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BONE INFECTION: A CLINICAL PRIORITY FOR CLINICIANS, SCIENTISTS AND EDUCATORS

T.F. Moriarty[1,*], G. Muthukrishnan[2], J.L. Daiss[1], C. Xie[2], K. Nishitani[1], Y. Morita[2], H. Awad[2], K.L. de Mesy Bentley[2], E. Masters[2], T. Bui[2], M. Yan[2], J. Owen[3], B. Mooney[2], S. Gill[2], J. Puetzler[4], J.C. Wenke[5], M. Morgenstern[6], W-J. Metsemakers[7,10], C. Noll[8], A. Joeris[9], R.G. Richards[1], E.M. Schwarz[2] and S.L. Kates[3]

[1] AO Research Institute Davos, Davos, Switzerland
[2] Center for Musculoskeletal Research, Department of Orthopaedics and Rehabilitation, University of Rochester Medical Center, Rochester, NY, USA
[3] Department of Orthopaedic Surgery, Virginia Commonwealth University, Richmond, VA, USA
[4] Clinic for General and Tumour Orthopaedics, University Hospital Muenster, Muenster, Germany
[5] Orthopaedic Trauma Research Department, U.S. Army Institute of Surgical Research, San Antonio, TX, USA
[6] Center for Musculoskeletal Infections, Department of Orthopaedic and Trauma Surgery, University Hospital Basel, Basel, Switzerland
[7] Department of Trauma Surgery, University Hospitals Leuven, Leuven, Belgium
[8] Institute of Medical Microbiology, University Medical Center Göttingen, Göttingen, Germany
[9] AO Innovation Translation Center, AO Foundation, Dübendorf, Switzerland
[10] KU Leuven - University of Leuven, Department of Development and Regeneration, Leuven, Belgium

Abstract

Bone infection has received increasing attention in recent years as one of the main outstanding clinical problems in orthopaedic-trauma surgery that has not been successfully addressed. In fact, infection may develop across a spectrum of patient types regardless of the level of perioperative management, including antibiotic prophylaxis. Some of the main unknown factors that may be involved, and the main targets for future intervention, include more accurate and less invasive diagnostic options, more thorough and accurate debridement protocols, and more potent and targeted antimicrobials. The underlying biology dominates the clinical management of bone infections, with features such as biofilm formation, osteolysis and vascularisation being particularly influential. Based on the persistence of this problem, an improved understanding of the basic biology is deemed necessary to enable innovation in the field. Furthermore, from the clinical side, better evidence, documentation and outreach will be required to translate these innovations to the patient. This review presents the findings and progress of the AO Trauma Clinical Priority Program on the topic of bone infection.

Keywords: Fracture-related infection, *Staphylococcus aureus*, biofilm, osteomyelitis, canaliculi, hydrogel.

***Address for correspondence**: T. Fintan Moriarty, AO Research Institute Davos, Clavadelerstrasse 8, 7270 Davos Platz, Switzerland.
Telephone number: +41 814142397 Email: fintan.moriarty@aofoundation.org

List of Abbreviations

AIGEC	Anti-Infection Global Expert Committee
ALBC	antibiotic-loaded bone cement
Amd	amidase
ASC	antibody-secreting cell
AUC	area under the curve
BMI	body mass index
CaP	calcium phosphate
CFU	colony-forming unit
CHIPS	chemotaxis inhibitory protein of *S.aureus*
CoNS	coagulase-negative staphylococci
CORS	Canadian Operational Research Society
CPP	Clinical Priority Program
CPS	calcium phosphate scaffolds
CRP	C-reactive protein
DAIR	debridement, antibiotic treatment and implant retention
DFI	diabetic foot infections

EBJIS	European Bone and Joint Infection Society
ESR	erythrocyte sedimentation rate
ESWT	extracorporeal shockwave therapy
FDA	Food and Drug Administration
FRI	fracture-related infection
FST	foot salvage antimicrobial therapy
G-CSF	granulocyte-colony stimulating factor
Gmd	glucosaminidase
ICM	international consensus meetings
IL	interleukin
IsdB	iron-regulated surface determinant protein B
KC	keratinocyte chemoattractant
mAb	monoclonal antibodies
MALDI-TOF	matrix-assisted laser desorption/ ionisation time-of-flight MS
MBEC	minimum biofilm eradication concentration
MENSA	medium enriched for newly synthesised anti-*S. aureus* antibodies
MIC	minimum inhibitory concentration
MRSA	methicillin-resistant *S. aureus*
MS	mass spectrometry
MSIS	MusculoSkeletal Infection Society
NOD-SCID	nonobese diabetic/severe combined immunodeficiency
NSG	NOD-SCID gamma
OLCN	osteocyte lacuno-canalicular network
ORS	Orthopaedic Research Society
OTA	Orthopaedic Trauma Association
PJI	periprosthetic joint infection
PLGA	poly(lactic-co-glycolic acid)
PMMA	polymethyl methacrylate
S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*
SEM	scanning electron microscopy
SSI	surgical site infection
T2D	type 2 diabetes
TEM	transmission electron microscopy
WBC	white blood cell

Introduction

Bone infection comprises a range of related, yet significantly distinct clinical fields including FRI, PJI, septic arthritis and diabetic foot osteomyelitis, amongst others. The burden of bone infection is often associated with high-risk populations – such as open fractures, immunocompromised patients and patients undergoing revision surgery. However, a bone infection may also occur in patients without any remarkable comorbidity or risk factor and is a potential outcome for any patient receiving an orthopaedic device. Regardless of the underlying reasons, bone infection can have significant consequences for the patient and can be a challenging complication for the multidisciplinary team of microbiologists, infectious-disease physicians and orthopaedic-trauma surgeons involved in caring for such a patient (Fig. 1). In recent years, consensus opinions derived from international expert panels have emerged to provide guidance on best practice in prevention, treatment and diagnosis, and advance from the previous eminence-based practice. This has been a welcome development for the field that promises to provide a framework for future prospective studies and internationally accepted evidence-based treatments in the coming years.

The persistence of bone infections, despite best practice, suggests that current concepts and interventions can be further improved. Some of the innovations that have relatively recently reached the clinic include biomaterials such as antibiotic-loaded synthetic bone substitutes (Pesch *et al.*, 2020), implant coatings (Metsemakers *et al.*, 2015), mechanical innovations such as the reamer irrigator aspirator (Tosounidis *et al.*, 2016), diagnostic innovations such as the alpha defensin assay (Marson *et al.*, 2018) and a small number of clinical trials on immunisation against *S. aureus* that remain either unpublished or without confirmed efficacy (Proctor, 2015). The penetration of some of these innovations in the clinic, and their impact, are yet to be determined, although significant further advances will certainly be required in the coming decades. Amongst the few to have reached the clinic, there is the alpha defensin test, which has been applied in a lateral flow format and used in synovial fluid for the detection of PJIs. To date, results suggest it has value, although it may not be superior to existing measures such as WBC and neutrophil count (Ivy *et al.*, 2021).

Targets for further research should include i) the inability to develop an effective immunisation strategy against the most common pathogen in bone infection, *S. aureus*; ii) antimicrobial resistance, which may render even current standards such as systemic and local antibiotic therapy increasingly ineffective; iii) the inability to target biofilm-growing bacteria or bacteria residing within the osteocyte-lacuno canalicular system. Whether these issues will eventually be addressed by improved diagnostics or surgical resection, *e.g.* debridement tools more potent and fit-for purpose antimicrobials or a combination thereof, remains to be seen. Importantly, translation to the clinic will require a balance between responsible use of antibiotics and convincing clinical data on potential to improve patient care. Despite the numerous options currently available in the clinic, the patients' needs are not fully met. The consequence is the continued and widespread use of "home-made solutions" using antibiotics in an off-label manner such as the application of antibiotic powders directly to the wound, irrigation with antibiotic solutions and self-fabricated antibiotic delivering spacers that are the best available option, though far from ideal and frequently based on a limited amount of scientific evidence.

The combined potential of basic science, clinical science and industry seems, nevertheless, well placed to make progress in the coming decades. The present review aims to provide an overview of new areas of research identified by expert consensus committees that will be required to impact patient care. Furthermore, the AO Trauma CPP on bone infection was convened to tackle this clinical need and the scientific outputs of this surgeon-driven, science-based consortium are also described.

New concerns from ICMs

In the 20th century, clinical treatment protocols for infection in orthopaedics were established by innovators responding to urgent needs and many of these protocols became "standard of care" without level 1 evidence to support them. While most of these protocols have proven their worth from clinical experience, the new era of value-based healthcare policies that demands evidence-based medicine has changed the cost-effectiveness of some, which have been the focus of transformative ICMs (Schwarz *et al.*, 2020; Schwarz *et al.*, 2019). At the commencement of the AO Trauma CPP on bone infection, one of the most controversial issues was the upper limit of BMI threshold for elective surgery, which provoked strong arguments between delegates to the point that the question was removed without an official vote at the 2013 ICM. However, this stimulated high level peer-reviewed research that facilitated "unanimous" agreement at the 2018 ICM, which voted that there is "strong" and "consensus" evidence that "a substantially increased risk is noticed in patients with a BMI > 40 kg/m^2 and the risks of surgery must be carefully weighed against its benefits in these patients" (Schwarz *et al.*, 2019). The 2018 ICM also revealed other major points of contention, the main one being the absence of a functional definition of "acute" and "chronic" infection with which to guide clinical decision (*i.e.* retention or removal of hardware), which could not be resolved by the general assembly or the biofilm workgroup (Saeed *et al.*, 2019). In support of modifying guidelines to define "acute" *versus* "chronic" infection based on histopathology features (neutrophils *vs.* macrophages and plasma cells respectively), the AO Trauma CPP published clinical data demonstrating that these features can co-exist in the same bone biopsy (Masters *et al.*, 2019b). The AO Trauma CPP also responded to several of the 38 "high-priority" research questions identified by the 2018 ICM (Schwarz *et al.*, 2019), including the efficacy of ALBC. A focussed ICM was held on this specific topic, highlighting several major concerns with the way ALBC is used (Schwarz *et al.*, 2020). The first is that there is no level 1 evidence demonstrating its proven efficacy to prevent or treat bone infections. The second is that the classical MIC used in an *in vitro* assay to evaluate and compare antibiotics has little if any value for clinical translation of novel therapies for established biofilm infections or those treated locally. Moreover, new assays to evaluate the MBEC that account for bacteria killing time (Castaneda *et al.*, 2016), biofilm age (Holmberg and Rasmussen, 2016) and host factors such as plasma and haeme (Cardile *et al.*, 2014) and can be translated to the clinical microbiology laboratory are needed. The third is that commonly used antibiotics in ALBC (*e.g.* gentamicin) can potentially lead to resistant

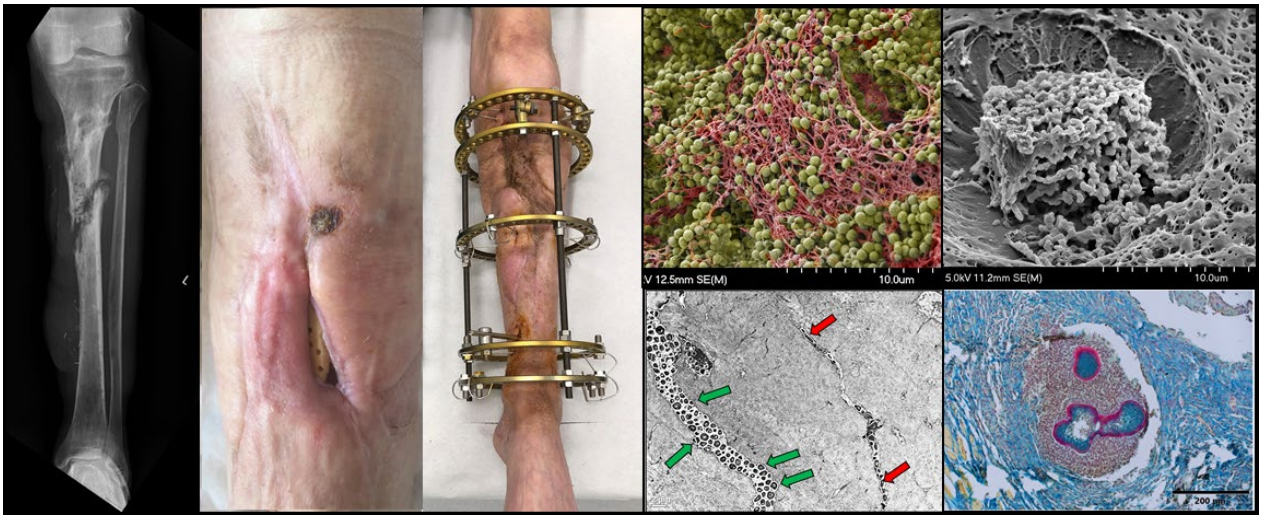

Fig. 1. Key phenomena in bone infection from the clinical and basic science perspective. Left-hand side, macroscopical images of an FRI. From left, radiographic image of a patient with an FRI revealing healing complications and periosteal reaction. Image reveals simultaneous failure of soft-tissues healing, revealing the pale avital bone through the unhealed soft tissue wound. Fixation of the fracture after treatment may require invasive procedures and prolonged presence of fixation devices, such as a ring fixator. Right hand-side, microscopical images of the basic biological features involved in bone infection as revealed by *in vitro* and laboratory animal studies. Upper left, an *in vitro* grown biofilm of *S. aureus*, including extracellular matrix and fibrin (red). Upper right, *Cutibacterium acnes* present in an osteocyte lacuna in a rabbit; lower left, *S. aureus* propagating through the OLCN in an infected bone; lower right, a staphylococcal abscess community in the bone marrow of an infected mouse.

isolates emerging (Neut *et al.*, 2003). These concerns serve as prime targets for future interventions.

Multidisciplinary approaches to address the clinical problem of bone infection

It seems unlikely that significant advances can be made without interdisciplinary collaboration across scientific, clinical and industrial stakeholders. From a basic-science perspective, improved understanding of the basic biology of bone infection will absolutely be needed to identify new targets that can prevent and treat bone infections. From a clinical perspective, the development of bone infection registries, collecting real-world data on a large scale as well as high-quality clinical studies to separate the interventions that have efficacy from those that do not are key to widespread adoption of new technologies. The AO Trauma CPP was established in a ground-up approach, whereby an obvious clinical problem required input from an interdisciplinary consortium. The structure of the consortium included careful consideration of the basic-science problems as well as clinical evidence and patient materials needed to provide a better understanding of the clinical problem. Finally, the structure included educational and outreach aspects to ensure best practices were presented to the medical and scientific community and ensure that the benefits of scientific advances could be moved from theory into practice. The following sections present the scientific highlights of the programme as well as educational aspects used to further improve current best practice.

Host-pathogen interactions in bone infection

Invasion of bone canaliculi by *S. aureus*

Recently, the understanding of the pathophysiology of bone infections has evolved arising from TEM imaging studies in infected bone of both human and murine origin. Historically, *S. aureus* was defined as 1 μm-diameter cocci incapable of motility. However, TEM studies have shown deformed and propagating rod-shaped bacteria, ranging in diameter between 0.2 to 0.8 μm, occupying the sub-μm OLCN in live cortical bone (Fig. 2) (de Mesy Bentley *et al.*, 2018; de Mesy Bentley *et al.*, 2017). Initiation of OLCN invasion is accomplished through asymmetric binary fission of daughter cells, proliferating at the leading edge while utilising demineralised collagen as a nutrient source. Through careful observation and serial sectioning, this deformed elongated shape was confirmed in both murine (de Mesy Bentley *et al.*, 2017) and human (de Mesy Bentley *et al.*, 2018) bone, proving not to be an artefact of sectioning, and was recently also reported by others (Zoller *et al.*, 2020).

Further studies have shown that *S. aureus* also infects living osteocytes and forms small colony variants in this niche *in vitro*; moreover, infection of osteocytes has also been shown in human patients (Yang *et al.*, 2018). As each osteocyte has approximately 40-50 canaliculi (Beno *et al.*, 2006; Tiede-Lewis *et al.*, 2017), these cells may effectively serve as OLCN "hubs" for continued bacterial invasion to neighbouring and distant osteocytes within bone tissue and may be a key factor in infection persistence.

The authors' hypothesis was that *S. aureus* invasion of the OLCN is driven by a novel genetic mechanism that enables bacterial cell deformation and propagation through canaliculi. To determine *S. aureus* genes involved in this novel phenotype, an *in vitro* platform was created featuring a nano-porous membrane to mimic the size and geometry of canalicular openings. Using this platform, *S. aureus* strains with mutations in genes hypothesised to be involved in OLCN invasion were screened for their ability to propagate through nanopores (Masters *et al.*, 2019a). This study identified the *S. aureus* gene *pbp4*, encoding an uncanonical cell-wall transpeptidase (da Costa *et al.*, 2018), as necessary for *S. aureus* deformation and propagation through 0.5 μm pores *in vitro*. Subsequent *in vivo* studies of implant-associated bone infection have proved that *pbp4* deletion results in decreased peri-implant bone loss due to decreased osteoclast activation as well as complete loss of OLCN invasion (Masters *et al.*, 2020). While continued research is required to completely describe the mechanism of *S. aureus* OLCN invasion, genes identified by Masters *et al.* (2020) represent possible targets for the development of novel antimicrobials to treat infections.

Fracture stability and infection

One of the unique features of FRIs that is not present in PJI, for example, is the role of biomechanical stability and fracture healing. Fracture healing has often been described using the "diamond concept", which includes osteogenic cells, an osteoconductive scaffold, growth factors and biomechanical stability (Giannoudis *et al.*, 2007). Immune responses have more recently emerged as an additional component of fracture healing, whilst simultaneously being crucial for defence against pathogens. Recently, Sabaté-Brescó *et al.* (2017b) have shown that a lack of biomechanical stability leads to a delay in clearance of *S. epidermidis* from bone osteotomies in mice. This supports, in general terms, the long-standing teaching that stability is required to reduce infection risk (Foster *et al.*, 2020c). This may be due to improved bone healing and protection of vascularisation under optimal stability but also inflammation and vascular damage under conditions of instability. Data suggested that fracture instability leads to a local increase in inflammatory cytokines such as G-CSF, KC and IL-6 and that this is exacerbated in the presence of *S. epidermidis* (Sabaté-Brescó *et al.*, 2021). *S. aureus* infections result in a much more severe inflammatory response, associated with increased secretion of pro-inflammatory cytokines and a rapid loss of stability, once again highlighting the significant differences in pathogenesis between

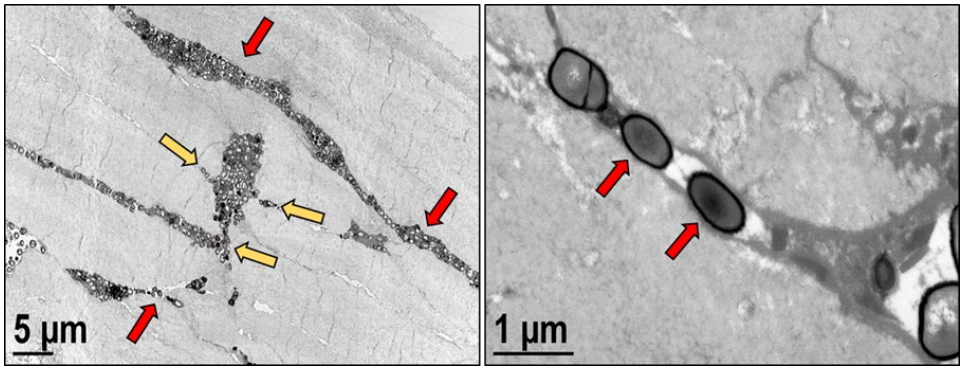

Fig. 2. TEM imaging of *S. aureus* invasion into the OLCN. Left: an osteocyte (yellow arrows) has been killed by *S. aureus* that have invaded into its lacunar space. Note, deformed bacteria are infiltrating into canaliculi emanating from the lacunar space (tips of yellow arrows). The OLCN connection between osteocytes facilitates continued invasion into neighbouring and distant osteocytes (red arrows) (×3,500). Right: a TEM image displaying *S. aureus* cocci deformation to accommodate the submicron space of an osteocyte's canaliculus (×30,000).

S. epidermidis and *S. aureus* (Sabaté-Brescó *et al.*, 2017a). This study offers an obvious link between surgical practice, stabilisation options and FRI risk and provides a mechanistic insight into long-standing clinical teaching based on clinical experience.

Impact of obesity and T2D on implant-associated osteomyelitis

Among other risks, obesity/T2D increase susceptibility to bone infections following orthopaedic surgery (Dowsey and Choong, 2009; Jamsen *et al.*, 2012; Wu *et al.*, 2014), not including ulcerative soft tissue infections that spread to underlying bone. Earlier data indicates that persistent osteomyelitis occurs in cases of obesity/T2D due to two concurrent mechanisms: 1) response of *S. aureus* to the diabetic-host microenvironment; 2) impairment of the immune responses due to the chronic inflammation (Farnsworth *et al.*, 2017). *S. aureus* upregulates fibrinogen adhesion proteins to exploit the increased fibrinogen levels circulating in the obese, diabetic host to initiate and propagate fibrin-encapsulating abscesses, which facilitate evasion of immune cells (Bembde, 2012; Farnsworth *et al.*, 2017; Kannel *et al.*, 1990). Obese/T2D hosts have also been historically characterised to have weakened neutrophil functions including adhesion, migration and phagocytosis, as shown in *ex vivo* experiments (Alba-Loureiro *et al.*, 2006; Delamaire *et al.*, 1997; Gallacher *et al.*, 1995; Kuwabara *et al.*, 2018). However, *in vivo* data from obese/T2D host foot ulcer and implant-associated mouse models suggest that innate immune cells are highly recruited to the site of infection despite diminished clearance of *S. aureus* (Farnsworth *et al.*, 2018a; Farnsworth *et al.*, 2018b). Continued recruitment of innate immune cells to infected bone during chronic infection suggests sustained inflammation that could inhibit normal bone repair processes, as indicated by increased periosteal reactive bone formation and enhanced osteolysis in obese/T2D hosts (Farnsworth *et al.*, 2018b). Functional deficits in both innate and adaptive immunity likely contribute to increased infection severity and susceptibility. Decreased T cell and B cell activation is associated with reduced production of *S. aureus*-specific IgG (Farnsworth *et al.*, 2018a; Farnsworth *et al.*, 2015). Immune-impairing hyperinflammation in the obese/T2D host is also linked to other complications of this disease, including liver dysfunction, islet inflammation and gut dysbiosis, which appear to be primarily mediated by obesity (Belkaid and Hand, 2014; Lumeng and Saltiel, 2011; Thingholm *et al.*, 2019). Thus, targeting the causes of inflammation in obese/T2D patients, such as the well-recognised gut dysbiosis, opens promising new avenues for decreasing susceptibility to the more severe implant-associated osteomyelitis and other complications in this disease population.

Host immunity against *S. aureus*: protective *vs.* susceptible immune proteomes

In a cohort of patients with bone infection, IsdB, a haeme-iron scavenging surface protein, was identified as the most immunodominant antigen during *S. aureus* bone infection (Nishitani *et al.*, 2015a). Remarkably, patients with high titres of circulating antibodies against IsdB were more likely to die from infection (Nishitani *et al.*, 2015a), indicating their role as susceptibility enhancement antibodies. That result was consistent with the failure of the phase IIB/III clinical trial of an IsdB active vaccine (Merck's V710), which was associated with an increased mortality rate due to sepsis in patients with SSI (Fowler *et al.*, 2013). Preclinically, a trojan-horse macrophage theory showed that anti-IsdB antibodies can facilitate *S. aureus* internalisation and survival in macrophages *in vitro* and mediate *S. aureus* dissemination to distal organs *in vivo*, indicating that *S. aureus* invades host macrophage using anti-IsdB antibodies and proliferates and disseminates in an immune-privileged environment (Nishitani *et al.*, 2020). In sharp contrast to anti-IsdB antibodies, antibodies against the Gmd protein

subunit of *S. aureus* autolysin were identified as protective antibodies utilising murine-osteomyelitis models (Varrone *et al.*, 2014; Yokogawa *et al.*, 2018). In a clinical study within the AO Trauma CPP Bone Infection Registry, it was found that only 6.7 % of osteomyelitis patients had high levels of circulating anti-Gmd antibodies and these high levels of anti-Gmd were associated with a nearly 3-fold increase in infection-control odds, suggesting that anti-Gmd passive immunisation therapy for osteomyelitis might be beneficial and a potential target for future intervention (Kates *et al.*, 2020b).

Development of a passive immunisation for *S. aureus* osteomyelitis

While vaccines are the most cost-effective intervention for infectious diseases, to date, all efforts to develop an immunisation against *S. aureus* have failed for various reasons (Proctor, 2015). The main challenges include lack of knowledge of protective *versus* antibody-dependent bacterial infection enhancement and the great variability in patient-specific immune responses, which are biased by prior exposures to this commensal (Muthukrishnan *et al.*, 2019a; Ricciardi *et al.*, 2018; Ricciardi *et al.*, 2020b). With this knowledge,

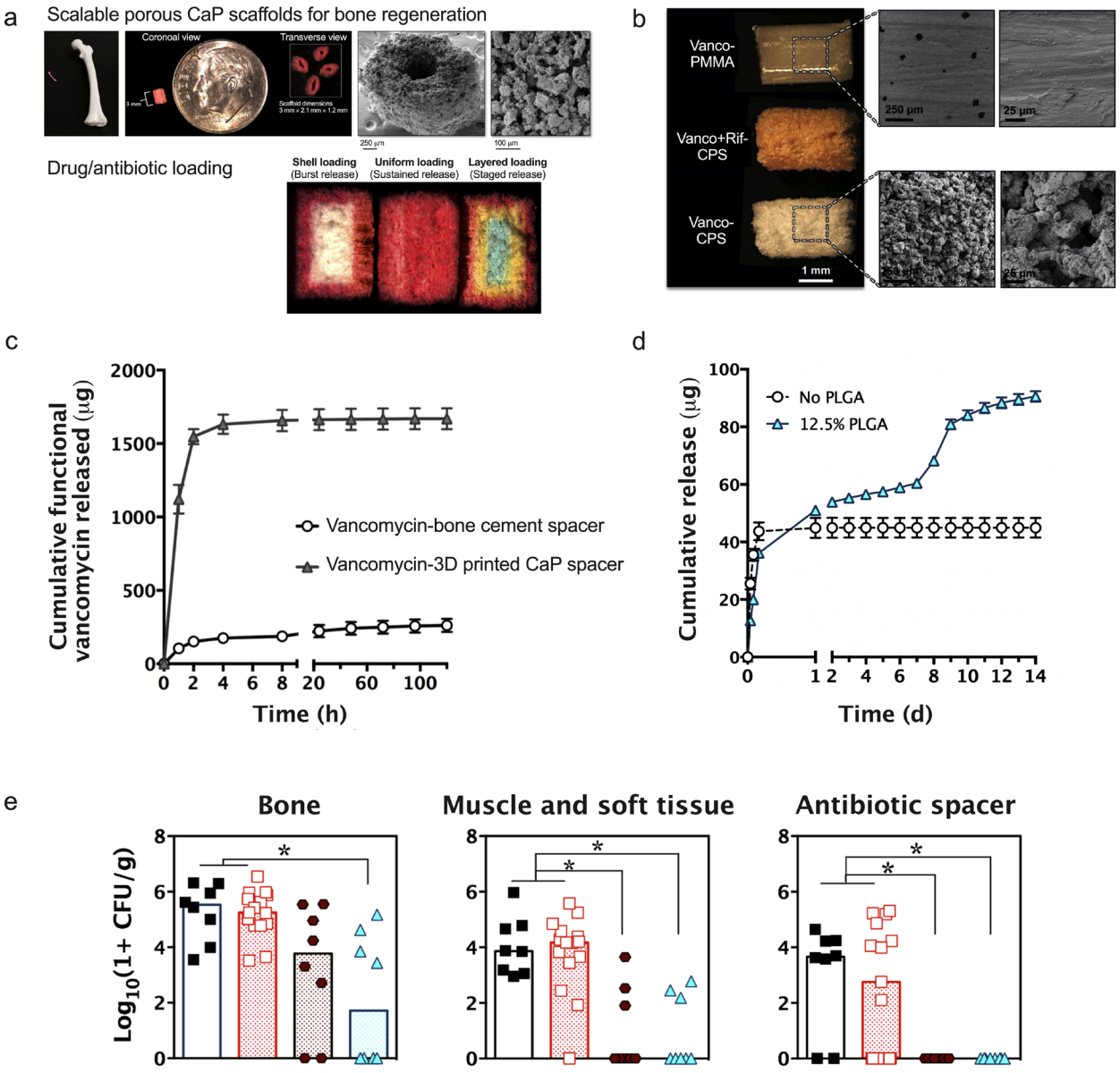

Fig. 3. 3D-printed CaP scaffolds. (**a**) CaP scaffolds for bone regeneration and infection eradication can be scaled from human to murine anatomy. SEM images of the CaP surface shows porous structure for efficient cell attachment. CaP scaffolds can be loaded with antibiotics specifically printed into different regions of the scaffold, indicated by different colours. (**b**) Differences in the surface morphology and porosity between 3D-printed CPS and PMMA spacers are shown by SEM images of various antibiotic-loaded scaffolds. (**c**,**d**) Antibiotic release of vancomycin-loaded spacers. (**e**) Bacterial colonisation, measured by end-point CFU assay, was reduced by low-dose local antibiotic delivery using 3D-printed CPS.

a preclinical research program was commenced to identify protective antigens in a murine model of implant-associated osteomyelitis (Li *et al.*, 2008; Nishitani *et al.*, 2015b) and an international registry of orthopaedic patients with culture confirmed *S. aureus* bone infections was developed to correlate patient-specific immune responses with clinical outcomes (Kates *et al.*, 2020a; Morgenstern *et al.*, 2020b). Independently, these research programs derived the same conclusions that immunity against the autolysin antigens (Amd and Gmd) is protective, while antibodies against the iron surface determinant (Isd) proteins are potentially pathogenic through induction of trojan-horse macrophages and sepsis following SSI (Masters *et al.*, 2019b; Nishitani *et al.*, 2020). Preclinically, passive immunisation with anti-Gmd mAb protects mice from MRSA infections (Varrone *et al.*, 2014) and prevents reinfection following a one-stage revision surgery (Yokogawa *et al.*, 2018). Also, these research programmes demonstrated the safety of a clinically relevant intravenous infusion of anti-Gmd mAb in sheep and estimated the circulating half-life to be 23.7 d. Using the patients' sera from the AO Trauma CPP Bone Infection Registry, Lee *et al.* (2020) demonstrated that endogenous anti-Gmd antibody levels in sera of osteomyelitis patients ranges from < 1 ng/mL to 300 μg/mL, with a mean concentration of 21.7 μg/mL, and estimated its circulating half-life to be 120.4 d. Thus, an anti-Gmd mAb passive immunisation to treat *S. aureus* bone infection, akin to bezlotoxumab (Zinplava™) passive immunisation to prevent recurrent *Clostridioides difficile* infection (Prabhu *et al.*, 2018; Wilcox *et al.*, 2017), is feasible. Additionally, implementing this approach rather than pursue an active vaccine would likely circumvent the problems of host immune variability, immunosuppression and post-infection phenotypic adaptation by the pathogen. However, this will require a companion diagnostic to identify patients who do not have endogenous neutralising antibodies and would be expected to benefit from this therapy. Furthermore, potential issues in translating experimental results from murine to human populations remains a challenge.

Humanised mice

The failure to have effective anti-*S. aureus* immunotherapies can partially be attributed to overreliance on murine model systems to study human immunology. Often, the knowledge acquired using mice models does not necessarily translate into useful vaccine candidates in humans. A case in point is the murine preclinical data of an IsdB-based immunogenic vaccine candidate that demonstrated reduced infection lethality and protection against bacteraemia in mice (Brown *et al.*, 2009; Kim *et al.*, 2010; Kuklin *et al.*, 2006; Torres *et al.*, 2006). Unfortunately, an active vaccination clinical trial based on these preclinical studies involving 8,000 patients failed to protect against *S. aureus* bacteraemia (Fowler *et al.*, 2013).

While being a common commensal, *S. aureus* is an acknowledged human pathogen with numerous virulence factors and bicomponent toxins with a high degree of tropism to receptors expressed on human leukocytes (Alonzo and Torres, 2013; Alonzo and Torres, 2014). Therefore, *S. aureus* does not necessarily exhibit its typical phenotype in murine *S. aureus* infections. A small rodent model with human specificity, correct receptor targets and relevant immune cells will be more suitable for studying a human-adapted pathogen such as *S. aureus*.

Humanised mice generated by engrafting human immune cells to immunodeficient NOD-SCID IL2Rγnull (NSG) mice (Ishikawa *et al.*, 2005; Lan *et al.*, 2006; Shultz *et al.*, 2005) are an attractive model to study *S. aureus* pathogenesis during bone infections. As part of the AO Trauma CPP research program priority areas, the utility of these humanised mice to study *S. aureus* bone infections was recently evaluated and it was examined if they elicited human immune responses due to *S. aureus* osteomyelitis (Muthukrishnan *et al.*, 2021). Interestingly, humanised mice suffered exacerbated osteomyelitis and engrafted human T cell responses correlated with infection severity in these humanised mice (Muthukrishnan *et al.*, 2021). A human-relevant preclinical small animal model would be beneficial to 1) evaluate active and passive *S. aureus* vaccine candidates and 2) compare inter-individual responses to infection based on donor-specific factors.

"The race for the surface" might be a sprint

The "race for the surface" concept has been the prevailing explanation for understanding the fate of implants where there is competition between bacterial colonisation and host-cell integration and protection (Gristina, 1987; Gristina *et al.*, 1988). If microbes reach the surface first, they will attach, replicate, form a biofilm and cause a recalcitrant infection. Conversely, host-cell integration occurring before bacteria colonisation will result in lower chances of infection and improved implant survival. A common anti-infection approach is to protect the implants against bacterial colonisation by an active (releasing antimicrobials) or passive coating but the timeline for the host to be able to fend for itself is still unknown. Therefore, the optimum antimicrobial release kinetics and release duration are not known and a common approach is to have an extended release, often for a month or more. Recently, a preclinical implant infection model was developed where the device implantation was uncoupled from bacterial challenge to provide an understanding of the role time plays in the cellular events that are required to prevent implant infection (Shiels *et al.*, 2020). In this bilateral intramedullary nail rat model, *S. aureus* was injected into the tail vein either immediately after or 1, 3 and 7 d following implant placement. 2 weeks following inoculation, implants and tissues were harvested for

bacterial quantification. As time between implant placement and bacterial challenge increased, infection rate and bioburden decreased. Almost all implants had measurable bioburden when challenged at day 1 but only two implants had recoverable bacteria when inoculated 7 d after implant placement. Additional animals were not inoculated but euthanised at 1, 3 or 7 d and the host cells adhered to the implant were identified. This protection against infection corresponded to a shift in host cell population surrounding the implant. Initially, cells present were primarily non-differentiated stem cells, such as bone marrow mesenchymal stem cells or immature haematopoietic cells. At day 7, there was a mature monocyte/macrophage population. Importantly, it appears that the initial cell population differentiated into the immune cells and the timeline for this appears to be fairly conserved across species. Studies in different anatomic locations, species and health status (comorbidities such as diabetes, advanced age, trauma, *etc*.) along with implant coating and antimicrobial release would help to understand further the timeline where the body can protect the implant and surrounding tissue against infections. Taken together, it appears that therapies and strategies may only need to protect implants against bacterial colonisation for approximately a week.

Preclinical studies into prophylaxis and treatment

3D-printed antimicrobial-loaded CaP

When a bone allograft or an orthopaedic device is infected, thorough debridement of the infected bone and soft tissue is standard of care and often a temporary hand-moulded ALBC spacer is also implanted to support treatment. ALBCs are commonly used despite lack of evidence for efficient drug delivery. Published data show that ALBC burst release about 5 to 15 % of the incorporated drugs within the first 24 h, with significantly less drug release in the following days followed by a prolonged tail of likely sub-inhibitory concentrations (Moojen *et al.*, 2008). Furthermore, ALBC requires revision surgery to first debride the infected bone and treat the infection, by temporarily installing the spacer, and then to remove it and treat a possible bone defect. Therefore, next generation biomaterial spacers are required that enable sustained and controlled delivery of the antibiotics over an extended period in single-stage procedures for infection management and bone repair. To meet these criteria, several groups (Inzana *et al.*, 2016; Trombetta *et al.*, 2017) have developed 3D-printing strategies to design bioactive ceramic spacers that can function as antibiotic-eluting devices and, concurrently, as biodegradable, osteo-inductive

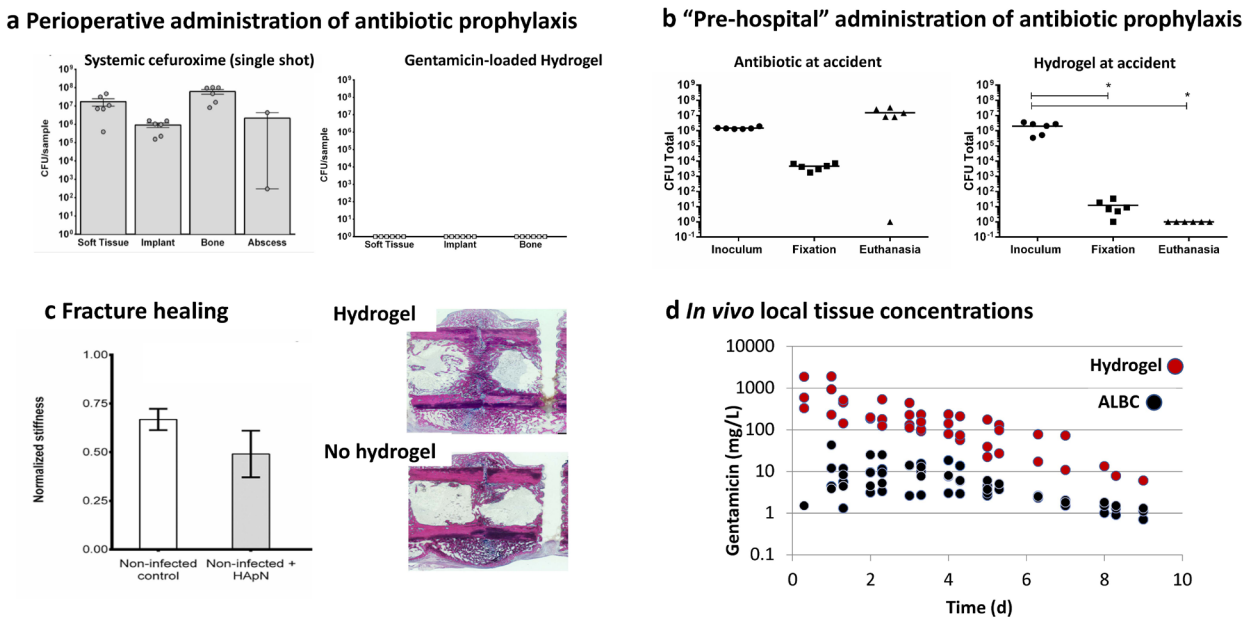

Fig. 4. Preclinical efficacy of an antibiotic-loaded thermo-responsive hyaluronic acid hydrogel. (**a**) In a rabbit humeral osteotomy and plate fixation model, the antibiotic loaded hydrogel achieved complete protection of all animals, whilst systemic delivery of a single shot of cefuroxime could not prevent infection in any. (**b**) The hydrogel formulation may also be applicable by first responders and, in this study, antibiotic therapy was given in a modified animal-model whereby the initial wound and inoculation occurred 4 h prior to fixation. In this model, the antibiotic-loaded hydrogel could completely prevent infection when given at the time of accident and washed-out during fixation surgery, whilst systemic antibiotics once again failed to prevent infection. (**c**) Fracture healing in the presence of the hydrogel showed no significant differences in mechanical strength of the bone at 4 weeks and histological sections confirmed ongoing healing that was equivalenting the presence or absence of the hydrogel. (d) Increased local tissue (intramedullary extracellular fluid) antibiotic concentrations achieved by the gentamicin-loaded hydrogel compared with gentamicin-loaded bone cement (ALBC) in a sheep model. Images adapted from Ter Boo *et al.* (2016), Ter Boo *et al.* (2018) and Vallejo Diaz *et al.* (2020).

scaffolds to elicit bone regeneration, without the need to remove them. Compared to the traditional bead-like PMMA spacer, this technology can build patient-specific scaffolds based on medical imaging (computed tomography or MRI) (Fig. 3**a**). The inclusion of CaP particles creates pores on the surface of the scaffold, which enable extrinsic or intrinsic cell infiltration (Fig. 3**b**). Further, the 3D printing process allows for the versatility to incorporate drugs in the form of i) surface- or shell-loading to enable quick, burst release; ii) homogenous loading for prolonged, sustained release; iii) layered loading with multiple drugs for staged release strategies (Fig. 3**a**). Drug-elution studies found that 3D-printed CaP spacers demonstrate significant improvements in antibiotic release (5-fold) over comparably loaded bone cement spacers (Inzana *et al.*, 2014; Inzana *et al.*, 2015b) (Fig. 3**c**). Drug elution was further improved by coating CaP scaffolds with drug-loaded PLGA (Fig. 3**d**). Finally, in an established murine model of osteomyelitis with DAIR, 3D-printed CaP scaffolds with PLGA coatings outperformed the traditional PMMA cement spacers by significantly reducing the bacterial burden and resulting in more than 50 % complete eradication of the infection (Fig. 3**e**) (Inzana *et al.*, 2015a; Inzana *et al.*, 2015b; Trombetta *et al.*, 2019a; Trombetta *et al.*, 2021; Trombetta *et al.*, 2019b) (Fig. 3**e**). Furthermore, bone regeneration was assessed and the 3D-printed scaffolds resulted in a greater than 3-fold increase in bone formation compared with PMMA spacers (Trombetta *et al.*, 2019b). However, this was limited to the bone-scaffold interface and did not result in bridging mineralised callus to result in complete healing. Future goals are to optimise the osteoinductive properties of these scaffolds to eliminate the need to remove them, thereby increasing clinical benefits.

Hydrogel as preventative and therapeutic modality for FRI

The application of antibiotics directly into surgical sites or infected wounds has remained an area of significant interest in recent years. By applying the antibiotic directly to the infected area, higher concentrations can be achieved compared to systemic administration, without some of the potential adverse effects such as renal toxicity (Hake *et al.*, 2015; Metsemakers *et al.*, 2020a). As already described, ALBC is still commonly applied as cement beads to a trauma wound or as a moulded cement spacer replacing a prosthetic joint, despite significant uncertainty about antibiotic release and efficacy. Currently, several innovative biomaterials are available as promising alternatives to bone cement for local application of antibiotics targeting bone infections (Foster *et al.*, 2020b; Inzana *et al.*, 2016; Ter Boo *et al.*, 2015). These biodegradable materials eliminate the need for follow-up removal surgeries and often have a more optimal and complete antimicrobial release profile (Inzana *et al.*, 2016; McKee *et al.*, 2010). In recent years, Ter Boo *et al.* (2016; 2018) have described a degradable thermo-responsive hyaluronic acid hydrogel, loaded with gentamicin, that has outperformed standard single-shot systemic cefuroxime in a rabbit FRI model, without significantly impacting osteotomy healing (Fig. 4). By applying such a gel locally, and not requiring repeated injections, this material displays a significant advantage over conventional systemic antibiotic therapy and has further advantages in terms of wound closure, ease of use and range of potential applications. The hydrogel may also be applied in a pre-hospital setting, as may be done for example by first responders at the scene of an injury. The benefit of this approach is that the material may then be removed during surgery. This approach was tested in a rabbit model and shown to be effective with only a few hours contact time (Vallejo Diaz *et al.*, 2020). More recently, the same hydrogel has been loaded with gentamicin and vancomycin and used in the treatment of a chronic MRSA bone infection in sheep, with performance equivalent to ALBC in successfully eradicating the infection, without the need for later removal of the carrier due to the biodegradable nature of the hydrogel (Boot *et al.*, 2021; Foster *et al.*, 2020a).

Extracorporeal shockwaves

Focused high-energy ESWT is a treatment modality used to enhance bone healing in fracture non-unions (Everding *et al.*, 2020). ESWT has also been described to have direct anti-bacterial effects (Gerdesmeyer *et al.*, 2005; Horn *et al.*, 2009; Inanmaz *et al.*, 2014; Qi *et al.*, 2016) and increase tissue vascularity (Wang *et al.*, 2003). ESWT was evaluated as an adjunctive treatment alongside conventional surgical debridement and systemic antibiotics *in vitro* and in a clinically relevant rabbit model of FRI (Arens *et al.*, 2015). After plate fixation of a humeral osteotomy in rabbits, infection was established with a clinical *S. aureus* isolate. A DAIR procedure was performed after 14 d. Then, rabbits received no further treatment (controls), shockwaves (4,000 impulses, 23 kV, at day 2 and 6 after revision), systemic antibiotics (rifampin and nafcillin) or the combination of antibiotics and shockwaves. Treatments were applied over 1 week and euthanasia was performed after another week without treatment to determine infection burden. In this model, the combination of ESWT and systemic antibiotics resulted in an average 100-fold reduction in total CFU compared with antibiotic treatment alone (Puetzler *et al.*, 2020). The reduction in bacteria was the greatest on the implants, which is of special interest as it suggests that shockwaves might facilitate non-invasive *in situ* eradication of biofilm on foreign bodies and, thus, expand the application range for implant retention. Various mechanisms are currently being discussed. It seems plausible that mechanical stresses (compression, tension and shear), as well as cavitation effects, play a role in direct biofilm disruption (Rassweiler *et al.*, 2011). Electron microscopy images of the implant surface

show many small craters after ESWT, which are likely caused by microjets created by imploding cavitation bubbles (Milstrey *et al.*, 2021). In addition to direct mechanical effects, mechanotransduction may also play a role. However, to what extent the shock waves induce biochemical signals that elicit specific cellular responses remains to be elucidated.

In the *in vivo* model, ESWT did not induce dissemination of bacteria into the bloodstream, suggesting that ESWT may not be a risk for bacteraemia, even when given with high energy in case of local, high bacterial loads. Future studies will be required to determine the effect of ESWT on eukaryotic cells at the implant interface as well as longer term *in vivo* studies.

Diagnosis

Rapid sensitive detection of pathogens causing bone infection

To improve the prognosis and provide focused therapy of bone infections, particularly those involving implants, methods for rapid and accurate identification of causative pathogens are highly relevant. With the introduction of MALDI-TOF MS into microbiological diagnostic laboratories about a decade ago, rapid identification of pathogens to the species level from microbial cultures became possible within a few minutes (Bizzini *et al.*, 2011; Borens *et al.*, 2012). For identifying microbial species directly out of liquid culture, MALDI-TOF MS workflows are already regularly used for blood culture specimens [reviewed in Morgenthaler and Kostrzewa (2015) and Ruiz-Aragon *et al.* (2017)] containing a variable degree of host cellular remnants. This has recently been adapted to joint specimens taken during surgery (Kuo *et al.*, 2020; Noll *et al.*, 2020). In addition, successful identification protocols bypassing a culture step and directly using liquid specimens, such as cerebrospinal fluid (Bishop *et al.*, 2018; Segawa *et al.*, 2014) and urine (Inigo *et al.*, 2016; Li *et al.*, 2019), have been published. In contrast, specimens associated with PJI have only low bacterial densities with concomitant cellular debris so that direct, culture-less bacterial identification out of these specimens is difficult (Lallemand *et al.*, 2016). In those cases, enrichment steps – *e.g.* in liquid medium, prolonged incubation, increase sensitivity and specificity (Font-Vizcarra *et al.*, 2010; Larsen *et al.*, 2012) – are still needed. Also, they allow for observance of slow-growing small colony variants (Bogut *et al.*, 2014) or fastidious bacteria and lead to improved diagnosis (Schafer *et al.*, 2008).

In view of the expected reduction in the time to diagnosis, the use of MALDI-TOF MS for species identification directly from synovial fluid or even host tissues is highly desirable. The most challenging parameters to overcome are the low bacterial load and detritus of human tissue cultures inhibiting the analyses.

Immune proteome studies

Diagnosis of bone infections remain a primitive art dependent on overt infection symptoms (weeping inflamed wounds) or combinations of direct culture tests and blood-borne biomarkers. Blood-based diagnostics have clear advantages over culture, as they are minimally invasive, less time consuming and easy to administer. Unfortunately, existing diagnostics such as CRP, ESR and WBC are not specific and force clinicians to administer empiric antibiotics until the pathogen is identified in culture (Ricciardi *et al.*, 2020a). To overcome this, the potential of anti-*S. aureus* antibody levels in serum was investigated (Fig. 5) (Muthukrishnan *et al.*, 2019b). Among the many immunodominant *S. aureus* antigens, Morgenstern *et al.* (2020a) focussed on eight cell-wall-associated or secreted proteins expressed by most virulent strains: cell-wall-modifying enzymes (Amd, Gmd), iron-regulated surface determinant proteins (IsdA, IsdB, and IsdH), toxins and immune evasion proteins (alpha-haemolysin, SCIN, CHIPS). Utilising patient samples from the AO Trauma CPP Bone Infection Registry, which included only *S. aureus* infected patients [see below for details (Morgenstern *et al.*, 2020a)], it was observed that antibody levels against these antigens rise during infection, providing a blood-based measure of active infection (Kates *et al.*, 2020c). The diagnostic utility of this immunoassay proved to be good (AUC > 0.9) (Nishitani *et al.*, 2015a), which is remarkable considering that most people have appreciable levels of circulating anti-*S. aureus* antibodies due to its commensal nature. However, serum levels remain elevated for months following an intervention, making serum antibodies poor measures for tracking therapy. To address this limitation, the measurement of circulating ASCs was explored (Fig. 5). ASCs are present in the blood only when an infection is ongoing; upon resolution, their levels drop to 0. Using these assays developed for serum antibodies to study ASCs, *S. aureus*-infected patients with multiple types of bone infections, tracked therapy and recurrence were studied (Muthukrishnan *et al.*, 2020; Oh *et al.*, 2018). Additionally, Sulovari *et al.* (2020) achieved reasonable success in simultaneous identification of *Streptococcus agalactiae* and *S. aureus* infections in the same immunoassay. However, a critical high priority area of future research is developing immunodiagnostic assays for reliable identification of polymicrobial infections, including *S. aureus*, *S. epidermidis*, *Streptococcus agalactiae*, *Cutibacterium acnes* and *Enterobacteriae*.

Clinical evidence generation

AO Trauma Registry

The AO Trauma CPP Bone Infection Registry was developed to improve the understanding of host-pathogen interaction by collecting clinical data, bacterial isolates and serum from patients with *S.*

aureus bone infection (Kates *et al.*, 2020a; Morgenstern *et al.*, 2020a). The prospective multinational registry with a 12-month follow-up included adult patients (18 years or older) with culture-confirmed *S. aureus* infection in long bones after fracture fixation or arthroplasty. Baseline patient attributes and details on infections and treatments were recorded. Blood and serum samples were obtained at baseline, 6 and 12 months. Clinical outcomes and patient-reported outcomes using the Short Form 36 Health Survey Questionnaire (version 2), Parker Mobility Score and Katz Index of Independence in Activities of Daily Living were assessed at 1, 6 and 12 months.

In total, 292 patients were enrolled between November 2012 and August 2017 in 18 centres from 10 countries in Europe, North America, South America and Asia. MRSA was detected in one third of the patients ($n = 82$, 28.4 %). Patients from North America

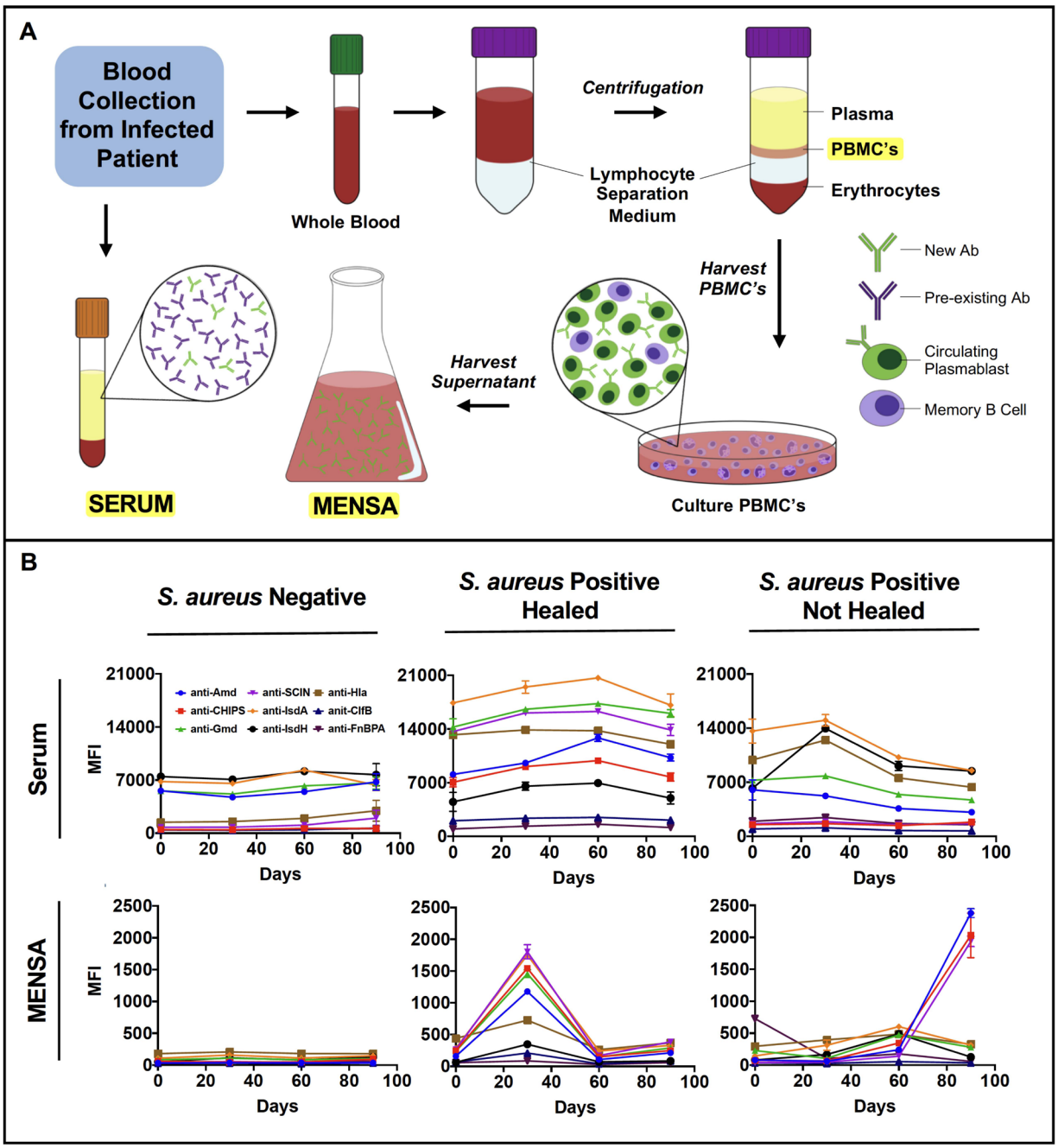

Fig. 5. A diagnostic and prognostic immunoassay for the measurement of anti-*S. aureus* antibody levels in patients with osteomyelitis. (**a**) Schematic illustration of production of serum and isolation of MENSA from peripheral mononuclear cells of patients with osteomyelitis. (**b**) Anti-*S. aureus* antibody levels in serum and MENSA were determined using a custom bead-based multiantigen Luminex immunoassay developed by the authors. Anti-*S. aureus* IgG responses were examined in serum and MENSA of DFI patients undergoing FST. The change in antibody titres over the course of FST of a representative patient whose DFI was negative for *S. aureus*, a patient with *S. aureus* infection that responded to FST and a patient with *S. aureus* DFI that failed FST are presented. Remarkably, MENSA levels faithfully reflected the *S. aureus* infection over time while serum levels remained unchanged (see Oh and Muthukrishnan *et al.*, 2018; Oh *et al.*, 2018). Reproduced with permission from Masters *et al.* (2019b).

sites had the highest proportion of MRSA infections (n = 39, 48.8 %), patients from Central European sites the lowest (n = 18, 12.2 %). An improvement in patient outcomes was found at 6 and 12 months when compared to baseline. Despite an improvement following infection treatment, fewer than two thirds of the patients were cured at the 1-year follow-up (118/194, 62.1 %). Patient-reported outcome scores at the 12-month follow-up were worse for patients with MRSA infections as compared to infections with methicillin-susceptible strains (Morgenstern *et al.*, 2020a). The biospecimens collected with the clinical data will allow for the analysis of relationships between patient demographics, comorbidities, treatment modality, patient-specific host immunity to the causal pathogen(s) and outcomes (Morgenstern *et al.*, 2020a), early examples of which are already published (Lee *et al.*, 2020).

In a prospective point-prevalence study, antibiotic resistance of commensal *S. aureus* and CoNS was investigated in an international cohort of 1,166 surgeons from 75 countries. The average *S. aureus* nasal colonisation rate was 28.0 % and MRSA rate 2.0 %. The observed regional variations were significant, with the highest rates of MRSA colonisation in Asia, Africa and Central America and the lowest in North America and Europe. High rates of methicillin-resistant CoNS nasal carriage of 21.4 % were observed with a similar geographic distribution. However, colonisation rates in patients receiving orthopaedic surgery are broadly equivalent to the general population. Recent use of systemic antibiotics was associated with higher rates of carriage of resistant staphylococci (Morgenstern *et al.*, 2016).

Standardised management guidelines for FRI

Until recently, management principles of FRI were based on research that has primarily been performed on PJI. Although there are similarities, FRIs have unique features (*i.e.* fracture, bone healing, soft-tissue injury, option to remove implant after healing) that need to be considered (Metsemakers *et al.*, 2018b). A recent literature review confirmed the lack of high-quality scientific evidence in the FRI field and stressed the importance of the development of standardised management guidelines (Bezstarosti *et al.*, 2019). Therefore, the FRI Consensus Group was created under the umbrella of the AO Foundation (*i.e.* AO Trauma CPP on Bone Infection, Technical Commission's AIGEC, AO Research Institute Davos) in collaboration with well-known international organisations (*i.e.* EBJIS, OTA, PRO-IMPLANT Foundation). The main aim of this group was to develop standardised principles for diagnosis and treatment based on scientific evidence and expert opinion.

During the first consensus meeting, the experts concluded – based on review of the literature (Metsemakers *et al.*, 2018a) – that although a well-established diagnosis is the first step in the treatment process of FRI, standardisation within this field has been poor. The lack of a definition based on diagnostic criteria for infection has hampered the development of treatment protocols that are based on comparable studies and outcomes. For these reasons, an international consensus definition for FRI was developed (Metsemakers *et al.*, 2018c) and recently updated (Govaert *et al.*, 2020). Two levels of certainty around diagnostic features were defined. Criteria for infection can be confirmatory (infection is present) or suggestive. The presence of one of the five confirmatory signs should prompt the initiation of treatment. Suggestive signs should motivate the medical team to further investigate the possibility of the presence of confirmatory signs (Metsemakers *et al.*, 2020b). This definition should not only improve the quality of published reports but also the overall management of these patients in daily clinical practice (Obremskey *et al.*, 2020). Careful attention to establish an adequate diagnosis of FRI allows for better surgical planning and pathogen-specific antimicrobial therapy, leading to an improved patient outcome (McNally *et al.*, 2020). Currently, multiple projects are being finalised to validate this FRI consensus definition, leading to a further improvement in the diagnostic pathway.

As a next step, a second consensus meeting was convened, focussing on management principles in general and assessment of outcome. At the centre of these recommendations was the implementation of a multidisciplinary team approach. This should lead to appropriate use of antimicrobials and standardisation of surgical strategies (Metsemakers *et al.*, 2020b). Two main surgical concepts were described. The first concept consists of implant retention and the second of implant removal (healed fracture) or implant exchange [unhealed fracture (Metsemakers *et al.*, 2020b)]. Furthermore, multiple key aspects for an optimal surgical treatment were presented. One of the cornerstones of every surgical approach being a judicious and well-planned debridement with removal of all dead tissues and acquisition of deep tissue biopsies for microbiology and histopathology. This should be followed by osseous stabilisation (if required), delivery of antimicrobial therapy (using local and systemic antimicrobials) and sufficient vital soft tissue coverage (Depypere *et al.*, 2020b). Guidelines regarding antimicrobial therapy (*i.e.* local and systemic) were developed and published separately (Depypere *et al.*, 2020a; Metsemakers *et al.*, 2020a). Finally, it was stressed that a minimum follow up of 12 months is critical. This should not only include clinical outcomes of fracture union and absence of infection recurrence but also standardised patient-reported outcome measures (Metsemakers *et al.*, 2020b).

Education

Because research needs to be disseminated and understood by clinicians and other scientists, it was

essential to develop a formal education programme on musculoskeletal infection as part of the CPP on Bone Infection. Using a backward-planning process, in conjunction with the AO Education Institute, the CPP Bone Infection team developed a formal AO infection course which has been offered to the surgical community since 2012. Initially offered in Davos, Switzerland, the course has been given in many regions including Europe, Asia, Latin America and the Middle East. The AO infection course has focused on dissemination of best practices in prevention, diagnosis and treatment of bone infections. Most of the focus has revolved around FRI but there has been limited coverage of PJI as well. The CPP Bone Infection team has also offered educational symposia at many large international scientific meetings including EBJIS, OTA, MSIS, ORS, CORS and others. Overall, the participant ratings at these educational offerings have been high and have reflected the overall need for such in person educational offerings. A textbook, Principles of Orthopedic Infection Management, was also produced by the AO Trauma CPP Bone Infection team and has been well received and widely read.

Future directions

Training the next generation of bone-infection investigators

While development of novel diagnostics and interventions for bone infection is the primary goal of the AO Trauma CPP on Bone Infection, it is likely that the greatest impact of this research will be the training of the next generation of scientists, biomedical engineers and surgeons, who must continue this work if it is to improve patient care. As cutting-edge research in this field requires great depth and breadth in microbial pathogenesis, osteoimmunology, surgery, drug-device development, *in vivo* modelling, regulatory science, clinical trials and cost-effectiveness outcomes, the AO Trauma CPP on Bone Infection has been remarkably successful in recruiting very talented young men and women within these sub-specialties to perform this work during their graduate and post-doctorate training. In additions to their research outcomes and deliverables, the AO Trauma CPP on Bone Infection also mentored dozens of trainees in team-based structured science, scholarship and oral presentation of their work at international meetings. In doing so, these future leaders now have the requisite knowledge, skills, confidence and networks to add onto the accomplishments of their mentors and hopefully solve any remaining and emerging problems in this field. Indeed, several of the initial trainees in the AO Trauma CPP on Bone Infection have already obtained leadership positions in academia, government and industry, which validates this research, education and training approach that should be emulated by other biomedical training programs.

Translation of technologies for bone infection to the clinic: still not an easy path but has COVID-19 changed anything?

Out of the numerous technologies being described in the scientific literature aimed at preventing or treating bone infection, a huge majority never makes it to clinical application. The obvious clinical need to provide better preventative and therapeutic interventions is to be balanced against the need for prevention of excessive use of antibiotics. However, at the present time, there appears to be a significant regulatory burden on the application of antimicrobial technologies in the orthopaedic trauma field, that has restricted developments from progressing from preclinical to the clinical stage (Moriarty *et al.*, 2014). To claim an anti-infective benefit, the clinical proof of efficacy places an extremely prohibitive high cost on any party innovating in this field. The result is that many surgeons apply off-label home-made solutions such as antibiotic-loaded bone cements fashioned into makeshift implant coatings or antibiotic powders being dosed directly into wounds. The outlook for innovations to make it to the clinic may require a balanced or stepwise approach where new technologies are first proven to be effective in high-risk cases, with subsequent advancement to more general populations once safety and efficacy are established. This has been the case with the development of the COVID-19 vaccines and monoclonal antibody passive immunisations, which went from concepts through FDA-approved emergency use authorisation in less than one year through an unprecedented contemporary partnership between government and industry for the greater good of humanity.

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Discussion with Reviewers

Reviewer: The authors have provided a strong account of the scientific endeavour of their programme aimed at better understanding and treating bone infections. One wonders if it will be equally important to demonstrate to governments and health systems the huge socioeconomic costs of these infections to elevate bone infection to a clinical priority.
Authors: We are fully in agreement with the comment of the reviewer. Demonstration of the huge socioeconomic costs of bone infections, or differently stated, demonstration of the potential of cost savings is something that should be addressed in future research projects within the CPP Bone Infection. This requires cross disciplinary expertise and long term follow-up to fully measure the impact of bone infection. This is not a simple undertaking but would provide the needed justification for continued research and development to address this clinical problem.

Reviewer: Passive immunisation as an approach to protect against *S. aureus* infections appears to be a viable option, as opposed to an active vaccine. It would seem to circumvent the problems of host immune variability, immunosuppression and rapid post-infection phenotypic adaptation by the pathogen. What are the barriers to the implementation of this approach?
Authors: While passive immunisation has the ability to overcome host immunity shortcomings including variability, immunosuppression and a lengthy process to develop protection, it has four major limitations compared to active vaccination that need to be considered. The first is the lack of robustness, as passive immunisation is limited to the monoclonal antibodies that are administered to the patient. Thus, antibodies against other antigens and all forms of cellular immunity are not included in this treatment. The second is the lack of durability, as efficacy from passive immunisation is limited to the 4-8 month half-life of the antibody, while active vaccination has life-long protection potential in some cases. The third is that passive immunisations are 25-50 times more expensive than active vaccines. Using the current COVID-19 examples, the mRNA lipid nanoparticle vaccines (BNT162 and MRNA1273) cost 15-20 USD per dose (Web ref. 1, Web ref. 2) and the passive immunisation (casirivimab and imdevimab) costs 66 USD per dose (Web ref. 3). Using non-pandemic examples, the FDA-approved active vaccine for herpes zoster (Shingrix) costs 155 USD per dose (Web ref. 4) and the passive immunisation for *Clostridioides difficile* (bezlotoxumab) costs 3,976.70 USD per dose (Web ref. 5). Fourth, passive immunisations are delivered intravenously in infusion centers, which presents major challenges for rural communities and 3rd world countries. Therefore, taken together, passive immunisation is broadly considered a therapy to treat active infectious disease, and not a prophylaxis such as active vaccination.

Reviewer: Chemical modification of metal alloys or coatings (*e.g.* silver) has been an area of intense interest. Are these being considered by the AO Trauma CPP?
Authors: At the present time, the AO Trauma CPP does not have a surface-coating technology in preclinical research that is targeted for translation.

Reviewer: A number of other approaches to combat orthopaedic-related infections include surface modification, such as creating nano-structures to either release antibiotics or physically kill bacteria; would you have concerns about surface integrity of the modified implants?
Authors: One of the primary concerns in developing a surface coating for an orthopaedic implant is indeed the surface integrity of the implant after sometimes forceful insertion into the patient. In addition to the implant surface integrity, also the integration of the implant with the local tissues is important, particularly the bone: implant interface in arthroplasty. Despite the large number of surface coatings or other surface modifications designed to impart antimicrobial functionality on orthopaedic devices, the application in the clinic is minimal. This is a result of a combination of simple economics on the commercialisation side and the burden for evidence of efficacy and safety on the regulatory side. This is a topic that has run for many years and it seems we are not lacking in the scientific ingenuity to address the problem but the practical route toward clinical application is the main barrier.

Sebastian Zaat: What is the time span required between blood samples' collections to unequivocally assess a rise in the antibody levels against the chosen

S. aureus antigens? Is this time span sufficiently short to make the technique feasible for a relevant clinical diagnosis?

Authors: These are intriguing questions that frame two of the fundamental under-addressed issues in orthopaedic infections. The first question asks whether measuring increases in antibody titres against *S. aureus* antigens can be used as a tool for clinical diagnosis. When we began this work in 2013, we too were concerned about this same issue. Specifically, every adult human has been infected with *S. aureus* sufficiently to elicit a substantial humoral response that, then, provides a varied and problematic background against which the response to an ongoing infection must be measured.

Our initial assumption was that made by many other investigators: the pre-existing antibody response is sufficiently high and varied that it would obscure the production of new antibodies, thereby making the antibody-based diagnosis of an ongoing infection impossible. To our surprise, this idea turned out to be largely false. As Nishitani *et al*. (2015) showed, patients bearing different types of orthopaedic infections tended to have elevated antibody titres compared to healthy controls. The antibody-based test was not perfect, but it was as good as many tests are during their initial evaluations. The AUC value in a receiver, operating characteristic curve comparing patients with confirmed *S. aureus* infections to healthy controls, was 0.9. To be clear, this level of performance required a single sample and no comparison of paired early and late samples, although that could lead to potential improvement.

Our patient population has been heterogeneous, some with initial infections a few weeks post-surgery and others having experienced serial infections, so timing of the intervals between infection, symptom onset and elevation of serum antibody levels has been difficult to measure directly. In the mouse transtibial pin model, Li *et al*. (2008) showed that the anti-*S. aureus* IgM serum response was measurable by day 6 post-primary infection and the IgG response by day 11 (although this may be slightly artificial because of the relatively high initial dose of *S. aureus* bacteria on the infection pin). That said, in humans, most primary viral and bacterial infections are detectable by serum antibodies 7-10 d post symptom onset (Carter *et al*., 2017, additional reference). Considering that anti-*S. aureus* humoral responses are essentially always secondary responses, this same 7-10 d estimate is probably conservative. Consequently, with some modest improvements, the serum-based anti-*S. aureus* immune response is readily measurable in most patients sufficiently early to be comparable or superior to the complicated methods currently recommended (Glaudemans *et al*., 2019, additional reference) for detection of a new *S. aureus* infection.

We have sought an improved method for detecting and tracking the success of therapy for orthopaedic *S. aureus* infection. How can we measure ongoing infections in "real time"? How can we measure an ongoing infection in such a way that we are not confounded by prior infections? How can we measure the success (or failure) of therapeutic interventions? How can we diagnose recurrences that occur shortly after an initial infection? To address these questions, we introduced the measurement of antibodies secreted by newly stimulated circulating ASCs. ASCs are stimulated in the bone marrow during an ongoing infection and mature into the circulation into plasma cells that either relocate to the bone marrow, where they become long-lived plasma cells, or produce antibody for days to weeks and then die. As a population, ASCs 1) emerge into the circulation prior to sero-conversion, 2) are the cells that will produce the serum antibody response, 3) are present in the circulation only during active infection (background is 0). These attributes make them attractive biomarkers for both detecting and tracking therapy of ongoing infections. Briefly, ASCs provide a good measure of nascent infections, although, surprisingly, the AUC values have been slightly lower than those for serum. In addition, ASC levels decline almost to baseline (0) following successful therapy and they rapidly re-emerge upon re-infection (Oh *et al*., 2018; Muthukrishnan *et al*., 2020).

Sebastian Zaat: Has the final bone regeneration in the presence of biodegrading 3D-printed spacers that are not removed been assessed? Isn't it necessary at some point when infection has been eradicated to remove the degrading material since it may compromise optimal bone repair?

Authors: We have assessed bone regeneration. In general, the 3D-printed scaffolds resulted in more than 3-fold increase in bone formation compared with PMMA spacers (Trombetta *et al*., 2019b). However, this was limited to the bone-scaffold interface and did not result in bridging mineralised callus to result in complete healing. Our intention is to optimise the osteo-inductive properties of these scaffolds to eliminate the need to remove them, as suggested by the Reviewer.

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Editor's note: The Guest Editor responsible for this paper was Henny Van der Mei.