A Hyaluronic Acid Hydrogel Loaded with Gentamicin and Vancomycin Successfully Eradicates Chronic Methicillin-Resistant *Staphylococcus aureus* Orthopedic Infection in a Sheep Model

Willemijn Boot,^a Tanja Schmid,^a Matteo D'Este,^a Olivier Guillaume,^{a,c} Andrew Foster,^{a,d} Laurent Decosterd,^b Robert G. Richards,^a David Eglin,^a Stephan Zeiter,^a Thomas F. Moriarty^a

^aAO Research Institute Davos, Davos Platz, Switzerland

^bFaculty of Biology and Medicine, University of Lausanne, Lausanne, Switzerland

^c3D Printing and Biofabrication Group, Institute of Materials Science and Technology, TU Wien, Vienna, Austria

^dDepartment of Orthopaedic Surgery and Jamieson Trauma Institute, Royal Brisbane and Women's Hospital, Queensland University of Technology, Brisbane, Australia

ABSTRACT Implantable orthopedic devices have had an enormously positive impact on human health; however, despite best practice, patients are prone to developing orthopedic device-related infections (ODRI) that have high treatment failure rates. One barrier to the development of improved treatment options is the lack of an animal model that may serve as a robust preclinical assessment of efficacy. We present a clinically relevant large animal model of chronic methicillin-resistant *Staphylococcus aureus* (MRSA) ODRI that persists despite current clinical practice in medical and surgical treatment at rates equivalent to clinical observations. Furthermore, we showed that an injectable, thermoresponsive, hyaluronic acid-based hydrogel loaded with gentamicin and vancomycin outperforms current clinical practice treatment in this model, eliminating bacteria from all animals. These results confirm that local antibiotic delivery with an injectable hydrogel can dramatically increase treatment success rates beyond current clinical practice, with efficacy proven in a robust animal model.

KEYWORDS MRSA, biofilms, gentamicin, implanted devices, local delivery, osteomyelitis, vancomycin

Approximately 2 million fracture fixation devices and 600,000 joint prostheses are implanted in the United States annually (1). These orthopedic devices restore joint function, reduce pain, correct deformities in children, and support early mobilization and healing of traumatic bone fractures. The most common complications related to the use of orthopedic devices include implant failure, implant loosening, healing delays, and infection, of which the latter can be one of the most challenging to resolve. The incidence of orthopedic device-related infections (ODRI) persists at approximately 2% (1) for elective joint replacement and increases to up to 5 to 25% following internal fixation of a fracture, depending on the severity of the injury (2, 3). Considering the high total number of implantations per year, ODRI has a significant burden for the patient, but also for health care systems at a cost ranging from approximately \$60,000 to \$150,000 per case (4).

Once a chronic infection is established, multidisciplinary management from both a surgical and medical perspective is required (5). Surgical strategies in these cases generally involve removal of the device and multiple revision procedures to resect infected, necrotic bone, which can result in large bone defects, prolonged hospital stays, and increased health care costs (6). A majority of ODRI are caused by

Citation Boot W, Schmid T, D'Este M, Guillaume O, Foster A, Decosterd L, Richards RG, Eglin D, Zeiter S, Moriarty TF. 2021. A hyaluronic acid hydrogel loaded with gentamicin and vancomycin successfully eradicates chronic methicillin-resistant *Staphylococcus aureus* orthopedic infection in a sheep model. *Antimicrob Agents Chemother* 65:e01840-20. <https://doi.org/10.1128/AAC.01840-20>.

Copyright © 2021 American Society for Microbiology. All Rights Reserved.

Address correspondence to Thomas F. Moriarty, fintan.moriarty@aofoundation.org.

Received 26 August 2020

Returned for modification 29 November 2020

Accepted 18 January 2021

Accepted manuscript posted online 1 February 2021

Published 18 March 2021

Staphylococcus species (7–10), including antibiotic-resistant strains such as methicillin-resistant *Staphylococcus aureus* (MRSA). Recommended antibiotic therapy usually consists of 2 weeks of intravenous therapy (e.g., vancomycin for MRSA), followed by a combination of orally administered antibiotics for up to 12 weeks (11). Oral therapy often involves a combination of a biofilm-active antibiotic (rifampin for staphylococci) and a second partner drug to reduce the risk of resistance developing during therapy. Despite these elaborate and costly treatment protocols, ODRI still suffer unacceptably high treatment failure rates (12). Reported failure rates for the staged revision approach to MRSA ODRI vary from 11 to 52% (13), with failure to follow appropriate surgical and medical algorithms associated with poorer outcomes. In general, patients with infections caused by MRSA have longer hospital stays and higher hospitalization rates than those with a methicillin-susceptible *S. aureus* (MSSA) infection (14). MRSA remains, therefore, a pathogen of concern in orthopedic surgery.

An adjunctive approach to improving treatment success rates in ODRI is the application of antibiotics directly to the infected area. This local application can achieve higher concentrations of antibiotics at the site of infection than systemic administration without some of the negative side effects of long-term systemic antibiotic use, such as renal toxicity (i.e., for vancomycin) (15). Historically, the most commonly used carrier material in orthopedic surgery has been bone cement. Antibiotic-loaded bone cement (ALBC) may, for example, be introduced as beads to a trauma wound or as a molded spacer replacing a prosthetic joint. The ALBC provides multiple days of local antibiotic release and both mechanical stability and dead space management. However, ALBC cannot be loaded with all antibiotics due to the exothermic reaction occurring during polymerization, which inactivates heat-sensitive antibiotics. Distribution of ALBC within the infected area is also limited, and a further drawback is the fact that it is nonbiodegradable, and, therefore, it requires a second surgery for removal (16, 17). Finally, the concentration of antibiotics released from bone cement is relatively high for the initial weeks, dropping to subinhibitory concentrations for prolonged periods of time thereafter, risking resistance development within exposed bacteria (16, 18, 19).

Many innovative options are available as promising alternatives to bone cement for local application of antibiotics for the prevention and treatment of ODRI (17, 20). Some of the more promising technologies are biodegradable materials, which can eliminate the need for follow-up removal surgeries and release the total drug payload (17, 21). We have described a degradable thermoresponsive hyaluronic acid hydrogel that may be loaded with antibiotics such as gentamicin, which has been shown to outperformed systemic antibiotics in the prevention of ODRI in rabbits (22). By applying such a gel locally and not requiring repeat 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.

At the present time, few antibiotic-loaded materials have been successfully translated to the clinic (23), and those that are, are primarily targeted at prevention of infection rather than treatment of ODRI. Treatment is a substantially different challenge than prevention in numerous practical aspects, including antibiotic selection, antibiotic dosing, bacterial burden, and antibiotic tolerance, as well as economic aspects such as patient numbers, competitive products, and regulatory requirements. Furthermore, the options currently used in the clinic often involve off-label use of antibiotics and have not evolved over the decades since implementation (e.g., bone cement) due to a combination of regulatory and commercial barriers (23, 24). Therefore, although customized, targeted solutions are required to improve patient care, none have been widely adopted to date.

One barrier to the translation of an innovative treatment strategy is predicting clinical efficacy. At the present time, there are few animal models that reliably recapitulate an MRSA ODRI or that progress through a treatment regimen

approaching best practice in human medicine, i.e., involving implant exchange, surgical resection, and combined local and systemic antibiotic therapy. This situation is true for both fracture-related infection (FRI) and periprosthetic joint infection (PJI), whereby a recent review highlights this crucial limitation (25). We have previously described a large animal model of a two-stage exchange of hardware due to infection with susceptible *S. aureus*. However, there remains a lack of an equivalent model of MRSA infection and one in which antibiotic therapy is administered according to human guidelines. In the absence of a robust preclinical *in vivo* assessment of efficacy, confidence in any new treatment strategy may be tempered.

The first aim of this study was to develop a clinically relevant large animal model of chronic MRSA ODRI treated with the current clinical standard of care (local ALBC and systemic antibiotics). The second aim was to use the current clinical practice regimen in this model as a benchmark to evaluate the efficacy of our antibiotic-loaded biodegradable thermoresponsive hydrogel loaded with gentamicin and vancomycin to treat chronic MRSA ODRI.

RESULTS

Tolerability and pharmacokinetics of systemic antibiotic therapy. In general, the sheep tolerated the infusions of vancomycin, rifampin, and co-trimoxazole. However, immediate postoperative infusion led to clinically apparent discomfort, which was not observed when a 24-h delay was introduced. Therefore, the infusions were withheld for 24 h after surgery in the infected animals to allow for postoperative recovery prior to infusion. Serum antibiotic concentrations peaked at 1 h postinfusion and decreased by 4 h in all cases and were further reduced in preinfusion samples (Fig. 1). Mean serum peak/trough concentrations were as follows: $35 \pm 21/6 \pm 3 \mu\text{g/ml}$ vancomycin, $15 \pm 5/2 \pm 2 \mu\text{g/ml}$ rifampin, and $79 \pm 28/22 \pm 8 \mu\text{g/ml}$ co-trimoxazole. The average serum trough values of vancomycin achieved when administered twice a day were below desired targets (15 to $20 \mu\text{g/ml}$ or area under the concentration-time curve [AUC] of $400 \mu\text{g}\cdot\text{hour/ml}$) (26). To more closely approach target clinical trough values, infusions were switched to three times daily after 4 days of infusions in sheep in the infection studies. In sheep receiving vancomycin infusions 3 times a day, average trough serum vancomycin values of $32 \pm 12 \mu\text{g/ml}$ and an average AUC of $571 \mu\text{g}\cdot\text{hour/ml}$ were achieved ($n=6$).

Bone and subcutaneous extracellular fluid (ECF) concentrations displayed persistently elevated concentrations, though greater variability was observed than in serum samples. Mean ECF concentrations were $13 \pm 4 \mu\text{g/ml}$ vancomycin, $1 \pm 2 \mu\text{g/ml}$ rifampin, and $8 \pm 3 \mu\text{g/ml}$ co-trimoxazole.

Radiographic and histological imaging of infection. Radiographs were taken after primary surgery, revision surgeries 1 and 2, and at euthanasia. The postoperative radiographs confirmed appropriate placement of the intramuscular nail and lack of iatrogenic fracture (Fig. 2A). Radiographs taken at revision surgery 1 exhibited signs of osteolysis and thickening of the cortical bone due to the infection, primarily localized to the area where the inoculum was introduced (asterisk in Fig. 2B). At revision 2 and euthanasia, each sheep had commenced or completed surgical and medical therapy, and no further signs of increased infection severity were observed (radiographs not shown).

Bone biopsy specimens were additionally taken from sheep for histopathology to confirm that a chronic infection was present in this model. After Brown and Brenn staining, positively stained coccoid bacteria were observed in numerous locations (Fig. 2C and D). Specifically, bacteria were found in bone marrow abscesses (Fig. 2C) and inside osteocyte lacunae (Fig. 2D). The histopathology stains do not reveal the viability of the bacteria at the time of biopsy and are therefore not considered diagnostic for infection (bacterial culture results shown below).

***In vitro* antibiotic release profile and antibacterial efficacy of hydrogel.** The release of gentamicin and vancomycin from the antibiotic-loaded hydrogel was

FIG 1 Local (ECF) and systemic (serum) antibiotic concentrations of vancomycin, rifampin, and co-trimoxazole in sheep. Sheep received antibiotic doses as follows. (A) One gram vancomycin was diluted administered over 1 h in 100 ml saline, twice a day. (B) Six hundred milligrams rifampin were administered over 1 h in 500 ml saline, twice a day. (C) Co-trimoxazole (15 mg/kg body weight, 0.6 ml per 10 kg per infusion) was administered as slow intravenous push. Extracellular fluid was taken twice per day by ultrafiltration for up to 14 days (filled circles, intramedullary probe samples; empty circles, soft tissue probe samples), and serum was taken at regular intervals.

measured *in vitro* (Fig. 3). In the first 24 h, 74% of the gentamicin and 65% of the vancomycin were released. A continued release of antibiotics was observed during the first 4 days, with the relative amount of vancomycin released being higher than gentamicin. The release rate diminished thereafter (Fig. 3A and B). After 14 days, most of the antibiotics were depleted from the hydrogel, with a residual amount extracted below 0.5%.

Antibacterial activity (Fig. 3C) was assessed by exposing bacteria to antibiotics in phosphate-buffered saline (PBS), empty hydrogel, or antibiotic-loaded hydrogel. When exposed to antibiotic-loaded hydrogel, no viable bacteria were detected at any time point. In the PBS and in the empty hydrogel, bacteria were present at all time points, and their number declined slightly over time. Representative images of a zone of inhibition surrounding 20 μ l of antibiotic-free (left) or antibiotic-loaded (right) hydrogel are shown in Fig. 3D.

Efficacy of antibiotic-loaded hydrogel in treatment of chronic MRSA ODRI.

Animal welfare and monitoring as well as weight change and white blood cell count (WBC) are presented in supplemental text and Fig. S1 in the supplemental material. The average inoculum given to each sheep was 1.6×10^7 ($\pm 0.57 \times 10^7$), with no differences seen between the three groups ($P \geq 0.05$). At revision surgery 1, where all groups were as yet untreated, bacteriological data revealed similar bacterial burden in all animals at each location (Fig. 4A). Furthermore, at revision surgery 2, no significant

FIG 2 Characterization of the infection induced in sheep, confirming radiographic (A and B) and histological (C and D) features of osteomyelitis. (A) Mediolateral projection radiographs illustrating the postoperative situation, with intramedullary nail and physiological appearance of bone. (B) Eight weeks later before revision surgery, clear radiographic signs of infection are visible, e.g., cortical thickening (asterisks) and periosteal reaction (arrowhead). Histological evaluation of biopsy specimens removed during debridement confirm classical features of osteomyelitis. (C and D) Bacteria were observed in bone samples taken from the distal bolt hole during revision surgery 1 in both the intramedullary space (C) and in the cortical bone (D). Scale bar in panels C and D, 20 μ m. Left images, Brown and Brenn staining; right images, hematoxylin and eosin staining.

differences were found between groups for total bacterial counts of hardware or bone marrow samples (Fig. 4B). At euthanasia (Fig. 4C), all samples from the 6 sheep in the negative-control group were culture positive, which proves that in this model, debridement, suboptimal systemic antibiotics (co-trimoxazole without vancomycin), device exchange, and absence of local antibiotic therapy could not treat the infection. The standard-of-care MRSA therapy resulted in 3 sheep being completely culture negative ($P=0.0259$ compared to experimental group). Seven out of a total of 24 biopsy specimens taken from the 6 sheep in this group were culture positive. The 6 sheep receiving antibiotic-loaded hydrogel were all culture negative at euthanasia, with none of the 24 taken samples positive for bacterial growth. All bacteria isolated from infected sheep at euthanasia were found to be *S. aureus* by latex agglutination test. A standard gentamicin zone of inhibition test on isolates recovered at revision surgery 2 and at euthanasia of the culture-positive sheep in the standard-of-care group yielded identical zones, indicating antibiotic susceptibility did not change during treatment (data not shown).

Systemic antibiotic concentrations in sheep receiving hydrogel. In the infected sheep, serum gentamicin and vancomycin concentrations arising from the hydrogel were measured after administration of the antibiotic-loaded hydrogel at revision surgery 1 (Fig. 5). Six hours postoperation, peak gentamicin values were measured ($0.85 \pm 0.33 \mu\text{g/ml}$). No gentamicin could be measured from 3 days after surgery. Vancomycin concentrations were around or below the detection limit in all samples.

DISCUSSION

Failure of current medical and surgical practices to reliably eradicate ODRI represents a personal burden for the affected patient and significant socioeconomic cost for health care systems. Improved treatment modalities targeting ODRI are therefore urgently required. In this study, we describe a large animal model of a chronic MRSA ODRI. Furthermore, the model combines the current best practice in medical and surgical treatment utilizing human implants and surgical instruments, a known systemic and local pharmacokinetic profile of current clinical antibiotic therapy, and,

FIG 3 *In vitro* release profiles and antibacterial activity of the antibiotic-loaded hydrogel. (A and B) Gentamicin (A) and vancomycin (B) release profiles from the hydrogel loaded with 1% gentamicin and 4% vancomycin. Columns depict the concentration of released antibiotic into the medium per milliliter; dotted lines show the cumulative release of antibiotics with the total released antibiotic set as 100%. (C) *In vitro* antibacterial efficacy of antibiotic-loaded hydrogel against planktonic MRSA exposed to either hydrogel loaded with both vancomycin and gentamicin, empty hydrogel, or PBS. Total bacterial counts were performed at 24, 72, and 168 h. Error bars depict the standard error of the mean. *, $P < 0.05$. (D) Zones of bacterial inhibition after exposure to empty hydrogel (left) or antibiotic-loaded hydrogel (right). Gent, gentamicin; Vanc, vancomycin.

importantly, a clinically realistic treatment failure rate when treated with current clinical practices. As such, the model reliably recapitulates chronic ODRI and the difficulty in treating MRSA infections. Using this model, we could show that the dual application of a gentamicin- and vancomycin-loaded hydrogel completely eradicated the infection as part of a combined medical and surgical treatment approach. As such, the data represent a robust preclinical assessment of efficacy.

The use of a hydrogel to deliver antibiotics brings several advantages for treatment of ODRI. It produces a high local antibiotic level at the target site and a safe systemic level. The material is easily handled and manipulated, degrades, and allows repeat administration. The hydrogel used in this study achieved a 100% eradication of infection that exceeds the success rates of the current best medical and surgical practice, i.e., ALBC. The increased efficacy of hydrogel over ALBC likely stems from two important features, drug release and degradability. In the first case, drug release from the hydrogel is rapid, as we show both *in vitro* and *in vivo*. As a concentration-dependent antibiotic, the killing efficacy of gentamicin is maximized with high concentrations rather than prolonged release. *In vitro*, a relatively rapid release from the hydrogel was observed, and *in vivo*, we see a short period of systemic exposure, also indicative of a burst release. Although release from ALBC also displays a burst release in the initial days, numerous *in vitro* studies have shown that only 10 to 20% of loaded gentamicin is released from ALBC (18). Therefore, despite equivalent loading amounts in hydrogel and ALBC, the ALBC will release a lower peak concentration, a lower total amount of released antibiotic, and, importantly, a longer tail of subinhibitory concentrations. The lower peak is likely to reduce the maximum bacterial killing activity; however, the longer tail in antibiotic release may be even more of an issue, as it is associated with increased risk of inducing resistance (19). In each of these parameters, the hydrogel outperforms ALBC according to our current understanding of ideal

FIG 4 Bacteriology outcome of treatment of ODRI infection in sheep. (A) Bacteriology after first revision surgery; (B) bacteriology after second revision surgery; (C) bacteriology after euthanasia. *, $P < 0.05$ compared to the negative control. HW, hardware; ST, soft tissue; CB, cortical bone; BM, bone marrow.

pharmacodynamics and generation of antibiotic tolerance/resistance and likely contributes to the observed improved *in vivo* efficacy of the hydrogel.

The second aspect we consider responsible for the outperformance of the hydrogel over ALBC lies in the degradability of the hydrogel. As a nondegradable material, ALBC must either be surgically removed or left *in situ* permanently. Permanent placement of

FIG 5 Systemic antibiotic concentrations in sheep receiving antibiotic-loaded hydrogel. Serum gentamicin (A) and vancomycin (B) concentrations were measured in infected sheep after administration of antibiotic-loaded hydrogel in the medullary canal of the tibia. Note that systemic antibiotic therapy with vancomycin commenced at 3 days.

ALBC is generally not done due to the aforementioned risk of development of resistance (19). However, as a degradable material, the hydrogel may be applied to any surgical site, allowing wound closure and no requirement to reoperate for removal. In a staged treatment approach of a chronic ODRI, where the interval between stages is ideally free of any foreign material, the hydrogel allows application at both initial revision surgery and a second application around the definitive fracture fixation device, as was performed in this study. In effect, the drug delivery vehicle clearance allows successive injections, doubling the total amount and total duration of antibiotic delivery to the local wound area.

It is not possible to differentiate between the elevated concentrations and the second application as the key to the eradication of the infection. Success of antibiotic therapy has traditionally recognized the importance of duration of treatment, and this may suggest that the repeated application is the key feature of success. However, it remains to be determined if the hydrogel may be applied once in a one-stage exchange procedure. Such experiments are required to determine this, but it would be an attractive prospect that would eliminate the need for the second revision surgery.

Additional benefits of using a hydrogel that are not explicitly addressed in this study include the ability to load it with a broad range of antimicrobials to potentially address a wide range of pathogens. ALBC undergoes an exothermic curing process that generates heat detrimental to many heat-sensitive antibiotics, including many beta-lactams and rifampin (27). Therefore, using a hydrogel as delivery vehicle greatly expands the range of antibiotics, with the possibility of adapting to the pathogen involved. This hydrogel has previously been loaded with the antibiotic rifampin and the antiseptic chlorhexidine (28). The use of salts such as gentamicin sulfate can interfere with the lower critical solution temperature (LCST) of the thermoresponsive element in the hydrogel, a phenomenon known as the Hoffmeister effect (29), and this is known to reduce the LCST of the hydrogel used in this study (30). However, a change in transition temperature does not impact the delivery or applicability of the hydrogel, as it remains injectable and solidifies at body temperature, and so, a wide range of antimicrobials are likely readily loaded within the hydrogel as described.

There are alternative bioresorbable delivery systems available on the market (20, 31). Calcium sulfate (CS) is the most commonly used resorbable material for local delivery of antibiotics in orthopedic medicine (17). The efficacy of local tobramycin delivered from CS pellets is comparable with handmade polymethylmethacrylate (PMMA) beads for treatment of chronic osteomyelitis or infected nonunion (21, 32). CS has advantages over PMMA in that the material can carry a wider range of antibiotics and does not need a second surgery to remove them, as CS pellets take approximately 2 to 3 months to radiographically resorb (32). The most commonly reported complication of the use of CS is aseptic seroma formation and drainage (33, 34), which we did

not observe with the use of a hydrogel in our model. It is also possible to apply antibiotics locally without any carrier material. Local aqueous gentamicin and tobramycin administration in wound cavities at the time of the index surgical procedure has been shown to lower infection rates in open fractures (35). Furthermore, aqueous antibiotic solution injected locally after wound closure is a simple delivery method that has demonstrated positive results in animal and clinical models (36). The main disadvantage of locally administered antibiotics without a carrier is that there is no controlled delivery of antibiotics directly into target tissues and no sustained release over time. Biodegradable carriers such as hydrogel overcome this issue.

One of the concerns of systemic antibiotic therapy is the risk of systemic exposure. In particular, gentamicin has been associated with oto- and nephrotoxicity at high concentrations (37).

In the present study, similar to the observed *in vitro* release, gentamicin serum concentration peaks at 24 h after hydrogel administration but was an order of magnitude below the clinical threshold for toxicity. After 3 days, systemic concentrations were below the detection threshold. This confirms that systemic exposure after local application does not pose a long-term risk for systemic toxicity. Locally, of course, concentrations would be much higher, potentially making local toxicity a concern. *In vitro* studies have shown limited cytotoxicity for vancomycin on osteoblasts; however, of the aminoglycosides, tobramycin was found to be less toxic than gentamicin (38), which may suggest tobramycin may be a more suitable candidate should local toxicity be a major concern. In a previous *in vivo* study, with a gentamicin-loaded hydrogel, we could not see any significant impact of hydrogel application on fracture healing (22), suggesting that the hydrogel is safe to apply both from a local and systemic perspective.

The *in vitro* release of gentamicin was more rapid during the first 24 h than vancomycin, which displayed a more sustained release, especially between day 1 and day 4. Both observations may be attributed to the smaller size and higher water solubility of gentamicin than vancomycin. It may be that vancomycin, as the larger molecule, takes more time to diffuse out of the molecular network. Despite the higher overall concentration (1% [wt/wt] for gentamicin and 4% [wt/wt] for vancomycin), systemic vancomycin levels were nearly undetectable in the serum. Again, this could be attributed to the molecular differences bringing to different diffusion through body tissues. At the end of the *in vitro* study, no antibiotic was detectable in the hydrogel, yet not all vancomycin initially loaded was detected. Vancomycin degradation has been reported *in vivo*, which can lead to an underestimation of vancomycin concentrations when high-performance liquid chromatography (HPLC) is used for quantification (39, 40).

In this study, we investigated the general pharmacokinetic parameters or targets for antibiotic therapy for MRSA ODRI. Since no such therapy had previously been applied to sheep, this was a necessary step to ensure all groups, particularly the one receiving current clinical practice, received adequate antibiotic therapy. This also ensured that treatment did not fail due to unexpected kinetics in sheep. Vancomycin is a key antibiotic in the treatment of MRSA infection. Adequate dosing is confirmed with trough concentrations of 15 to 20 $\mu\text{g/ml}$ or a 24-h vancomycin AUC of 400 $\mu\text{g}\cdot\text{hour/ml}$ (41), with dose adjustment based on AUC considered more accurate (41, 42) and safer with regard to nephrotoxicity risk (42). A vancomycin 24-h AUC of $\geq 677 \mu\text{g}\cdot\text{hour/ml}$ has been associated with an approximate 3- or 4-fold increased nephrotoxicity risk in humans (43). In our animal model, AUC values were below dosing guidelines when vancomycin was administered twice a day but reached target AUC when given three infusions per day (571 $\mu\text{g}\cdot\text{hour/ml}$). With the three times daily dosing, the average trough serum values of $32.3 \pm 11.9 \mu\text{g/ml}$ is above the human target range, though as mentioned above, AUC-based dosing may be a more reliable indicator. Nevertheless, renal function was not examined in this study and remains a limitation of the study.

The pharmacokinetic evaluation of rifampin is more challenging due to the lack of widely acknowledged targets. Peak serum concentrations of rifampin in humans after an

oral dose of 600 mg are around 10 $\mu\text{g/ml}$ (44, 45), which matches the concentrations measured in our model ($15.0 \pm 4.9 \mu\text{g/ml}$), suggesting that rifampin therapy is broadly similar to the human standard of care, even when given intravenously (i.v.) rather than via oral route. A particularly valuable data set from this study is the local ECF concentrations of antibiotics measured by ultrafiltration. The concentrations measured in the intramedullary (i.m.) canal reflect an average value over the sampling period and do not reveal peaks and troughs in high resolution; however, the average concentrations of systemically delivered vancomycin and co-trimoxazole of 10 to 15 $\mu\text{g/ml}$ are well above MIC values and indicative of adequate antibiotic therapy. The lower concentrations of rifampin are likely underestimated due to the lack of preservative (to prevent oxidation) in the standard ultrafiltration tubes.

Although the present model is quite far advanced in comparison with others in the field, a number of other models for treatment of ODRI have been described, most notably in mice, rats, and rabbits (25, 46). Issues with many of the described models include the absence of antibiotic-resistant pathogens, the use of a short-term infection development phase and thus lack of a chronic infection, and the use of nonstandard antibiotic selection, dosing, or duration. Typically, these animal models do not involve implant exchange, another cornerstone of treatment of chronic ODRI, as it removes any biofilm-colonized implants and necrotic tissue. In general, the closer the animal model recapitulates the clinical condition, the more robust the assessment of efficacy (47). Choosing a sheep model over smaller species offers a number of key benefits, but also some challenges. Advantages of using large animals include the opportunity to use clinically available human implants and surgical instruments. Sheep have a body weight similar to humans, so human-scale antibiotic therapy could be applied. The scale and dimensions of infected tissue and an 8-week maturation phase mirror the clinical condition where infection cannot be easily localized to a predefined area. This relates quite closely to human conditions. However, the tolerability of sheep for certain antibiotics used in human clinics is unknown, and oral antibiotics are an issue for ruminants, as their uptake in the stomach is different than in humans. Lastly, acquiring and housing sheep is more resource intensive than smaller animals and requires animal caretakers and surgeons to have the appropriate expertise.

In developing this model, certain compromises were made to minimize the burden to the animals. First, there was no fracture and soft tissue trauma involved, as soft tissue and fracture healing would significantly complicate all aspects of the surgical approaches, although soft tissue and fracture healing can influence the local inflammation and impact upon infection (48, 49). On the other hand, the absence of soft tissue trauma and a fracture increases reliability and the relatability of the model to other forms of ODRI. A second compromise was that antibiotic therapy did not start immediately after surgery, as may occur for a human patient, but after 24 h to allow sheep to recover from surgery. This was deemed necessary for animal welfare reasons. The duration of antibiotic exposure in this study was a total of 6 weeks. Clinical guidelines currently suggest from 6 to 12 weeks of antibiotic therapy for chronic ODRI. It may be that treatment would show better efficacy if extended to 12 weeks; however, the comparison between hydrogel and ALBC is instructive at the tested duration and may indicate that shorter antibiotic exposure times could be used in conjunction with local antibiotics using a hydrogel as delivery vehicle. Shorter systemic antibiotic exposure would have both cost and patient benefits (50), as extended antibiotic therapy is known to impact the host microbiome (51), which is crucial for many host health aspects (52). One final limitation of the present data set is that hydrogel degradation kinetics and the fate of its component hemagglutinin (HA) and poly(*N*-isopropylacrylamide) (pNIPAM) were not directly evaluated. As expected, no remnants of the hydrogel were macroscopically visible at the end of the study. Unmodified hyaluronic acid is degraded and resorbed within hours or days upon injection due to enzymatic digestion (53, 54), whereas the thermoresponsive component pNIPAM is nonbiodegradable. Furthermore, given the brush copolymer structure of hydrogel and the noncovalent nature of the temperature-sensitive physical cross-linking, HA degradation is sufficient

FIG 6 Overview of timeline and treatment protocols for the two-stage exchange treatment of chronic MRSA ODRI in sheep. (A) An intramedullary nail served as orthopedic device placed in sheep tibia, and MRSA was inoculated into the intramedullary cavity and allowed to develop for 8 weeks. (B) Negative-control and standard-of-care groups received treatment that included removal of the nail and placement of a temporary ALBC spacer (green) for 2 weeks followed by spacer removal and reimplantation of a new i.m. nail (purple). (C) In the hydrogel group, treatment included removal of the nail and placement of 20 ml of antibiotic-loaded hydrogel (blue) for 2 weeks, after which a new nail was also introduced alongside a second application of hydrogel. (D) All groups received systemic antibiotic therapy as shown. *, previously applied treatment known to be tolerated but not pathogen-adapted (56); **, pathogen (MRSA)-adapted antibiotic therapy according to Depypere et al. (11); RSx1, first revision surgery to remove implant and debride i.m. canal; RSx2, second revision surgery to place a new i.m. nail; Gent, gentamicin; Vanc, vancomycin.

to trigger the gel dissolution, and past *in vitro* studies have confirmed that the hydrogel is not cytotoxic to human fibroblasts or human carcinoma cells (54, 55).

MATERIALS AND METHODS

Study design. In developing a clinically relevant large animal model of chronic MRSA ODRI with medical and surgical treatment matching human guidelines, the first step was to perform an antibiotic tolerability and pharmacokinetic study to ensure that adequate antibiotic administration was given to the sheep. Furthermore, in order to confirm that the key features of chronic ODRI pathology were seen in this model, we evaluated radiographic and histological features of the infection 8 weeks after inoculation.

The second aim of this study was to evaluate treatment efficacy of an antibiotic-loaded thermoresponsive, hyaluronic acid-based hydrogel in this model. This included *in vitro* evaluation of drug release and antibacterial efficacy, followed by an *in vivo* study comparing treatment of chronic MRSA ODRI (outlined in Fig. 6). The three treatment groups included a negative control, previously shown to be an effective treatment against methicillin-sensitive *S. aureus* (MSSA) and expected to fail in this MRSA model (56); an MRSA-targeted antibiotic regimen based on the current standard of care for human patients with systemic antibiotics and ALBC (39); and a group that received the same MRSA-targeted systemic antibiotic therapy and the antibiotic-loaded hydrogel. A group size of 6 was selected to allow an effect size of 70% with 80% power and a significance level of 0.05. All available data are presented in the manuscript, and no outliers or otherwise excluded for any reason. Animal welfare conditions and exclusion criteria are described below and in supplemental text.

Ethical approval and animal care. Institutional review board approval for this study was granted by the ethical committee of the canton of Grisons in Switzerland (34/2013, 41/2016, and 07/2018). The experiments were performed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved facility and according to Swiss laws and regulations for animal protection. A total of 24 skeletally mature (2 to 4 years old) female Swiss Alpine sheep were included in this study, including 6 in the safety and pharmacokinetics study (average preoperative weight, 58.6 kg; range, 55.5 to 66.5 kg) and 19 in the infection study (average preoperative weight, 73.1 kg; range, 60 to 86 kg). One sheep in the infection study was replaced after the first surgery due to a postoperative complication (constipation) unrelated to the study goals and was replaced. Prior to inclusion, each sheep underwent physical examination, including complete blood count and a radiographic screening to ensure the tibial canal could accommodate the i.m. nail. The sheep were group housed for at least 2 weeks before

surgery for acclimatization to the housing conditions. The sheep were kept in an environment with daily cycles of 12 h light and dark and were fed twice per day with hay, a mineral lick, and hand-fed grain.

Safety and pharmacokinetics of antibiotic therapies. In order to evaluate tolerability and pharmacokinetics of antibiotic therapy, 6 sheep received infusions of a regimen appropriate for human treatment of MRSA ODR1, which includes vancomycin (1 g per infusion; Sandoz Pharmaceuticals AG, Rotkreuz, Switzerland), rifampin (600 mg per infusion; Labotec Pharma SA, Meyrin, Switzerland), and co-trimoxazole (trimethazol, 200 mg/ml sulfamethoxazole, and 40 mg/ml trimethoprim; 0.6 ml per 10 kg per infusion; Werner Stricker AG, Zollikofen, Switzerland). Rifampin is usually administered orally in humans, which is not suitable for sheep (ruminants), and so, all sheep received i.v. infusions of all 3 antibiotics. For the infusions, venous access was maintained throughout the study with a 16G catheter inserted into the jugular vein using the Seldinger technique. Vancomycin was diluted in 10 ml sterile water for injections and administered over the course of 1 h in 100 ml 0.9% saline (1 g per infusion). Rifampin was diluted in 10 ml sterile water and administered over 1 h in 500 ml 0.9% saline (600 mg per infusion). Aqueous co-trimoxazole was given as a slow intravenous push (0.6 ml per 10 kg per infusion).

Sheep were closely monitored by veterinarians for adverse effects during each infusion. Blood was taken daily before infusion and 1 and 4 h postinfusion. At each time point, blood was collected from the jugular vein in serum-separating tubes. After centrifuging the blood for 10 min at 2,500 rpm, serum was collected and stored at -20°C . In addition, extracellular fluid from the i.m. canal and adjacent subcutaneous tissue was collected in the morning and evening for a maximum of 10 days through ultrafiltration membranes (BASi Bioanalytical Systems, West Lafayette, IN, USA) placed surgically prior to the start of infusions. In brief, under general anesthesia and analgesia, a small skin incision was made on the medial side, and a 4.3-mm hole was drilled in the tibia slightly distal to proximal orientation. The filtration loops were inserted in the bone, and the distal end was tunneled through the skin to exit on a point lateral and distal to the incision. The exit points were covered with gauze pads and a bandage. The collection tubes were replaced every morning and evening for as long as possible or for a maximum of 10 days and then stored at -20°C .

Antibiotic quantification in serum and extracellular fluid. The antibiotic concentration in serum or extracellular fluid samples was measured in a single batch after thawing. Each sample was mixed with acetonitrile, vortexed, and then centrifuged at $4,000 \times g$ at 9°C for 10 min in order to precipitate proteins. The clear supernatant was diluted with an equal volume of PBS and filtered.

For gentamicin quantification, 50 μl of each sample were added to 100 μl of 2-propanol and 50 μl of o-phthalaldehyde (OPA) reagent and gently shaken for 45 min at room temperature protected from light (57). The OPA reagent was prepared by dissolving 20 mg of OPA in 1 ml of ethanol and adding to 19 ml of 0.4 M boric acid solution, pH 10.4, and 0.4 ml of 2-mercaptoethanol. The absorbance at 332 nm was read with a plate reader and the concentration determined through a calibration curve of freshly prepared gentamicin sulfate standards. Each sample was analyzed in duplicate.

Vancomycin was measured using a high-performance liquid chromatography (HPLC) Agilent 1260 Infinity unit with quaternary pump, autosampler, UV-visible (UV-vis) detector, and column temperature controller. Each sample was filtered before being loaded into the autosampler. The analysis was run based on previously published methods (39). The mobile phase was NH_4PO_3 (pH 4)/acetonitrile 92/8, isocratic elution at 1 ml/min. The column was a Phenomenex Kinetex 5- μm XB-C18 100A, 150 by 4.6 mm. The detection wavelength was 220 nm, and column temperature was 30°C . The concentration was determined via a calibration curve of freshly prepared vancomycin standards.

For co-trimoxazole, the mobile phase consisted of a mixture of Milli-Q water/methanol/acetic acid (79/20/1 [vol/vol/vol]) at 1.0 ml/min for 15 min; otherwise, the same protocol as for vancomycin was used. Co-trimoxazole contains two active ingredients, sulfamethoxazole (at 200 mg/ml) and trimethoprim (at 40 mg/ml). Trimethoprim and sulfamethoxazole were eluted after ± 6.0 and 11.0 min, respectively, and detected with a UV detector at 254 nm. Concentrations presented represent the sum of both antibiotics.

For rifampin, the mobile phase consisted of a mixture of Milli-Q water (with 0.2% trifluoroacetic acid [TFA]) and acetonitrile (50/50 [vol/vol]) at a flow rate of 1.0 ml/min for 10 min. Rifampin was eluted after ± 5.6 min and detected with a UV detector at 345 nm. To prevent rifampin oxidation, serum samples were supplemented with ascorbic acid at 100 $\mu\text{g/ml}$ (58). Supplementation of the vacuum tubes for ultrafiltration collection was not possible.

Preparation of thermoresponsive hyaluronic acid-based hydrogel. The poly(*N*-isopropylacrylamide)-grafted thermoresponsive hyaluronic acid hydrogel was prepared as described previously (22, 30). Briefly, pNIPAM with an M_n of 23.5 kDa was grafted to HA with an M_w of 1.64 MDa via amide formation in dimethyl sulfoxide (DMSO) to achieve a molar degree of substitution (DS) of 4%. The lyophilized product was sterilized with ethylene oxide. The antibiotic-loaded hydrogel was prepared by dissolving the polymer to a final concentration of 11% (wt/wt) using PBS supplemented with 1% (wt/wt) gentamicin sulfate and 4% (wt/wt) vancomycin under sterile conditions and mixing overnight at 4°C . Identical preparations were used for the *in vitro* and *in vivo* studies.

***In vitro* antibiotic release from hydrogel.** Approximately 500 mg of the antibiotic-loaded hydrogel was weighed and placed into a dialysis tube with a molecular weight cutoff of 12 to 14 kDa. Tubes were submerged in 8 ml of PBS and gently shaken at 37°C . At each time point, 1 ml of solution was withdrawn and stored at -20°C , and 1 ml PBS added to replace the sampled fluid. Samples were taken at 3 h, 6 h, 24 h, 1 day, 2 days, 4 days, 7 days, and 14 days. The gentamicin sulfate concentration in the samples was determined with an OPA assay (57), and the vancomycin concentration was determined via HPLC as described above. Results were normalized to the effective weight of each sample, accounting for the

dilution of the release medium at each time point. At the final time point, the hydrogels were disrupted in water to measure residual antibiotic within the hydrogel. Each sample was analyzed in triplicate.

In vitro antibacterial activity of antibiotic-loaded hydrogel. The *in vitro* antibacterial efficacy of the antibiotic-loaded hydrogel was evaluated by inoculating 198 μ l of hydrogel with 2 μ l bacterial suspension ($2.7 \times 10^7 \pm 1.8 \cdot 10^7$ CFU) and incubating at 37°C. Hydrogels were either empty or loaded with antibiotics at the concentration used in the *in vivo* study. Antibacterial efficacy was assessed at days 1, 3, and 7 by adding 1.5 ml cold PBS (to transition the hydrogel to a liquid state) to the hydrogel and immediately vortexing, serially diluting in PBS, and plating on blood agar plates. Experiments were repeated 3 times, with 3 replicates each time.

Sheep model of chronic MRSA ODRI and antibiotic treatments. The surgical protocols for the two-stage exchange of hardware due to MRSA infection have been adopted from our previous large animal model (lacking MRSA-adapted antibiotic therapy) (56). Briefly, after inducing anesthesia and analgesia (as previously reported), a unicortical hole (4.3-mm drill bit; DePuy Synthes, Bridgewater, NJ, USA) was made through the medial cortex of the left tibia to facilitate introduction of the bacterial inoculum into the i.m. canal. A medial parapatellar approach was utilized to gain access to the extrasynovial entry point for the i.m. nail. The entry point for the nail was cranial to the joint between the patellar tendon and the anterior edge of the medial meniscus. The medullary canal was opened using a cannulated awl (10 mm; Depuy Synthes). A 190-mm-long, 7.5-mm-diameter unreamed solid humeral nail (UHN; Depuy Synthes) was inserted, and the distal of the two proximal locking bolt holes received an interlocking bolt (3.9 mm; Depuy Synthes). The collagen sponge with the inoculum (prepared as described below) was placed into the inoculation hole, and all tissues were sutured closed.

At the first revision surgery, the entry point in the tibial plateau was reopened with a cannulated awl, and the locking bolt and nail were removed. Debridement of the i.m. canal of the tibia was performed using a femoral canal brush (Smith and Nephew AG, London, UK). The brush, nail, and bolt were processed for quantitative bacteriology to confirm infection was present in each animal upon commencement of treatment. Treatments were applied in 6 sheep per group as per Fig. 6.

At the second revision surgery, the spacers/hardware were removed and sampled for microbiological analysis. The i.m. canal was debrided as described for revision surgery 1, and the brush was used for quantitative bacteriology of the bone marrow. A new sterile nail was inserted, and the more proximal of the two proximal locking bolt holes received an interlocking bolt.

Antibiotic-loaded bone cement. The ALBC nails were fabricated by coating a 1.1-mm stainless steel cleaning stylet (DePuy Synthes, Switzerland) with polymethylmethacrylate (PMMA; Copal G+V; Heraeus). The cement was prepared according to the manufacturer's instructions, and a 6.5-mm endotracheal tube was used as a mold. The cement nails contained approximately 170 mg gentamicin and 670 mg vancomycin. The exact amount depended upon the final length of the spacer, which was tailored to the length of the tibia of each individual sheep.

Thermoresponsive hyaluronic acid-based hydrogel. For the *in vivo* study, the i.m. canal of the left tibia was filled with 20 ml antibiotic-loaded hydrogel, prepared as described above. A small amount of hydrogel was displaced from the i.m. canal during nail insertion. Twenty milliliters of hydrogel contain 200 mg gentamicin and 800 mg vancomycin.

Systemic antibiotic therapy. All sheep received ceftiofur (2.2 mg/kg i.v.) after induction, prior to the primary surgery, with additional doses administered during surgeries lasting longer than 1.5 h.

The sheep in the negative-control group received an antibiotic regimen previously used to treat MSSA infection in this model. These sheep received 2 weeks of systemic co-trimoxazole. Infusions were given every 24 h (subcutaneous [s.c.], 15 mg/kg body weight; Borgal 24%; MSD Animal Health GmbH, Switzerland).

The standard-of-care and hydrogel groups received MRSA-targeted systemic therapy and received vancomycin infusions (1 g per infusion) twice a day, starting 24 h after revision surgery 1. From days 5 to 14, sheep received three vancomycin infusions per day (every 8 h) based on therapeutic drug monitoring recommendations arising from the pharmacokinetics (PK) study. After revision surgery 2, sheep received rifampin and co-trimoxazole infusions, as described in the safety and pharmacokinetics study above, for 4 weeks.

All sheep were euthanized after a 2-week antibiotic holiday to ensure no residual antibiotics were left in the tissues that may lead to false-negative results.

Bacteriology. The bacteria used to inoculate all sheep was MRSA EDCC 5443 (German collection of microorganisms, DSM number 29134). This is a human clinical isolate and is resistant to amoxicillin and clavulanic acid and susceptible to gentamicin, vancomycin, and co-trimoxazole. The inoculum was prepared as previously described (56), whereby 20 μ l of a bacterial suspension was added to a collagen sponge (TissuFleece E; Baxter AG, Volketswil, Switzerland) yielding an expected target inoculum of 1.0×10^7 CFU per animal. Quantitative bacteriological analysis was performed on the bacterial solution that was used for each inoculum.

Quantitative bacteriology was also performed on biopsy specimens at both revision surgeries and euthanasia. At revision surgery 1, the interlocking bolt and i.m. nail (collectively termed hardware) and the femoral canal brush were removed intraoperatively and placed in sterile PBS. At revision surgery 2, any hardware and the femoral canal brush were similarly placed in sterile PBS. After euthanasia, soft tissue samples and cortical bone biopsy specimens were taken from the interlocking bolt holes (distal and proximal) and the inoculation point. Furthermore, bone marrow was collected. In all cases, the bone biopsy specimens and bone marrow samples were weighed and homogenized in PBS using an Omni-TH handheld homogenizer (LabForce AG, Switzerland). All hardware was sonicated for 5 min. The samples were 10-fold serially diluted and plated onto blood agar (BA) plates (Oxoid AG, Pratteln, Switzerland)

containing 5% defibrinated horse blood (TCS Biosciences Ltd., Buckingham, UK). Agar plates were incubated at 37°C, and colonies were counted after 24 and 48 h of incubation. CFU counts were obtained from each sample, and data from the same material or source were combined into single values (i.e., hardware) to simplify data presentation.

In vivo observations. Sheep were weighed and blood samples were taken just before primary surgery, the first and second revision surgeries, and euthanasia. Total white blood cell count (WBC) was performed on whole blood samples within 1 h after collection using an electronic cell counter (Vet abc; scil animal care, Viernheim, Germany). Mediolateral and craniocaudal radiographs of the left tibia were taken before and after each surgery and then every other week and at euthanasia. Radiographs were taken while sheep were sedated (0.04 mg/kg i.m. of detomidine; Dr. E. Graeb AG, Bern, Switzerland). A clinical scoring system was used to assess the burden based upon key parameters, including systemic signs of infection, ongoing lameness, elevated temperature, WBC, or loss of more than 10% body weight. Animals reaching predefined scores would be euthanized.

Histology. Bone biopsy specimens were taken from the proximal bolt hole at the first revision surgery. The biopsy specimens were fixed in 70% methanol, decalcified with EDTA, and finally embedded in paraffin. Sections were cut and stained with hematoxylin and eosin (H&E) for tissue morphology and Brown and Brenn (BB) for bacteria.

Statistical analysis. The *in vitro* experiment, weights, WBC, and bacteriology were analyzed with a Kruskal-Wallis test with Dunn's multiple-comparison test using GraphPad (version 8.1.0). The primary outcome parameter was total bacterial numbers at euthanasia. For all tests, *P* values below 0.05 were considered significant.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.02 MB.

ACKNOWLEDGMENTS

We thank all the staff of the Preclinical Facility at ARI Davos for their help in conducting this study. We also thank Virginia Post, Iris Keller, and Pamela Furlong from ARI for technical support.

This work was supported by AOTrauma as part of the Clinical Priority Program (CPP) Bone Infection (grant number AR2016_09).

W.B. performed laboratory analysis and data analysis and cowrote the manuscript. T.S. performed surgical procedures, performed antibiotic administration and animal welfare monitoring, and cowrote the ethics application. M.D. performed laboratory analysis, analyzed data, and cowrote the manuscript. O.G. performed laboratory analysis and cowrote the manuscript. A.F. performed laboratory analysis, supported antibiotic administration, and cowrote the manuscript. L.D. performed laboratory and data analyses and approved the final manuscript. R.G.R. administered project funding, coapplied for funding, and approved the final manuscript. D.E. cowrote the grant application, oversaw laboratory studies, and cowrote the manuscript. S.Z. cowrote the grant application; oversaw all animal experiments, including ethical application, surgery, animal, and welfare; and cowrote the manuscript. T.F.M. cowrote the grant application, oversaw laboratory studies, and cowrote the manuscript.

We declare there are no competing interests of significance to the presented work.

REFERENCES

1. Darouiche RO. 2004. Treatment of infections associated with surgical implants. *N Engl J Med* 350:1422–1429. <https://doi.org/10.1056/NEJMr035415>.
2. Trampuz A, Zimmerli W. 2006. Diagnosis and treatment of infections associated with fracture-fixation devices. *Injury* 37:S59–S66. <https://doi.org/10.1016/j.injury.2006.04.010>.
3. Papakostidis C, Kanakaris NK, Pretel J, Faour O, Morell DJ, Giannoudis PV. 2011. Prevalence of complications of open tibial shaft fractures stratified as per the Gustilo-Anderson classification. *Injury* 42:1408–1415. <https://doi.org/10.1016/j.injury.2011.10.015>.
4. Schwarz EM, Parvizi J, Gehrke T, Aiyer A, Battenberg A, Brown SA, Callaghan JJ, Citak M, Egol K, Garrigues GE, Ghert M, Goswami K, Green A, Hammond S, Kates SL, McLaren AC, Mont MA, Namdari S, Obremskey WT, O'Toole R, Raikin S, Restrepo C, Ricciardi B, Saeed K, Sanchez-Sotelo J, Shohat N, Tan T, Thirukumaran CP, Winters B. 2019. 2018 International Consensus Meeting on Musculoskeletal Infection: research priorities from the general assembly questions. *J Orthop Res* 37:997–1006. <https://doi.org/10.1002/jor.24293>.
5. Metsemakers WJ, Morgenstern M, Senneville E, Borens O, Govaert GAM, Onsea J, Depypere M, Richards RG, Trampuz A, Verhofstad MHJ, Kates SL, Raschke M, McNally MA, Obremskey WT, Fracture-Related Infection (FRI) Group. 2020. General treatment principles for fracture-related infection: recommendations from an international expert group. *Arch Orthop Trauma Surg* 140:1013–1027. <https://doi.org/10.1007/s00402-019-03287-4>.
6. Trampuz A, Widmer AF. 2006. Infections associated with orthopedic implants. *Curr Opin Infect Dis* 19:349–356. <https://doi.org/10.1097/01.qco.0000235161.85925.e8>.
7. Arciola CR, An YH, Campoccia D, Donati ME, Montanaro L. 2005. Etiology of implant orthopedic infections: a survey on 1027 clinical isolates. *Int J Artif Organs* 28:1091–1100. <https://doi.org/10.1177/039139880502801106>.

8. Portillo ME, Salvado M, Trampuz A, Siverio A, Alier A, Sorli L, Martinez S, Perez-Prieto D, Horcajada JP, Puig-Verdie L. 2015. Improved diagnosis of orthopedic implant-associated infection by inoculation of sonication fluid into blood culture bottles. *J Clin Microbiol* 53:1622–1627. <https://doi.org/10.1128/JCM.03683-14>.
9. Zitron R, Wajsfeld T, Klautau GB, da Silva CB, Nigro S, Mercadante MT, Polesello GC, Cury RP, Salles MJ. 2016. Concentration of sonication fluid through centrifugation is superior to membrane filtration for microbial diagnosis of orthopedic implant-associated infection. *J Clin Microbiol* 54:788–790. <https://doi.org/10.1128/JCM.02427-15>.
10. Trebse R, Pisot V, Trampuz A. 2005. Treatment of infected retained implants. *J Bone Joint Surg Br* 87:249–256. <https://doi.org/10.1302/0301-620X.87B2.15618>.
11. Depypere M, Kuehl R, Metsemakers WJ, Senneville E, McNally MA, Obrebsky WT, Zimmerli W, Atkins BL, Trampuz A, Fracture-Related Infection Consensus Group. 2019. Recommendations for systemic antimicrobial therapy in fracture-related infection: a consensus from an international expert group. *J Orthop Trauma* 34:30–41. <https://doi.org/10.1097/BOT.0000000000001626>.
12. Campoccia D, Montanaro L, Arciola CR. 2006. The significance of infection related to orthopedic devices and issues of antibiotic resistance. *Biomaterials* 27:2331–2339. <https://doi.org/10.1016/j.biomaterials.2005.11.044>.
13. Parry MC, Duncan CP. 2014. The challenge of methicillin resistant staphylococcal infection after total hip replacement: overlooked or overstated? *Bone Joint J* 96-B:60–65. <https://doi.org/10.1302/0301-620X.96B11.34333>.
14. Engemann JJ, Carmeli Y, Cosgrove SE, Fowler VG, Bronstein MZ, Trivette SL, Briggs JP, Sexton DJ, Kaye KS. 2003. Adverse clinical and economic outcomes attributable to methicillin resistance among patients with *Staphylococcus aureus* surgical site infection. *Clin Infect Dis* 36:592–598. <https://doi.org/10.1086/367653>.
15. Hake ME, Young H, Hak DJ, Stahel PF, Hammerberg EM, Mauffrey C. 2015. Local antibiotic therapy strategies in orthopaedic trauma: practical tips and tricks and review of the literature. *Injury* 46:1447–1456. <https://doi.org/10.1016/j.injury.2015.05.008>.
16. Inzana JA, Trombetta RP, Schwarz EM, Kates SL, Awad HA. 2015. 3D printed bioceramics for dual antibiotic delivery to treat implant-associated bone infection. *Eur Cell Mater* 30:232–247. <https://doi.org/10.22203/ecm.v030a16>.
17. Inzana JA, Schwarz EM, Kates SL, Awad HA. 2016. Biomaterials approaches to treating implant-associated osteomyelitis. *Biomaterials* 81:58–71. <https://doi.org/10.1016/j.biomaterials.2015.12.012>.
18. van de Belt H, Neut D, Schenk W, van Horn JR, van der Mei HC, Busscher HJ. 2000. Gentamicin release from polymethylmethacrylate bone cements and *Staphylococcus aureus* biofilm formation. *Acta Orthop Scand* 71:625–629. <https://doi.org/10.1080/000164700317362280>.
19. Neut D, van de Belt H, Stokroos I, van Horn JR, van der Mei HC, Busscher HJ. 2001. Biomaterial-associated infection of gentamicin-loaded PMMA beads in orthopaedic revision surgery. *J Antimicrob Chemother* 47:885–891. <https://doi.org/10.1093/jac/47.6.885>.
20. Foster AL, Moriarty TF, Trampuz A, Jaiprakash A, Burch MA, Crawford R, Paterson DL, Metsemakers WJ, Schuetz M, Richards RG. 2020. Fracture-related infection: current methods for prevention and treatment. *Expert Rev Anti Infect Ther* 18:307–321. <https://doi.org/10.1080/14787210.2020.1729740>.
21. McKee MD, Li-Bland EA, Wild LM, Schemitsch EH. 2010. A prospective, randomized clinical trial comparing an antibiotic-impregnated bioabsorbable bone substitute with standard antibiotic-impregnated cement beads in the treatment of chronic osteomyelitis and infected nonunion. *J Orthop Trauma* 24:483–490. <https://doi.org/10.1097/BOT.0b013e3181d9f1d9>.
22. Ter Boo GJ, Schmid T, Zderic I, Nehrbass D, Camenisch K, Richards RG, Grijpma DW, Moriarty TF, Eglin D. 2018. Local application of a gentamicin-loaded thermo-responsive hydrogel allows for fracture healing upon clearance of a high *Staphylococcus aureus* load in a rabbit model. *Eur Cell Mater* 35:151–164. <https://doi.org/10.22203/ecm.v035a11>.
23. Grainger DW, van der Mei HC, Jutte PC, van den Dungen JJ, Schultz MJ, van der Laan BF, Zaat SA, Busscher HJ. 2013. Critical factors in the translation of improved antimicrobial strategies for medical implants and devices. *Biomaterials* 34:9237–9243. <https://doi.org/10.1016/j.biomaterials.2013.08.043>.
24. Busscher HJ, Alt V, van der Mei HC, Fagette PH, Zimmerli W, Moriarty TF, Parvizi J, Schmidmaier G, Raschke MJ, Gehrke T, Bayston R, Baddour LM, Winterton LC, Darouiche RO, Grainger DW. 2019. A trans-Atlantic perspective on stagnation in clinical translation of antimicrobial strategies for the control of biomaterial-implant-associated infection. *ACS Biomater Sci Eng* 5:402–406. <https://doi.org/10.1021/acsbomaterials.8b01071>.
25. Vanvelk N, Morgenstern M, Moriarty TF, Richards RG, Nijs S, Metsemakers WJ. 2018. Preclinical in vivo models of fracture-related infection: a systematic review and critical appraisal. *Eur Cell Mater* 36:184–199. <https://doi.org/10.22203/ecm.v036a14>.
26. Rybak M, Lomaestro B, Rotschafer JC, Moellering R, Jr., Craig W, Billeter M, Dalovio JR, Levine DP. 2009. Therapeutic monitoring of vancomycin in adult patients: a consensus review of the American Society of Health-System Pharmacists, the Infectious Diseases Society of America, and the Society of Infectious Diseases Pharmacists. *Am J Health Syst Pharm* 66:82–98. <https://doi.org/10.2146/ajhp080434>.
27. Samara E, Moriarty TF, Decosterd LA, Richards RG, Gautier E, Wahl P. 2017. Antibiotic stability over six weeks in aqueous solution at body temperature with and without heat treatment that mimics the curing of bone cement. *Bone Joint Res* 6:296–306. <https://doi.org/10.1302/2046-3758.65.BJR-2017-0276.R1>.
28. Perez-Kohler B, Linardi F, Pascual G, Bellon JM, Eglin D, Guillaume O. 2020. Efficacy of antimicrobial agents delivered to hernia meshes using an adaptable thermo-responsive hyaluronic acid-based coating. *Hernia* 24:1201–1210. <https://doi.org/10.1007/s10029-019-02096-3>.
29. Freitag R, Garret-Flaudy F. 2002. Salt effects on the thermoprecipitation of poly-(N-isopropylacrylamide) oligomers from aqueous solution. *Langmuir* 18:3434–3440. <https://doi.org/10.1021/la0106440>.
30. Ter Boo GA, Arens D, Metsemakers WJ, Zeiter S, Richards RG, Grijpma DW, Eglin D, Moriarty TF. 2016. Injectable gentamicin-loaded thermo-responsive hyaluronic acid derivative prevents infection in a rabbit model. *Acta Biomater* 43:185–194. <https://doi.org/10.1016/j.actbio.2016.07.029>.
31. Metsemakers WJ, Fragomen AT, Moriarty TF, Morgenstern M, Egol KA, Zalavras C, Obrebsky WT, Raschke M, McNally MA, Fracture-Related Infection Consensus Group. 2020. Evidence-based recommendations for local antimicrobial strategies and dead space management in fracture-related infection. *J Orthop Trauma* 34:18–29. <https://doi.org/10.1097/BOT.0000000000001615>.
32. Ferguson JY, Dudareva M, Riley ND, Stubbs D, Atkins BL, McNally MA. 2014. The use of a biodegradable antibiotic-loaded calcium sulphate carrier containing tobramycin for the treatment of chronic osteomyelitis: a series of 195 cases. *Bone Joint J* 96-B:829–836. <https://doi.org/10.1302/0301-620X.96B6.32756>.
33. McLaren AC. 2004. Alternative materials to acrylic bone cement for delivery of depot antibiotics in orthopaedic infections. *Clin Orthop Relat Res* 427:101–106. <https://doi.org/10.1097/01.blo.0000143554.56897.26>.
34. Kumar CY, Bn K, Menon J, Patro DK, Hb B. 2013. Calcium sulfate as bone graft substitute in the treatment of osseous bone defects, a prospective study. *J Clin Diagn Res* 7:2926–2928. <https://doi.org/10.7860/JCDR/2013/6404.3791>.
35. Lawing CR, Lin FC, Dahners LE. 2015. Local injection of aminoglycosides for prophylaxis against infection in open fractures. *J Bone Joint Surg Am* 97:1844–1851. <https://doi.org/10.2106/JBJS.O.00072>.
36. Cancienne JM, Burrus MT, Weiss DB, Yarbboro SR. 2015. Applications of local antibiotics in orthopedic trauma. *Orthop Clin North Am* 46:495–510. <https://doi.org/10.1016/j.joc.2015.06.010>.
37. Wargo KA, Edwards JD. 2014. Aminoglycoside-induced nephrotoxicity. *J Pharm Pract* 27:573–577. <https://doi.org/10.1177/0897190014546836>.
38. Rathbone CR, Cross JD, Brown KV, Murray CK, Wenke JC. 2011. Effect of various concentrations of antibiotics on osteogenic cell viability and activity. *J Orthop Res* 29:1070–1074. <https://doi.org/10.1002/jor.21343>.
39. Jesus VM, Lopez FG, Navarro AS. 2008. Development and validation of an HPLC method for vancomycin and its application to a pharmacokinetic study. *J Pharm Biomed Anal* 48:835–839. <https://doi.org/10.1016/j.jpba.2008.05.040>.
40. Morse GD, Nairn DK, Bertino JS, Jr., Walshe JJ. 1987. Overestimation of vancomycin concentrations utilizing fluorescence polarization immunoassay in patients on peritoneal dialysis. *Ther Drug Monit* 9:212–215. <https://doi.org/10.1097/00007691-198706000-00015>.
41. Meng L, Wong T, Huang S, Mui E, Nguyen V, Espinosa G, Desai J, Holubar M, Deresinski S. 2019. Conversion from vancomycin trough concentration-guided dosing to area under the curve-guided dosing using two sample measurements in adults: implementation at an academic medical center. *Pharmacotherapy* 39:433–442. <https://doi.org/10.1002/phar.2234>.
42. Neely MN, Kato L, Youn G, Kraler L, Bayard D, van Guilder M, Schumitzky A, Yamada W, Jones B, Minejima E. 2017. Prospective trial on the use of trough concentration versus area under the curve to

- determine therapeutic vancomycin dosing. *Antimicrob Agents Chemother* 62:e02042-17. <https://doi.org/10.1128/AAC.02042-17>.
43. Zasowski EJ, Murray KP, Trinh TD, Finch NA, Pogue JM, Mynatt RP, Rybak MJ. 2017. Identification of vancomycin exposure-toxicity thresholds in hospitalized patients receiving intravenous vancomycin. *Antimicrob Agents Chemother* 62:e01684-17. <https://doi.org/10.1128/AAC.01684-17>.
44. Garnham JC, Taylor T, Turner P, Chasseaud LF. 1976. Serum concentrations and bioavailability of rifampicin and isoniazid in combination. *Br J Clin Pharmacol* 3:897-902. <https://doi.org/10.1111/j.1365-2125.1976.tb00644.x>.
45. Burman WJ, Gallicano K, Peloquin C. 2001. Comparative pharmacokinetics and pharmacodynamics of the rifamycin antibacterials. *Clin Pharmacokinet* 40:327-341. <https://doi.org/10.2165/00003088-200140050-00002>.
46. Reizner W, Hunter JG, O'Malley NT, Southgate RD, Schwarz EM, Kates SL. 2014. A systematic review of animal models for *Staphylococcus aureus* osteomyelitis. *Eur Cell Mater* 27:196-212. <https://doi.org/10.22203/ecm.v027a15>.
47. Moriarty TF, Harris LG, Mooney RA, Wenke JC, Riolo M, Zaat SAJ, Moter A, Schaer TP, Khanna N, Kuehl R, Alt V, Montali A, Liu J, Zeiter S, Busscher HJ, Grainger DW, Richards RG. 2019. Recommendations for design and conduct of preclinical in vivo studies of orthopedic device-related infection. *J Orthop Res* 37:271-287. <https://doi.org/10.1002/jor.24230>.
48. Sabate Bresco M, O'Mahony L, Zeiter S, Kluge K, Ziegler M, Berset C, Nehrbass D, Richards RG, Moriarty TF. 2017. Influence of fracture stability on *Staphylococcus epidermidis* and *Staphylococcus aureus* infection in a murine femoral fracture model. *Eur Cell Mater* 34:321-340. <https://doi.org/10.22203/ecm.v034a20>.
49. Merritt K, Dowd JD. 1987. Role of internal fixation in infection of open fractures: studies with *Staphylococcus aureus* and *Proteus mirabilis*. *J Orthop Res* 5:23-28. <https://doi.org/10.1002/jor.1100050105>.
50. Dudareva M, Kumin M, Vach W, Kaier K, Ferguson J, McNally M, Scarborough M. 2019. Short or Long Antibiotic Regimes in Orthopaedics (SOLARIO): a randomised controlled open-label non-inferiority trial of duration of systemic antibiotics in adults with orthopaedic infection treated operatively with local antibiotic therapy. *Trials* 20:693. <https://doi.org/10.1186/s13063-019-3832-3>.
51. Blaser MJ. 2016. Antibiotic use and its consequences for the normal microbiome. *Science* 352:544-545. <https://doi.org/10.1126/science.aad9358>.
52. D'Argenio V, Salvatore F. 2015. The role of the gut microbiome in the healthy adult status. *Clin Chim Acta* 451:97-102. <https://doi.org/10.1016/j.cca.2015.01.003>.
53. Lepperdinger G, Fehrer C, Reitingner S. 2004. Biodegradation of hyaluronan, p 71-82. In Garg HG, Hales CA (ed), *Chemistry and biology of hyaluronan*. Elsevier Science Ltd, Oxford, UK. <https://doi.org/10.1016/B978-008044382-9/50035-2>.
54. Mortisen D, Peroglio M, Alini M, Eglin D. 2010. Tailoring thermoreversible hyaluronan hydrogels by "click" chemistry and RAFT polymerization for cell and drug therapy. *Biomacromolecules* 11:1261-1272. <https://doi.org/10.1021/bm100046n>.
55. Vihola H, Laukkanen A, Valtola L, Tenhu H, Hirvonen J. 2005. Cytotoxicity of thermosensitive polymers poly(N-isopropylacrylamide), poly(N-vinylcaprolactam) and amphiphilically modified poly(N-vinylcaprolactam). *Biomaterials* 26:3055-3064. <https://doi.org/10.1016/j.biomaterials.2004.09.008>.
56. Moriarty TF, Schmid T, Post V, Samara E, Kates S, Schwarz EM, Zeiter S, Richards RG. 2017. A large animal model for a failed two-stage revision of intramedullary nail-related infection by methicillin-resistant *Staphylococcus aureus*. *Eur Cell Mater* 34:83-98. <https://doi.org/10.22203/ecm.v034a06>.
57. Heriot M, Nottelet B, Garric X, D'Este M, Richards GR, Moriarty FT, Eglin D, Guillaume O. 2019. Interaction of gentamicin sulfate with alginate and consequences on the physico-chemical properties of alginate-containing biofilms. *Int J Biol Macromol* 121:390-397. <https://doi.org/10.1016/j.ijbiomac.2018.10.025>.
58. Guillaume O, Garric X, Lavigne JP, Van Den Berghe H, Coudane J. 2012. Multilayer, degradable coating as a carrier for the sustained release of antibiotics: preparation and antimicrobial efficacy in vitro. *J Control Release* 162:492-501. <https://doi.org/10.1016/j.jconrel.2012.08.003>.