenty-six methicillin resistant *S. aureus* isolates were identified and antibiotic susceptibility to 28 antibiotics was determined. Isolates were tested for their ability to form a biofilm, their haemolytic activity and staphyloxanthin production. They were typed by *agr* (accessory gene regulator) group and genes coding for the 13 most relevant MSCRAMMs, Panton-Valentine leukocidin (PVL), PIA (polysaccharide intercellular adhesin), beta-haemolysin, the five most relevant Staphylococcal enterotoxins (SEA-SEE), exfoliative toxins A and B (ETA and ETB) and toxic shock protein (TST) were screened for by PCR. Spa- typing and MLST was undertaken. One MRSA strain was selected for complete genome sequencing as it was found to be rifampicin resistant.

**RESULTS:** All MRSA isolates were multiply antibiotic resistant and 30% (8/26) and 27% (7/26) of the MRSA were resistant to gentamicin and tetracycline, respectively. One MRSA gentamicin resistant isolate was also rifampicin resistant. Most isolates (69%) were haemolytic. The majority of isolates (80%, 21/26) did not produce a biofilm. Virulence genes *hlgC/B* (encodes gamma-haemolysin), *icaA* (PIA), *eno* (encodes laminin binding protein), *clfA* (encodes adhesin binding to fibrinogen), *clfB* (encodes adhesin binding to fibrinogen) and *sdrC* (encodes adhesin binding to beta-neurexin) were exhibited by all isolates. Both Panton-Valentine leukocidin (PVL)+ MRSA carriers came from India. Isolates were identified to belong to well-known clonal lineages and epidemic outbreak HA- or CA-MRSA strains. The rifampicin resistant MRSA that was sequenced was identified as CC8 ST8- t064 strain that is not only epidemic amongst human but also in animals.

**DISCUSSION & CONCLUSIONS:** All of the 26 MRSA isolates were multiply antibiotic resistant and possessed a wide range of virulence factors. The strains were identified to belong to several different clonal lineages such as USA400 and USA500 that have caused epidemic outbreaks in the past. This highlights the importance of MRSA screening systems especially in clinical and healthcare settings.

**ACKNOWLEDGEMENTS:** The authors would like to acknowledge the contribution of Llinos Harris, PhD from the University of Swansea for assisting with the whole genome analysis.
Eradication of bacterial biofilms from titanium implants by vancomycin: beyond the reach of common local delivery.

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INTRODUCTION: The formation of biofilm is one of the key elements in implant-associated infections, allowing the causative bacteria to resist to the immune system and persist despite antibiotic treatment, often requiring removal of the implant [1]. Local delivery of antibiotics is commonly used in the treatment of implant related infections. The antibiotic release profile of antibiotics is considered to improve therapy as high local concentrations can be reached which should increase bacterial killing, even of resistant bacteria, or biofilm [2, 3]. However, it is unclear what release profile would be required to achieve an effective elimination of biofilm. No data are available if prolonged exposure might allow complete eradication of biofilm from an implant surface. Furthermore, without this data, it is impossible to identify performance targets for antibiotic carrier materials.

METHODS: Bacterial biofilms were grown on 13 mm titanium-7% aluminium-6% niobium (TAN) discs in Mueller Hinton broth (MHB, Oxoid) which was incubated shaking at 100 rpm at 37 °C for 7 days. Starting inocula was standardised to \(~10^7\) CFU/ml. Once grown, the specimens were then incubated statically at 37 °C in MHB containing vancomycin (source) at concentrations of 0, 100, 200, 500, 1000 and 2000 mg/L. After 0, 7, 14, 21 and 28 days, separate triplicate sets of samples were retrieved for quantification of residual biofilm and compared with untreated controls. From each timepoint, one additional sample disc was taken for scanning electron microscopy. Biofilm from all remaining samples were quantified by sonication and subsequent serial dilution onto MHA plates. Each experiment was performed in triplicate.

RESULTS:

![Figure 1. SEM image of UAMS-1 biofilm after 7 days of incubation at 37 °C. UAMS-1 biofilm completely covers the TAN disc surface (A) x3’000, scale bar 10.0 µm and (B) x10’000, scale bar 5.00 µm.](image)

![Figure 2. Viable CFU counts retrieved from the TAN discs. Treatments were performed for 7 days (d7), 14 days (d14), 21 days (d21) and 28 days (d28).](image)

DISCUSSION & CONCLUSIONS: We have shown that \(S.\) aureus biofilm eradication from TAN implants is possible by vancomycin, if sufficiently high concentrations are achieved for sufficiently long exposure times. However, the duration of exposure required is crucial and may not be achieved by the standard local delivery vehicles [4].

ACKNOWLEDGEMENTS: The authors thank P. Furlong for taking the SEM images.
Titanium-copper-nitride coated implants as a protection against colonizing bacteria

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INTRODUCTION: Implant related infections are caused by bacteria that invade the body peri-OP. Invading bacteria will colonize the implant surface and eventually form a biofilm, causing an infection. In clinical practice, implants often have to be removed in order to treat the infection. By adding a coating with an antimicrobial function to the implant surface, the colonization of the implant surface by bacteria and thus the formation of a biofilm can potentially be reduced. The goal is that the antimicrobial coating containing copper ions as antimicrobial substance does not harm the function of the body cells while maintaining a protection against colonizing bacteria.

METHODS: Animal studies: 5 healthy mature female rabbits weighing over 3.5 kg were used to perform osteosynthesis studies. Osteosynthesis plates half Titanium-Plasma-Spray (TPS) coated and half polished were Physical Vapor Deposited (PVD) with titanium-copper-nitride (TiCuN) with an amount of copper of about 1µg/mm². One plate per animal was fixed via two orthopaedic screws on the lateral side of the left femoral diaphysis.

Bactericidal tests: The tests have been carried out with staphylococcus aureus. Therefore 3 TiCuN-plates were coated with an amount of copper of about 1µg/mm² and were placed in a 10 well-culture-plate and immersed each in 4 ml culture medium with bacteria in a concentration of about 10⁷ cfu/ml and incubated 2, 4, 6, 8 and 24 h at 37°C. After these time points the incubation fluids were removed, the plates were carefully rinsed with 10 ml of sterile PBS-buffer and sonic agitated for 3 min to remove adhering bacteria. Serial dilutions of the rinsed fluid were also plated of mannit agar and incubated 24 h at 37°C to quantify the adhering bacteria.

RESULTS: Animal studies: After removing screws, the plates were fixed at the bone surface and it was hard to separate it from the bone surface due to new ossification below it. After removing the plates for further investigation - one half of contact surface of the plate was rough and the other half was polished, left a prominent impression on the new ossification surface (callus, for example see Fig 1B). Peritoneal new hard tissue formation below and surrounded the plate is visible.

DISCUSSION & CONCLUSIONS: According to macroscopic and histological findings and under the conditions of this study the femoral diaphysis at the implantation area of polished and rough parts in control group and test groups reveal a prominent osteosynthesis in form of lamellar segments filling out the acute area among orthopaedic fixed plate and femoral diaphysis. Copper in defined concentrations is suitable for implementation onto implant surfaces to support both prevention of infection and stimulation of osteogenesis.
In vitro release of cefazolin and vancomycin from three types of impregnated bone chips quantified using UPLC-DAD chromatography

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INTRODUCTION: Antibiotic (AB) -impregnated bonechips from a human morselized femoral head allograft (BCs) are widely used in orthopaedic surgery to prevent or treat infections. Literature suggests that BC are efficient carriers, but due to the diversity in the type of ABS, of BCs and of method used to evaluate AB release, a uniform impregnation protocol has not been draughted so far. In our hospital, a protocol on the use of AB-impregnated BCs was introduced based upon literature data with cefazolin (CFZ) as prophylactic AB and vancomycin (VAN) as documented AB treatment. However, as no studies have been performed with the type of BC used in our hospital and only limited data are available for CFZ, we performed an in vitro study to examine the release of both ABs from these impregnated BCs. Quantification was performed using a fully validated chromatographic method.

METHODS: For CFZ, impregnation with varying concentrations (0,2; 2; 20 and 200 mg/mL), durations (10, 30 and 240 min) and types of BCs (fresh frozen, processed frozen and processed lyophilized bonechips) was performed; for VAN only the impregnation concentration and duration were investigated. After impregnation, BCs were rinsed with saline in order to determine only the absorbed AB. Elution was performed in newborn calf serum at 37°C. Eluted AB concentrations were determined using Ultra Performance Liquid Chromatography – Diode Array Detection (UPLC-DAD). This chromatographic method, enabling measurement of VAN and CFZ, was fully validated according to FDA-criteria. In addition an elution study was performed on the commercially available Osteomycin®, bone chips containing VAN.

RESULTS: CFZ: In vitro experiments suggest that an impregnation concentration of 20 mg/mL is optimal, that AB uptake increases with impregnation time and that frozen BCs release significantly more AB than lyophilized. Frozen BCs impregnated with 20 mg/mL CFZ for 4 hours deliver the desired local concentration (therapeutic window 2 - 200 mg/L) for max. 4 hours, independent of the BC type (fresh frozen vs processed frozen). Fresh-frozen BCs elute significantly more within the first 2 hours. VAN: using processed frozen BCs, an impregnation-concentration of minimum 100 mg/mL during 10 minutes delivers the desired local concentration (therapeutic window 25 - 1000 mg/L) for 3 days. Longer impregnation time at this concentration had no effect. Osteomycin® delivers the desired local concentration for 8 days in our experimental setting.

DISCUSSION & CONCLUSIONS: Literature suggests that freshfrozen BCs can be used as carrier for CFZ [1] and VAN [1,3]. Due to limited knowledge of their release characteristics from processed BCs [2] we performed elution studies on processed BCs compared with fresh-frozen BCs and a commercial product . To facilitate comparison, our protocol was based on that of Mathyssen et al., but with direct quantification of elution concentrations. In contrast to the results of Mathyssen et al., the impregnation concentration had to be increased to 20 mg/mL to reach the desired local concentration which is however reached for only 4 hours. Impregnation with VAN 100 mg/mL during 10 minutes results in a release above the desired concentration for 3 days. Osteomycin® shows a substantially longer elution . Further research is mandatory to obtain better AB-impregnated BC carriers.

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Chemical and morphological surface modification titanium for tissue integration and bacteria adhesion and proliferation prevention

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INTRODUCTION: Bacterial infections are still one of the most serious complications that may impair clinical success and osteointegration of implantable medical devices, causing prosthesis removal and replacement and consequent health and social costs. Although antibiotics are the most used solution, alternative strategy could be surface modification against bacteria adhesion and proliferation. This study presents a surface modification able to improve eukaryotes adhesion (for osseointegration and soft tissue sealing) and to hinder bacteria colonization. An innovative titanium surface modification treatment, based on Anodic Spark Deposition electrochemical technique, was developed and tested using an animal model. Gallium, silicon, calcium, phosphorous and sodium were stably entrapped in an electrochemically thickened and nanostructured titanium oxide. Gallium was considered for its antibacterial properties.

METHODS: The following three materials were tested:
- ASD, Anodic Spark Deposition nanostructured titanium surface, containing Si, P, Ca and P, as described elsewhere [1];
- ASD+Ga, Anodic Spark Deposition nanostructured titanium surface [1], enriched with Gallium ions;
- CRT: acid-etched titanium surface.

The obtained surfaces were EDS characterized and compared by in vivo tests. Titanium rods were placed in the distal epiphysis of femur of twelve skeletally mature outbred male New Zealand white rabbits, considering a bilateral approach. Animals were randomly divided into two groups: normal model (not infected) and experimentally induced peri-implantitis model (inoculation of a severe dose of S. aureus). One and two weeks experimental times were considered. Histological and histomorphometric evaluations were performed on animals after sacrifice. The data were analyzed with variance (ANOVA) and Sidak correction. All analyses were performed with IBM SPSS statistics software v.21. The confidence level was set at 95%.

RESULTS: The surface chemical composition qualitatively detected by EDS analysis show pure titanium for CRT material, while Si, Ca, and P where visible on ASD. ASD+Ga, besides Ca, Si and P show also some Ga presence. In the normal model at one week, ASD and ASD+Ga show histomorphometric parameters comparable to the CTR. On the induced peri-implantitis model, an acute supplicative inflammation was observed. Compared to CTR and ASD, in ASD+Ga the peri-implant tissue was more safeguarded. Histomorphometric results at one week showed a better osseointegration of the surfaces ASD and ASD+Ga compared to CTR. At two weeks, an ASD+Ga surface-induced protective effect was testified by a lower tissue erosion than in CTR and higher than ASD. Moreover, ASD+Ga implants show a peri-implant tissue more preserved from bone degeneration.

DISCUSSION & CONCLUSIONS: A faster primary fixation in ASD and ASD+Ga implants, compared to CTR surface was assessed on the normal model. In peri-implantitis model, ASD+Ga surface was found to provide an osteoprotective action on bone peri-implant tissue at both the considered experimental time. It seems that ASD+Ga surface could enhance osseointegration and fixation and prevent bacteria adhesion and colonization. ASD+Ga treatment it seems to be a promising treatment for next generation implantable medical device.

ACKNOWLEDGEMENTS: This work has been supported by Eurocoating S.p.A. through a grant of Provincia Autonoma of Trento (project “Nemo”).
Development of novel synthetic antimicrobial and antibiofilm peptides (SAAPs) using the thrombocidin-1-derived peptide L3 as a scaffold

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INTRODUCTION: Biomaterial-associated infection (BAI) is a major cause of failure of indwelling medical devices. *Staphylococcus aureus* and coagulase-negative staphylococci are the most common causative agents of these infections. Bacteria can cause infection by either adhering to foreign bodies and subsequent biofilm formation and/or by colonizing the surrounding tissue surrounding these materials. Due to the combined presence of biomaterial and bacteria, the local immune response is compromised, leading to inability of host immune cells to kill phagocytosed bacteria. Treatment of these infections with antibiotics may fail due to the localization of the bacteria, and their low metabolic state. The increasing frequency of antimicrobial drug resistance underscores the need to develop novel antimicrobial agents. Antimicrobial peptides are considered promising candidates.

The EU consortium Biofilm Alliance (BALI) performs multidisciplinary research to design Synthetic Antimicrobial and Antibiofilm Peptides (SAAPs) and a controlled release coating, which can be applied to the surface of biomaterials as a coating. In this study we developed novel SAAPs with optimized *in vitro* antimicrobial and antibiofilm activities using peptide L3, a 15 amino acid peptide (LRCMCIKTTSGIHPK) derived from thrombocidin (TC)-1, the major cationic antimicrobial peptide of human blood platelets, as a scaffold.

METHODS: Based on NRM analyses, various derivatives of L3 were produced by optimizing both the cationic and hydrophobic domains. We determined *in vitro* microbicidal activity against various (antibiotic-resistant) bacterial strains. Moreover, the antibiofilm activity was assessed by incubating the peptides with *S. aureus* in BM2 medium for 24h. The ability of the lead peptide TC19 to prevent *S. aureus* colonization of implants *in vivo* was assessed in the experimental mouse subcutaneous BAI model. The peptide was injected in free form along the titanium implants.

RESULTS: Enhancing the cationicity of the C-terminus by lysine substitutions, or adding hydrophobic residues at the N-terminus improved activity of L3 against *S. aureus*, however, only in low salt buffer. Replacement of both threonine residues by hydrophobic tryptophan residues (i.e. peptide TC19) enhanced the activity in low salt buffer, PBS, as well as in 50% human plasma.

TC19 killed a wide spectrum of Gram-positive and -negative (antibiotic resistant) bacteria at concentrations ranging from 1.6 – 8 μM in PBS. In presence of 50% human plasma the bactericidal concentrations were 2 – 64-fold higher, depending on the tested strain. *S. aureus*-biofilm formation was prevented by TC19 at concentrations of 6.4 – 12.8 μM. TC19 showed low toxicity against human cells. The peptide neutralized bacterial inflammatory molecules, e.g. incubation of lipopolysaccharide (LPS) and UV-killed *S. aureus* with TC19 resulted in a reduction of the production of IL-12 and IL-8 by leukocytes in whole blood. Moreover, TC19 significantly reduced *S. aureus* colonization of titanium implants in the mouse BAI model after 1 day.

DISCUSSION & CONCLUSIONS: The promising characteristics and activity of TC19 indicate a strong potential to prevent biofilm formation. Experiments with TC19 incorporated in a controlled release coating in the mouse BAI model and in a rabbit humerus intramedullary nail infection model are currently undertaken.

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Biocompatible calcium-phosphorous-boron doped titanium coating

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INTRODUCTION: Surface treatments in implants are broadly studied to improve the cell-material interaction and to avoid bacterial attachment and proliferation. Calcium and phosphorous improve the osseointegration whereas boron is a bactericidal agent which participates in the bacteria quorum sense1, 2. It is possible to incorporate elements to titanium implant coatings by plasma electrolytic oxidation (PEO). This work aims to develop a Ca-P-B doped coating over titanium, evaluate its biocompatibility and its bactericidal activity.

METHODS: Titanium grade 4 samples were grounded with #600 SiC paper and ultrasonically cleaned. PEO was carried out at 350 V for 60 s. Calcium acetate and calcium glycerophosphate were used as base electrolyte. In order to evaluate the boron influence other group was oxidized adding disodium tetraborate in base electrolyte. Surface morphology and chemical composition were evaluated by scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS) respectively. Biocompatibility of surfaces was analyzed by measuring fibroblast cells viability with the methyltetrazolium test (MTT) after 1, 4 and 7 days of incubation. Bactericidal activity will be evaluated against Staphylococcus aureus. Unmodified titanium samples were used as control for all the tests.

RESULTS: PEO treated samples shows a homogeneous porous oxide layer, where calcium, phosphorous and boron were incorporated from the electrolyte, as show in Table 1 and Fig. 1. MTT essay showed higher cell viability for Ca-P and Ca-P-B doped samples compared to untreated titanium control, especially after 7 days, Fig. 2. Boron from the Ca-P-B samples could increase the RNA synthesis in fibroblasts cells, showing higher viability than Ca-P samples.

DISCUSSION & CONCLUSIONS: Boron was incorporated without changing the coating morphology. Boron doped coating increased the fibroblast activity. Future bacterial essays will guide to the optimum amount of boron needed to inhibit biofilm formation.

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Fluorescence in situ hybridization as a tool for the evaluation of microparticles-biofilms interaction

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INTRODUCTION: Polymeric microparticles (MP) as drug delivery systems can be a key strategy to enhance the antibiofilm effectiveness of clinically available antibiotics [1]. As a first stage in pharmaceutical development, in vitro proof experiments could be crucial to determine the most promising systems to be further tested by in vivo approaches. Within this context the authors have previously shown the interest of isothermal microcalorimetry, as a real-time, sensitive and accurate method to evaluate the microbiological activity of antibiotic-loaded microparticles [1]. To gain further knowledge on the interaction between MP and bacteria, fluorescence in situ hybridization (FISH) could be an interesting imaging tool [2]. The present study aimed at optimizing FISH parameters to evaluate the effect of polymeric microparticles on staphylococcal biofilms.

METHODS: Mature staphylococcal biofilms (S. aureus ATCC 43300 (MRSA) and S. epidermidis 8400, 1-5×10^8 CFU/mL) were grown on polyurethane pieces of fixed dimensions [3]. Different conditions were tested, namely incubation time, agitation, broth volume and its supplementation with glucose and/or calcium. Characterization of the effect of the microparticles on the biofilm size, structure and metabolic state was performed by FISH, using the pan-bacterial EUB338 FITC and staphylococci-specific STAPHYFITC probes as well as DAPI. Biofilms were incubated for 24 h with poly(methyl methacrylate) (PMMA) microparticles [1].

RESULTS: Table 1 summarizes the optimized parameters. The effect of MP on staphylococcal biofilms is illustrated in Fig. 1.

Table 1. Optimized conditions for in vitro growth of staphylococcal biofilms.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Agitation</th>
<th>Glucose</th>
<th>Calcium</th>
<th>Volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus</td>
<td>(+)</td>
<td>(+)</td>
<td>(-)</td>
<td>0.5</td>
</tr>
<tr>
<td>S. epidermidis</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>0.5</td>
</tr>
</tbody>
</table>

The MP caused an increase in FISH-labeled cells within S. epidermidis biofilms (Fig. 1 bottom right) compared to the control. No such effect was observed in S. aureus biofilms.

DISCUSSION & CONCLUSIONS: The optimal conditions for in vitro growth of staphylococcal biofilms differ depending on the bacterial strain. Both agitation and glucose-supplemented broth were beneficial to the growth of S. aureus and S. epidermidis biofilms. However an inoculum volume lower than 0.5 mL limited biofilm growth. In addition, broth supplementation with calcium has an overall negative effect on S. aureus biofilms, which is in accordance to the literature [4]. In conclusion, it was possible to grow mature staphylococcal biofilms with adequate size and thickness for testing of polymeric microparticles.

Elevated C reactive protein level is an indicator of infection in rabbits in a contaminated fracture model

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INTRODUCTION: Trauma and infection are both considered to lead to a rapid increase of the C-reactive protein (CRP) concentration in blood. Although an increase in CRP is not specific for infection, we wanted to determine if the magnitude or duration of this increase can differentiate infection from trauma in a contaminated rabbit osteotomy model.

METHODS: Twelve skeletally mature female New Zealand White rabbits were used in this study. After a craniolateral approach to the humerus and osteosynthesis with a locking compression plate, a complete mid-diaphyseal osteotomy was created underneath the central empty plate hole, and a Staphylococcus aureus inoculum applied to the surgical site. Six animals received no antibiotic treatment and six received a collagen fleece with gentamicin sulphate over the plate. Serum C-reactive protein was measured (Rabbit CRP ELISA Kit, ICL Inc.) preoperatively and postoperatively at 3hrs, 6hrs, and then daily. All animals were euthanized after 7 days. Statistical differences at different time points between the two groups were analysed using a Mann-Whitney U test.

RESULTS: In untreated animals, the procedure resulted in a 100% infection rate; bone, implant and soft tissue samples were all culture positive, whilst the local antibiotic treatment prevented infection with S. aureus. In all animals, CRP levels peaked between 24 and 72 hours post-surgery (Figure 1).

In the untreated group, the peak reached an average of 757µg/ml and remained elevated at an average of 214µg/ml on day 7. The treated group reached an average peak of 366µg/ml and CRP concentration returned to baseline from day 7.

DISCUSSION & CONCLUSIONS: The average peak CRP value was approximately twice as high in rabbits developing infection, as in the treated rabbits, although this did not reach statistical significance on day one or two.

Surgical trauma and the associated stress alone clearly influence the CRP level at these early time points. The true peak in CRP may have been missed with daily measurements since CRP has a serum half-life of only 4.5 hours. For a more precise determination of the time to reach the peak level and absolute peak level CRP concentration, more frequent samples would be necessary, particularly within the first 72 hours. On day 7 non-infected animals returned to baseline levels whereas the CRP remained elevated in infected animals with an average of 213µg/ml. We can conclude that in this rabbit contaminated osteotomy model, CRP levels on day 7 after surgery are diagnostic for establishment or successful prevention of an infection.
Novel composite material for delayed release of antibiotics

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INTRODUCTION: Beta-tricalcium phosphates (β-TCP) has proved as suitable material concerning mechanically solidity and biodegradability [1]. Scaffolds made of this material show a progressive resorption and a replacement by bone [2]. The aim of this study was to produce a composite of β-TCP, alginate and antibiotics to prolong the release behavior of antibiotics.

METHODS: Using the flow chamber developed by us, porous ceramics in a directional vacuum were filled with alginate of different composition containing 50 mg/ml of antibiotics. After cross-linking the alginate by calcium ions, incubation took place in 10 ml double-distilled water for 4 weeks at 37 °C. At defined times (1, 2, 3, 6, 9, 14, 20 and 28 days), the liquid was completely exchanged and analyzed by capillary zone electrophoresis and microtiter trials. For statistical purposes, the mean and standard deviation were calculated and analyzed by ANOVA.

RESULTS: The release of Vancomycin (VAN) from alginate was carried out via an external calcium source over the entire period with concentrations above the minimal inhibitory concentration (MIC). The burst release measured 35.2 ± 1.5%. We only observed the release of VAN from alginate with an internal calcium source over 14 days. The burst release here was 61.9 ± 4.3%. The release of clindamycin (CLI) from alginate with an external calcium source was detected over the release period. The native alginate’s burst release was 54.1 ± 7.8%; that of the sterile alginate 40.5 ± 6.4%. The alginate from an internal calcium source was released for only 9 days with a burst release of 65.9 ± 3.7%. The microtiter experiments revealed efficacy over the entire study period for VAN. The MIC value was determined in the release experiments as well in a range of 0.5-2.0 µg/ml against Staphylococcus aureus. CLI effectiveness lasted 20 days with values in the range of 0.06 - 0.5 µg/ml against Staphylococcus aureus.

DISCUSSION & CONCLUSIONS: We successfully increased the drug-release time up to 30 days using our novel composite material consisting of a microporous β-TCP ceramic and alginate for the delayed release of antibiotics. Our release-kinetics results from this composite reveal a significant improvement in the duration and antimicrobial activity compared with a previous study’s findings [4]. Even after 30 days we were still detecting microbially-effective concentrations that did not adversely affect cell morphology or cell growth. Our study provides evidence that our novel composite is a viable alternative to traditional methods such as the use of PMMA (Septopal chains), which also comes with the advantage of a degradable carrier and makes a secondary surgical intervention (to remove or replace the non-degradable PMMA carrier) unnecessary. However, further investigation is needed which should consider the in-vivo effects and investigate potential accumulation in the surrounding tissue.

Fig. 1: Release kinetics of VAN out of the composite (N=10) [3].
Bacterial inactivation using a light-activated methylene blue embedded in colloidal materials

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INTRODUCTION: Nowadays colloidal materials with embedded sensitizers represent prospective agents from environmental and medical viewpoints, where a direct use of photoactive solutions appears to be problematic [1-3]. The objective of this experimental study was to analyse in vitro inactivation of Staphylococcus aureus using a LED light-activated antimicrobial agent methylene blue (MB) and MB embedded in clay mineral of saponite type including that modified with dodecylammonium chloride (C12). The purpose was to compare effectiveness of both systems in photodynamic inactivation of bacteria.

METHODS: MB (LOBA, Feinchemie, Austria, $M_r = 319.86$) was used as sensitizer and as a component in the colloidal systems. Synthetic layered silicate of saponite type, with product name Sumecton, was purchased from Kunimine Ind., Japan. Cationic surfactant C12 (chloride salt, $M_r = 263.8$) was obtained from Sigma Aldrich (Germany). Monochromatic red LED light (16.7 W.m$^{-2}$) was positioned just under transparent inoculated 24-well polystyrene plates. Absorption spectroscopy was measured on a Cary 100 UV/VIS Spectrometer (Varian) with water as a reference substance. Reference strain S. aureus CCM 3953 (Czech Collection of Microorganisms, Czech Republic) and clinical isolate S. aureus DHN 18516 (HPL spol. s.r.o., Slovakia) were used in the experiments.

RESULTS & DISCUSSION: The colloidal dispersions effectively absorbed the LED light and upon illumination with visible light, generate singlet oxygen in aqueous solution. This was proved by EPR spectroscopy using a spin trapping agent. Modification of saponite with the C12 surfactant led to the significant enhancement of MB photophysical activity. The presence of C12 cations with disinfectant biocide properties could lead to suppression of dye molecular aggregation. The results of this study suggest that photosensitization with MB embedded in clay minerals using LED light illumination (1.67 mW.cm$^{-2}$, wavelength 576-672 nm and fluence 15 J.cm$^{-2}$) is effective at killing the Gram-positive bacteria. The cell survival (CS) of S. aureus CCM 3953 and S. aureus DHN 18516 were 21 and 25%, respectively. Additionally, complex MB with colloids of saponite–C12 showed higher antibacterial effectiveness on CS of S. aureus than determined for MB pure solution; CS of S. aureus CCM 3953 and S. aureus DHN 18516 were 12 and 21%, respectively. Therefore the choice of the silicate particles and saponite–C12–MB ratio influenced antimicrobial properties of colloidal systems. Another important feature of colloidal material observed was a high adherence of bacteria to these materials what was documented by optical and confocal laser scanning microscopy.

CONCLUSIONS: Experiments revealed that the clay mineral of saponite type with C12 surfactant is the adequate carrier for an immobilization of cationic MB molecules. This study exhibited that the interaction of MB with clay minerals could lead to the formation of new materials with effective antibacterial properties.

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Patterns of bone evolution near infected implants
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INTRODUCTION: Infection is one of the principal causes of orthopedic implant revisions. A dynamic process of bone formation and resorption takes place around an orthopedic implant directly after insertion. Despite its importance, the effect of bacterial infection on the temporal pattern of periprosthetic remodeling is still unknown. The aim of this study was to evaluate the morphological changes of bone adjacent to an implant in the presence of infections caused by various bacterial species.

METHODS: Twenty-four three-month-old female Wistar rats were used in this study. The surgical model is a single screw in the proximal part of the right tibia (Figure 1). Six rats received a sterile screw, six received a screw incubated with Staphylococcus epidermidis (10⁶ CFU), six with Propionibacterium acnes Type IA (10³ CFU), and finally six with P Acnes Type III (10³ CFU). The self-tapping cancellous bone screws were custom made of PEEK containing 20% of BaSO₄ for a better microCT contrast. They were 2mm in outer diameter and 5mm in length.

RESULTS: In the sterile group, we observed a steady BIC, BV/TV and strength increase until day 14 then plateaus. In the P. acnes IA and III, BIC and BV/TV were slightly lower until day 9, but then caught up with sterile specimens. In the S. epidermidis group, we observed a decrease in BIC, BV/TV and strength until day 9, at which time BIC plateaued, but BV/TV and strength increased again to the level of the other groups (Figure 3).

The analysis of bone formation and resorption over time revealed that the differences between groups are only a result of differences in resorption rates, while bone formation is left unaffected.

DISCUSSION & CONCLUSIONS: The initial bacteria populations on the screws were different by three orders of magnitude between the S. epidermidis and the P. acnes groups. This results from the differences in the affinity for PEEK of the two species. Future studies will adjust the inoculation procedure and equalize the bacterial dose between groups. However, the observed patterns are qualitatively interesting. The decrease/increase probably reflects an early osteomyelitis followed by an immune response and bone healing. At the bone level, these differences were only due to changes in resorption rates.
‘Dip and dry’ micropattern-capable bioactive coatings for biomaterial surface modification in treating implant related infection and inflammation

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INTRODUCTION: The demand for implantable medical devices is increasing. However such implants can trigger inflammation and infection at the interface, which left untreated, can lead to implant failure. α-Melanocyte stimulating hormone (αMSH) is an endogenous peptide hormone reported for its potent anti-inflammatory and anti-microbial properties. Our initial work has focused on delivering a synthetic sequence of αMSH to glass using calixarenes [1]. Most recently we are incorporating the peptide on aryl azide functionalised silanes offering an established capability for micropatterning via photolithography [2]. The aim is to provide a direct method in surface biomaterial modification to improve implant viability.

METHODS: For our initial inflammation studies, glass coverslips were coated with calixarene-PEG-3-GKP(D)V. On going work focuses on the biological evaluation of a modified azido-PEG-6-GKP(D)V tether for micropatterning. The KP(D)V peptide sequence was subjected to Gram-positive and Gram-negative bacteria.

RESULTS: XPS and SIMS confirm GKP(D)V was immobilised onto calixarene treated surfaces. Our initial work demonstrates that calixarene-PEG-GKP(D)V coated glass decrease TNF-α stimulated NF-κB activation by 23% ± 4% (n=3, p=0.001) [1]. This is comparable to untreated glass stimulated with both TNF-α and GKP(D)V at 10⁻⁹M (39% ± 3%; n=3, p=0.003). Modified azido-PEG-6-GKP(D)V tether exhibits similar inhibition at 10⁻⁸M (36% ± 4%; n=2, p=0.01) with studies on bacterial viability and the effects of micropatterning on aryl azide silane surfaces are ongoing. The isolated KP(D)V sequence exhibits bactericidal properties over a broad range of concentrations [3]. S. aureus reduction 16% ± 6% at 10⁻⁹M (p=0.026). E. coli reduction 34% ± 7% at 10⁻⁹M (p=0.001).

Fig. 2: KP(D)V reduces viability of S. aureus (a) and E. coli (b); n=3, *p≤0.05, **p≤0.001, ***p≤0.005.

DISCUSSION & CONCLUSIONS: Initial data indicates that the KP(D)V sequence exhibits anti-inflammatory and anti-microbial properties. When tethered by calixarene onto glass surfaces it maintains its anti-inflammatory properties creating viable bioactive surface coatings. By combining our methods of micropatterning with KP(D)V alongside other bioactive substrates we aim to create new multifunctional coatings in treating implant related infection and trauma.

ACKNOWLEDGEMENTS: We thank the BBSRC and EPSRC for funding this work.
**Antibiotic loaded micro porous oxide coatings for titanium-based dental implants**

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**INTRODUCTION:** Microbial colonization on implant surfaces is currently one of the most critical concerns and antimicrobial coatings are being proposed as potential solutions before and after implantation. Biocompatible compounds containing porous oxide coatings formed by microarc oxidation (MAO) process can be designed to act as a reservoir for releasing antimicrobial agents over extended time periods. In the present study, a broad-spectrum antibiotic (Targocid™) was loaded into the pores of the silver containing oxide coating on commercially pure titanium (Cp-Ti) samples in order to inhibit microbial infections on the surface of the biomedical devices.

**METHODS:** Coatings were deposited on Grade 4 quality Cp-Ti samples (10 mm in diameter and 4 mm in thickness). They were subjected to MAO process with an applied voltage of 400 V in an electrolyte containing calcium acetate hydrate, disodium hydrogen phosphate anhydrous and silver acetate. Following oxidation, a group of samples was immersed into a Targocid™ containing solution in a vacuum impregnation system. The morphology and mean elemental composition of the MAO exposed surfaces were evaluated by utilizing scanning electron microscopes (SEM, Hitachi TM-1000, Jeol JSM-6510 and Jeol NeoScope JCM-6000) equipped with energy dispersive spectrometers (EDS). The phase composition of the coatings was identified by conducting X-ray diffraction (XRD, GBC, MMA 027) analysis. Antibacterial activities of the samples were evaluated against Staphylococcus aureus (S. aureus) ATCC®6538p strains.

**RESULTS:** Micro pores were homogeneously distributed on the surface of the oxidized samples while also calcium and phosphorus containing compounds were detected on the surfaces by SEM and XRD analyses. The EDS analyses conducted during SEM surveys confirmed deposition of Ca and P which generated biocompatible compounds identified as HA and CaTiO₃. The results of the disc diffusion antibacterial tests of S. aureus colonies are shown in Fig. 1.

**DISCUSSION & CONCLUSIONS:** Despite high efficiency against bacteria colonization, Ag content of the implant surfaces should tightly be controlled because of its cytotoxicity. Therefore antibiotics assisted silver containing surfaces may improve antimicrobial efficiency without concern of silver cytotoxicity. Antimicrobial assays revealed that antibiotic loaded samples significantly inhibited the growth of methicillin-resistant *Staphylococcus aureus* when compared with the unoxidized samples.

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Release of gentamicin from a thermo-responsive hyaluronan hydrogel in an in vivo contaminated fracture model

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INTRODUCTION: Antibiotic-loaded biomaterials (ALBs) are able to offer proven additional antimicrobial protection beyond that provided by systemically delivered antibiotics [1]. Although the introduction of the first ALB was almost 50 years ago [2], there is still the need for improvement in terms of carrier degradation, release kinetics and handling [3]. In this study gentamicin release from an injectable thermo-responsive (HApNIPAm) hydrogel was compared with a commercially available collagen fleece in vitro and in an infected osteosynthesis model.

METHODS: In vitro release: Collagen fleece (Gentafleece, Resorba Medical (D)) (4 cm² pieces containing 8 mg gentamicin-sulphate) was put in vials containing 8 ml of PBS. For the hydrogel samples, 800 μl of a 15 w/v% HApNIPAm solution in PBS with 1 w/v% gentamicin sulphate (8 mg) were added to 8 ml pre-warmed PBS at 37°C to form cohesive hydrogel. The release was tracked by withdrawing 1 ml of the medium at every time-point and replacing it with an equal volume of PBS. Each condition was repeated 3 times.

In vivo release: Twenty New Zealand White Rabbits received a defined inoculum of Staphylococcus aureus into an osteotomy of the humerus, fixed with steel fixation plates. Ten rabbits were treated with collagen-fleece with gentamicin and 10 rabbits were treated with 800 μl of a 15 w/v% HApNIPAm solution with 1 w/v% gentamicin sulphate (8 mg gentamicin-sulphate). Gentamicin in PBS was measured by an o-phthaldialdehyde colorimetric assay and using an immunoassay method for the bloodplasma samples.

RESULTS: In vitro collagen fleece and the HApNIPAm hydrogel show an initial burst release of gentamicin (Fig 1). After 6 h, the cumulative release for both is similar, at approximately 75% release. The remaining 25% is more gradually released over the next 90 h for both collagen and HApNIPAm. Peak serum concentrations in vivo were seen after 3 h and were below 3 μg/ml for collagen fleece and below 1.5 μg/ml for HApNIPAm (Fig 2). Gentamicin concentration fell below the detection limit at 96 h for HApNIPAm and 168 h for Col-Gen.

DISCUSSION & CONCLUSIONS: In vivo quantification of gentamicin levels is complicated by the short half-life (t1/2=1h) of gentamicin in rabbits, and since administration is locally, only a small fraction of the total antibiotic enters the bloodstream. Nonetheless, systemic toxicity is not a risk factor for both tested ALBs.

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Preparation and antimicrobial activity of novel porous collagen/bioactive nanocomposite biomaterials

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INTRODUCTION: The design of new antimicrobial biomaterials is a challenge in the current material science. Collagen based biomaterials are of interest for tissue engineering, surface coatings, difficult healing wounds dressing, etc. Aim of this study was the preparation and antimicrobial activity evaluation of porous antimicrobial collagen based hybrid biomaterials employing novel antimicrobial nanocomposites.

METHODS: The following, prepared by us, nanocomposites were used in this study as antimicrobial agents: 1) Zinc Titanate (ZnTiO\(_3\)); 2) Ag/ZnO/ZnTiO\(_3\) nanocomposite; 3) reduced graphene oxide (RGO); 4) Ag/graphene nanocomposite; 5) Ag/Si/graphene nanocomposite; 6) graphene/Si nanocomposite; 7) ZnTiO\(_3\)/Si nanocomposite; 8) ZnTiO\(_3\)/Si/graphene nanocomposite.

Fibrillar collagen/antimicrobial agent porous hybrids were prepared by adsorption of the antimicrobial agent from suspension during the fabrication of a porous collagen matrix as described in [1].

The test bacterial strains: *C. lusitaniae* 74-4, *S. enterica* 2333, *E. coli* 264, *P. putida* 1090, *S. epidermidis* 3486, *B. cereus* 1095 and *L. monocitogenes* were cultured in the most suitable for each one media.

The cytotoxicity was evaluated by microscopic observation of the osteoblast cells morphology, MTT and LDH assay.

RESULTS: The antimicrobial activity (sterile zone, mm) is specific for each species as it is evident form Table 1.

The cell morphology and LDH assay indicate varying and specific toxicity of the antimicrobial samples although their significantly higher total cellular activity (MTT assay) as compared to that of the control (porous collagen matrix without antimicrobial agent).

DISCUSSION & CONCLUSIONS: Some samples show very strong antibacterial and antifungal activity and are promising as antinfective biomaterials.

Optimal balance between antimicrobial activity and toxicity could be achieved by speculation with the antimicrobial agent concentration.

Delivering antimicrobial agent, corresponding to the infection causing microbial, such collagen based composites improve the performance and enlarge the application of conventional collagen bio-materials in bone tissue regeneration, antimicrobial coatings to orthopaedic implants, surgery drains, wound dressing, etc.

ACKNOWLEDGEMENTS: Scientific Fund, Bulgaria (grant No. DDVU 02/100/20.12.2010.) and COST Action TD1305 are gratefully acknowledged for their financial support.

Table 1. Antimicrobial activity of the collagen/antimicrobial agent (1-8) porous composite as sterile zone, in mm, around the piece of material

<table>
<thead>
<tr>
<th>Sample</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
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<tr>
<td><em>C. lusitaniae</em></td>
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<td>4.0</td>
<td>17.0</td>
<td>4.0</td>
<td>14.0</td>
<td>0</td>
<td>0</td>
<td>4.0</td>
</tr>
<tr>
<td><em>S. enterica</em></td>
<td>0</td>
<td>1.25</td>
<td>0</td>
<td>0.25</td>
<td>1.0</td>
<td>0</td>
<td>6.0</td>
<td>0</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>2.5</td>
<td>7.5</td>
<td>0</td>
<td>4.0</td>
<td>3.5</td>
<td>0</td>
<td>9.0</td>
<td>1.0</td>
</tr>
<tr>
<td><em>P. putida</em></td>
<td>0</td>
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<td>0</td>
<td>0</td>
<td>2.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
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<td>2.5</td>
<td>0</td>
<td>0</td>
<td>2.0</td>
<td>0</td>
<td>15.0</td>
<td>0</td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td>3.5</td>
<td>2.0</td>
<td>0</td>
<td>1.0</td>
<td>2.0</td>
<td>0</td>
<td>4.5</td>
<td>0</td>
</tr>
<tr>
<td><em>L. monocitogenes</em></td>
<td>1.0</td>
<td>1.0</td>
<td>0</td>
<td>1.0</td>
<td>2.5</td>
<td>0</td>
<td>0</td>
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</tr>
</tbody>
</table>
Osteocyte participation in the response to orthopaedic surgery-related Staphylococcus aureus infection

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INTRODUCTION: Total hip replacement (THR) is a principal surgical procedure for advanced osteoarthritis (OA). However, peri-prosthetic bacterial infection following surgery can result in the need for revision surgery. In other tissues, localised bacterial infections activate innate immune responses, resulting in the release of mediators such as pro-inflammatory chemokines and cytokines. In the context of bone, some pro-inflammatory cytokines are known to stimulate osteoclast formation and activity. However, the effect of bacterial infections on the osteocyte, the most numerous bone cell type and which plays key roles in the regulation of physiological bone remodelling, remains unclear. In this study, we are examining bone biopsies from infected patients and osteocyte responses to experimental infection.

METHODS: Bone samples were collected from patients undergoing either THR for primary OA or revision THR for post-operative infection, with informed written patient consent. Swabs from each infection case indicated S. aureus infection. Small trephine bone biopsies were collected from the acetabulum and from the iliac wing as a control. Bone samples were rinsed in PBS and homogenised in TRIzol reagent for the extraction of total RNA and the analysis of gene expression by real-time RT-PCR. Human primary osteocyte-like cells were generated by long-term culture under differentiating conditions\textsuperscript{(1)} and exposed acutely to S. aureus at varying ratios of colony forming units/cell.

RESULTS: Gene expression analysis of infected patient bone biopsies indicated elevated relative expression of SOST mRNA, compared with primary OA bone samples harvested from corresponding anatomical sites (Fig. 1).

DISCUSSION & CONCLUSIONS: Our preliminary analyses have revealed the acetabular bone from patients with post-operative S. aureus infection has significantly increased expression of the osteocyte-related gene SOST, in comparison to bone biopsies from non-infected controls, consistent with our previous finding of pro-inflammatory cytokine-driven SOST/sclerostin expression in human osteocytes and bone\textsuperscript{(2)}. S. aureus exposed osteocytes also increased their expression of SOST, as well as chemokines typical of the innate immune response. Since increased sclerostin expression is associated with bone formation inhibition and bone loss, these findings suggest that in the situation of orthopaedic infection, osteocytes may contribute to implant loosening.

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Antibacterial silver-doped nanoscale hydroxyapatite paste: an injectable bone graft substitute

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INTRODUCTION: Bone infections remain a major clinical problem associated with biomaterial implantation. Currently, no bone augmentation materials are capable of stimulating bone regeneration whilst eliminating bacterial growth. Silver has been considered for use to prevent medical infections due to its’ broad spectrum of antimicrobial activity. Recent evidence suggests that nanoscale hydroxyapatite (nHA) has potent osteoconductive properties, with several nHA pastes now available on the market. Therefore the addition of silver ions to a nHA paste may present a promising new strategy for combating bone infections. The aim of this project was to synthesise a silver-doped nHA paste and assess its’ materials characteristics and antibacterial capabilities.

METHODS: A modified wet precipitation method¹ was used to prepare a range of silver-doped nHA pastes (0, 2, 5 and 10 mol. %). nHA suspensions were dried until a paste of approximately 85 % water content was achieved. Powdered samples were analysed using x-ray diffraction (XRD), x-ray fluorescence (XRF) and transmission electron microscopy (TEM). The powders were also heat treated at 1000 °C in order to further assess their material characteristics. The antibacterial activities of the pastes were then tested against Staphylococcus aureus and Pseudomonas aeruginosa using agar diffusion and broth dilution methods.

RESULTS: nHA was successfully produced in all silver-doped samples. The amount of silver incorporated into the product increased with the amount of silver used in the synthesis method. The silver-doped nHA had a lower thermal stability demonstrated by an increasing amount of β-tricalcium phosphate following thermal treatment at 1000 °C. Silver-doped pastes displayed antibacterial activity against S. aureus and P. aeruginosa in a dose dependent manner. The level of silver detected in the agar increased with the silver-dopant level of the paste.

![Fig. 1: Agar diffusion assay for 0 and 10 mol. % silver-doped nHA paste. Scale bar = 0.5 cm.](image)

DISCUSSION & CONCLUSIONS: The antibacterial activity of the silver-doped nHA paste was at least partially due to the action of diffusible silver ions. For the suspension cultures, a reduction in bacterial growth observed for the sample without silver doping may be due to the adherence of the bacteria to the nHA particles and subsequent precipitation. Nevertheless, decreased bacterial growth was observed in suspension culture for the silver-doped samples with the 10 mol. % silver-doped paste inhibiting growth the most, followed by the 5 and 2 mol. % pastes respectively. Therefore silver-doped nHA paste may be considered as a novel biomaterial which has the potential to reduce bone infections rates whilst encouraging optimal bone healing.

ACKNOWLEDGEMENTS: The authors would like to thank Ceramisys Ltd. and the EPSRC for funding this project.