Antimicrobial Mechanisms and Preparation of Antibiotic-impregnated Cement-coated Locking Plates in the Treatment of Infected Non-unions

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ABSTRACT

Background: Antibiotic-impregnated cement-coated plates (ACPs) have been used successfully for temporary internal fixation between stages in the two-stage treatment of infected non-unions. We describe our approach of using an ACP in the staged treatment of a methicillin-resistant *Staphylococcus aureus* (MRSA)-infected distal femoral non-union below a total hip prosthesis. In addition, we present the results of an *in vitro* experiment to provide an in-depth insight into the capacity of ACPs in (i) treating residual biofilm and (ii) preventing bacterial recolonisation.

Materials and methods: In the first stage, we used a titanium LISS plate coated with hand-mixed PALACOS with vancomycin (PAL-V) for temporary internal fixation combined with commercially prepared COPAL with gentamicin and vancomycin (COP-GV) to fill the segmental defect. In the second stage, the non-union was treated with double-plate fixation and bone grafting.

A Kirby–Bauer agar disc diffusion assay was performed to determine the antimicrobial activity of both ACPs and a drug-release assay to measure antibiotic release over time. A biofilm killing assay was also carried out to determine if the antibiotic released was able to reduce or eradicate biofilm of the patient's MRSA strain.

Results: At one-year follow-up, there was complete bone-bridging across the previous non-union. The patient was pain-free and ambulatory without need for further surgery. Both ACPs with COP-GV and PAL-V exerted an antimicrobial effect against the MRSA strain with peak concentrations of antibiotic released within the first 24 hours. Concentrations released from COP-GV in the first 24 hours *in vitro* caused a 7.7-fold log reduction of colony-forming units (CFU) in the biofilm. At day 50, both COP-GV and PAL-V still released concentrations of antibiotic above the respective minimal inhibitory concentrations (MIC), likely contributing to the positive clinical outcome.

Conclusion: The use of an ACP provides stability and infection control in the clinical scenario of an infected non-union. This is confirmed *in vitro* where the release of antibiotics from ACPs is characterised by an early burst followed by a prolonged sustained release above the MIC until 50 days. The burst release from COP-GV reduces CFU in the biofilm and prevents early recolonisation through synergistic activity of the released vancomycin and gentamicin.

Clinical significance: An antibiotic-impregnated cement-coated plate is a useful addition to the surgeon's armamentarium to provide temporary internal fixation without the disadvantages of external fixation and contribute to infection control in an infected non-union.

Keywords: Antibiotic, Cement, Infection, *In vitro*, Non-union, Osteosynthesis, Plate.

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INTRODUCTION

An infected non-union of the femur is a major complication that can lead to severe mental, physical and socioeconomic problems.[1,2] Treatment requires multiple surgical interventions and frequent hospital admissions.[3] A successful strategy consists of eradication of the infection using meticulous debridement and antibiotics whilst maintaining a mechanically stable environment.[4] Once the infection is cleared, attention is directed towards obtaining osseous healing with revision osteosynthesis with or without bone grafting. This implies a strategy with a multidisciplinary team.[5]

Stability between the stages of treatment is often provided by external fixation because it minimises hardware within the infected area and thereby reduces the risk of biofilm formation and persistence of infection.[6] The disadvantages of external fixation are poor control of periarticular fragments, difficult pin placement around periprosthetic non-unions and ensuing stiffness of adjacent joints. From a patient's perspective, external fixation is cumbersome, especially on the femur. As an alternative, it is possible to use the low-profile technique of supercutaneous plating with locking screws

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inserted percutaneously into the bone with reported good results in the tibia.[7,8] For a femur, the placement of a supercutaneous locking plate is not practical, given the relatively thick muscular envelope.

Another well-established technique is coating an intra-medullary nail (IMN) with polymethylmethacrylate (PMMA) cement containing a high concentration of antibiotics. The coated IMN provides stability, and the local antibiotics treat the infection and reduce the risk of biofilm formation.[9–11] However, an IMN has no role in a metaphyseal-infected non-union or in non-unions where a prosthesis is already in place. In such clinical scenarios, Liporace et al. were the first to describe the use of a locking plate coated with antibiotic-impregnated cement.[12] Since then, multiple reports have been published on the use of ACPs in the treatment of various infected bone defects.[13–18] Such series have demonstrated high infection clearance and bone union rates with this approach.[13,14] Despite the overall clinical success, 9–17% of patients have recurrence of infection after the first stage and require additional surgical intervention. The antimicrobial mechanisms of the ACP may play an important role in determining treatment failure or success. Therefore, to better understand the mechanisms that contribute to the clinical results associated with the use of ACPs, we aimed to assess (1) the role of ACPs in treating residual biofilm present after debridement and (2) the capacity of ACPs to prevent novel colonisation of the ACP. Combined with an illustrative case report, we investigated antimicrobial mechanisms by assessing the release kinetics and biofilm-killing capacities of two types of ACPs that we have used in our own clinical practice in a 'bedside-to-bench' *in vitro* experiment.

## Illustrative Case

Patient History

Our patient was a 62-year-old female who was referred to our institution with an infected non-union of the right distal femur below a total hip prosthesis. She had sustained the initial fracture one and a half years before and had already undergone 3 failed surgeries at another hospital. At the time of her presentation to us, she was wheelchair-bound. The non-union was stabilised with an external fixator (Fig. 1A). She had a CRP of 34 mg/L, an erythrocyte sedimentation rate (ESR) of 58 mm/hr and a leucocyte count of 8×10^9/L. Cultures of the purulent fluid from the pin sites grew methicillin-resistant *Staphylococcus Aureus* (MRSA). A two-staged revision was planned.

Technique – Stage One

In this first stage, the non-union was debrided thoroughly, and this included opening the medullary cavities on either side of the non-union. There were large holes and defects in the distal femur (Fig. 2A). Deep tissue cultures were obtained and 500-mg IV vancomycin was administered. A titanium LISS plate was coated with PALACOS cement (Heraeus Nederland BV, Breda, The Netherlands), to which we added 2.5 gm of vancomycin per 40 gm of cement. The choice of the plate depends on the location of the non-union and local availability and costs. We chose a titanium LISS plate as this plate is readily available in our institution, relatively cheap and provides sufficient stability for temporary internal fixation. The entire plate is covered with cement of approximately 3–4 mm thick. A cement gun is used to apply the cement directly on both sides of the plate, and then the cement is distributed over the plate manually and with a spatula. The cement should be 'doughy' to facilitate easy application. To protect obstruction of the screw
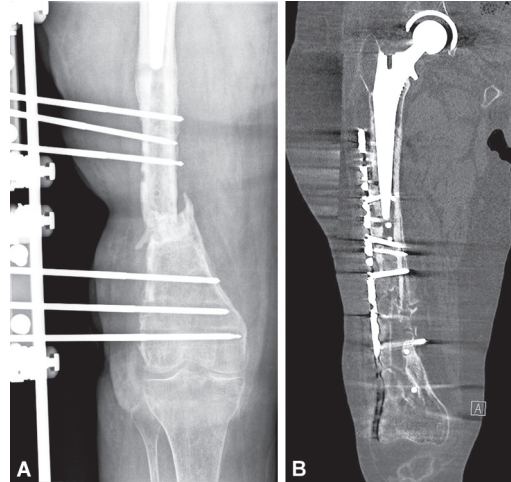
Figs 1A and B: (A) Anteroposterior radiograph of the right femur with the external fixator *in situ* at the first presentation to our institution; (B) Coronal CT-scan image taken 2 years after the second stage. There is bone bridging across the non-union site

holes, locking drill sleeves are applied to the plate (Fig. 2B). In order to coat the entire plate with a single batch of cement, it is important to ensure that sufficient drill sleeves are available, and this may require opening a second tray to access extra drill sleeves. The plate is then fixed to the aligned non-union with locking screws. At this stage, the screw heads are not yet coated as the cement has already hardened. In our case, we used commercially prepared antibiotic COPAL G+V (Heraeus Nederland BV, Breda, The Netherlands) to fill the segmental bone defect and to cover the screw heads (Fig. 2C). The choice of antibiotic cement should depend on the likely sensitivities of the suspected bacteria. The coating on the plate is used primarily to prevent novel biofilm formation. Although the release may exert an antibiotic effect in the soft tissues, we do not expect the antibiotics to reach the medullar cavity. The cement that is used to fill the segmental defect is—in combination with systemic antibiotics—primarily used as an antibiotic delivery agent to treat the infection. Postoperatively, our patient received 3 gm/24 hours of IV vancomycin. Two days after the surgery, all five culture samples grew MRSA. Intravenous vancomycin was continued for 1 week and then switched to oral levofloxacin 500-mg 2dd and rifampicin 450-mg 2dd for a total duration of 12 weeks.

Technique – Stage Two

The second stage is usually performed 6–8 weeks after the first stage.[19] However, due to the COVID-19 pandemic, the second stage was postponed and eventually took place 10 months after the first stage. At the time of the second stage, the wound had healed completely and the patient was pain-free. In the second stage, the plate and cement were removed (Fig. 3A). A small osteotome was used to 'chip-off' the cement from the plate. Removal of screws may be difficult; it is important to have an extraction set with various
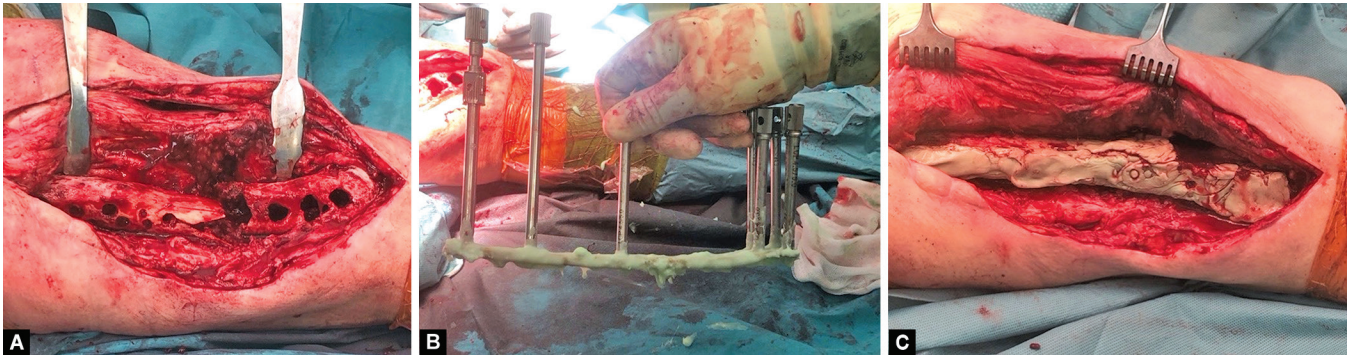
Figs 2A to C: Intraoperative images of the first stage. (A) The holes left by the external fixator and previous internal fixation are clearly visible; (B) The plate is covered with cement, and the screw holes are 'protected' with a locking drill sleeve to prevent obstruction of the holes; (C) The antibiotic-impregnated cement-coated locking plate *in situ*
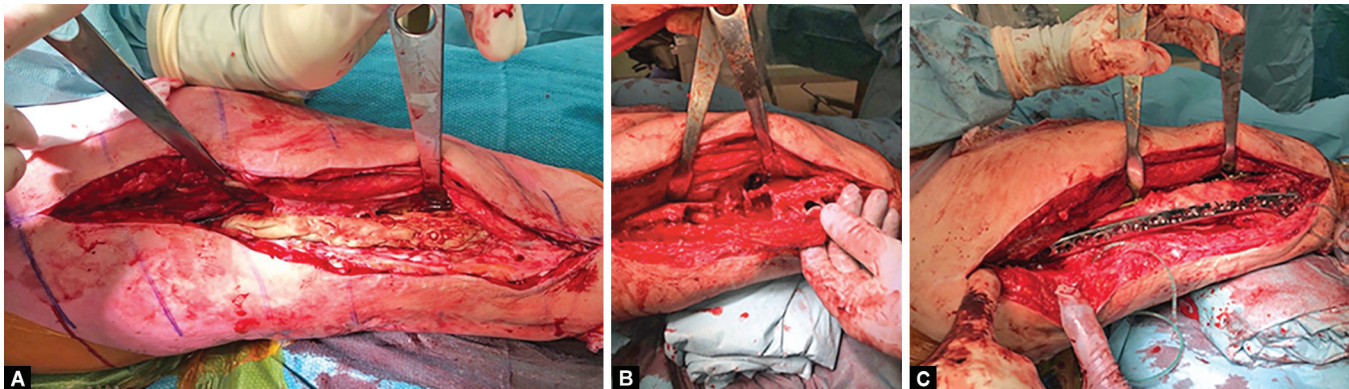
Figs 3A to C: Case I: Intraoperative images of the second stage. (A) Before removal of the antibiotic plate; (B) After removal of the plate and opening of the medullary cavities. One of the cortical bone grafts is shown in the hand of the surgeon; (C) After placement of the stainless-steel VA distal femur LCP and demineralised bone matrix around the non-union

instruments to facilitate removal available. After implant removal, deep culture samples were obtained. A routine dose of 2 gm of IV cefazolin was administered prophylactically. The medullary cavities were opened again, and a Judet quadricepsplasty was carried out to release adhesions. Two large cortical grafts were harvested from both anterior iliac crests, and an allograft femoral head was cut to size. The grafts were placed intramedullary in and across the non-union and into the anterolateral defect (Fig. 3B). A stainless-steel VA distal femur LCP (DePuy Synthes) was fixated laterally with hybrid fixation, and a titanium 4.5 mm LCP was placed anteriorly for fixation of the bone grafts (Fig. 3C). Finally, 10-cc demineralised bone matrix was placed around the non-union. Postoperatively, 3 gm/24 hours of IV vancomycin was administered for 2 weeks. As all cultures from the second stage were negative, no further antibiotic treatment was needed thereafter.

Outcome
At 2-months follow-up, the patient did not experience pain, the wound had healed and the CT-scan images showed early bone bridging (Fig. 1B). At 1 year follow-up, there was complete bone bridging. The patient remained pain-free and was ambulatory.

## MATERIALS AND METHODS
Material Preparation
Ethical approval for this study was waived by the Medical Ethics Review Committee of the Academic Medical Center (reference number W21_582 #22.026), and this study was conducted in accordance with the Declaration of Helsinki. Stainless steel one-third tubular plates (DePuy Synthes, Amersfoort, the Netherlands) were cut into two-hole plate segments. To standardise the amount of antibiotic cement on each plate segment, a custom-made aluminum mould with 10 compartments, each fitting a single-plate segment, was designed together with silicone moulds that fitted the tubular shape and screw holes of the plate segments (Fig. 4).

We prepared three formulations of cement, two with antibiotics similar to the cements used *in vivo* and one as control without antibiotics: (1) standard PALACOS LV (Heraeus Nederland BV, Breda, The Netherlands) mixed with 2 gm of vancomycin (Hikma Pharmaceuticals, London, United Kingdom) per 40 gm of cement powder, (2) commercially prepared antibiotic COPAL G+V (Heraeus Nederland BV, Breda, The Netherlands) cement containing 0.5 gm of gentamicin and 2.0 gm of vancomycin per 40 gm of cement powder and (3) standard PALACOS LV (Heraeus Nederland BV, Breda, The Netherlands) without antibiotics. The batches were mixed under sterile conditions with a mixing system without a vacuum and then applied with a small surplus into each compartment containing a plate segment. Pressure was applied with a glass plate to drive out excess cement (Fig. 4C). After hardening, the ACPs (Fig. 4D) were sterilised for 45 minutes under UV light.

Kirby–Bauer Assay
A Kirby–Bauer agar diffusion assay was performed to determine the antimicrobial activity of the ACPs against the MRSA strain from
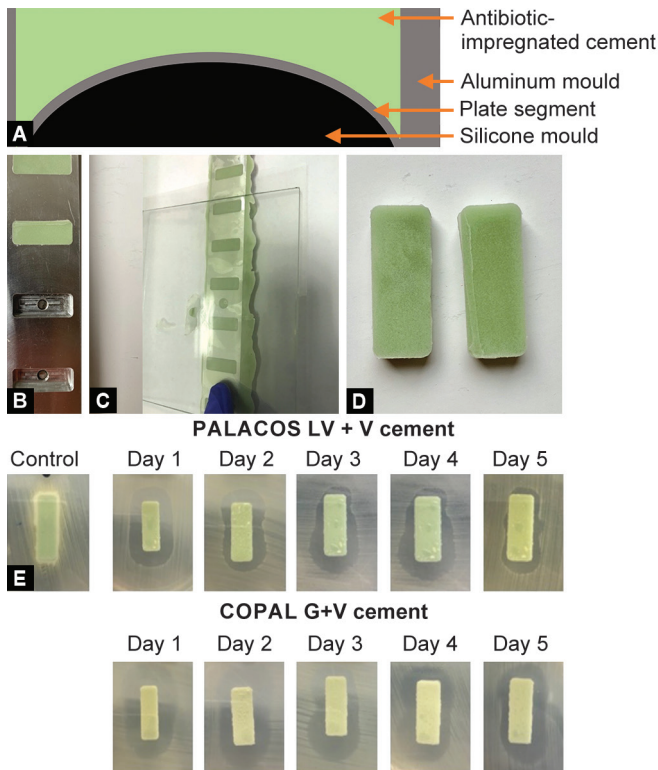
Figs 4A to E: (A) Graphical example of the set-up. A silicone mould is placed on the bottom of each compartment to prevent leaking of excess cement through the screw holes of the plate segments; (B) Custom-made aluminum mould with two coated plate segments in situ as an example. On the bottom of each compartment a screw is placed to press out the plate segment after coating; (C) Pressure is applied on the mould with a glass plate to drive out excess cement; (D) Antibiotic-impregnated cement-coated plate segments; (E) Visual example of the zones of inhibition for the plate segments coated with PALACOS LV + V cement or COPAL G+V cement. Pictures are taken from the bottom side of the agar plate. The coated plate segments were transferred daily to fresh agar plates seeded with the MRSA strain isolated from our patient. Results for the first 5 days of the experiment are shown

the non-union of the patient as measured by the zone of inhibition (ZOI).[20,21]

A suspension of five fresh colonies of the MRSA strain from a blood agar plate in 5 mL of phosphate buffer saline (PBS, ThermoFisher) was prepared. Mueller Hinton agar plates were seeded in two directions with a swab soaked in the bacterial suspension. Five ACPs per cement formulation (15 ACPs in total) were carefully placed on separate agar plates using tweezers and incubated at 37 °C for 24 hours. After 24 hours, inhibition zones at eight positions around the plate were measured using a calliper and photographed (Figs 4E and 5A) and averaged for each ACP. The average values were used to calculate the average and standard deviations for the five ACPs per cement formulation. Subsequently, the ACPs were placed on new agar plates that were prepared as above. This process was repeated daily for 28 days.

In Vitro Drug Release Assay

A drug release assay in 3 mL of PBS was performed to measure the amount of gentamicin and vancomycin released from COPAL G + V and PALACOS LV + V over time, and to compare the concentrations released with the minimal inhibitory concentration (MIC) of the strain from our patient as a measure of antimicrobial capacity, and with the minimum biofilm eradication concentration (MBEC) as a measure of biofilm-killing capacities. Three ACPs for each antibiotic of the antibiotic-impregnated cement formulations (9 ACPs in total) were prepared. All ACPs without and with cement were weighed with a microbalance (Sartorius, Germany) before starting the procedure. Each ACP was subsequently placed in a 15 mL Falcon tube with 3 mL of PBS, fully covering the ACP with liquid, and then incubated at 37°C and 120 rpm. The PBS was exchanged at 1 hour, 6 hours, 24 hours, 2 days, 3 days, 4 days, 6 days, 8 days, 10 days, 12 days and 15 days, and subsequently with 4-day intervals until 50 days. The eluates were stored at –20°C for further analysis. The amount of released vancomycin was quantified with quartz cuvettes by a UV–Vis spectrometer based on UV absorption at 280 nm. The amount of gentamicin was measured with the O-phthalaldehyde reagent (Sigma-Aldrich, US). The eluates from the plates without antibiotics served as controls. A calibration curve was plotted for vancomycin (10–200 μg/mL with $R^2 = 0.9974$) and gentamicin (1–50 μg/mL with $R^2 = 0.9912$) to estimate the concentration of drug released (Fig. 4C). Moreover, MICs for gentamicin and vancomycin for the MRSA strain were determined by VITEK.

Checkboard Biofilm-killing Assay

A checkboard biofilm-killing assay was performed to determine if specific concentrations of vancomycin and gentamicin were able to reduce and eradicate the biofilm. A colony taken from the MRSA isolate of the patient from a blood agar plate (Oxoid, United Kingdom) was suspended in 5 mL of Tryptic Soy Broth (TSB, Oxoid, Germany) and incubated overnight at 37°C and 120 rpm. The next day, a mid-logarithmic growth-phase culture was made by inoculating 100 μL of the overnight culture into a fresh tube with 5 mL of TSB, which was incubated for 3 hours at 37°C and 120 rpm. An inoculum suspension of 10^7 CFU/mL in TSB with 1% of glucose (Merck, USA) was prepared from the mid-logarithmic culture based on the optical density at 620 nm. From this bacterial suspension, 100 μL were pipetted into each well of a flat-bottom 96-well plate (Greiner bio-one, Austria), and the plate was incubated at 37 °C in a metal box with humidified atmosphere for 24 hours. The next day, each well was carefully washed twice with PBS to remove planktonic bacteria, and subsequently, 200 μL of concentrations of vancomycin and gentamicin in TSB with 1% of glucose were added to the remaining biofilms following a checkboard pattern. The plate was incubated at 37°C in the metal box with humidified atmosphere for 24 hours. After this incubation, the medium was removed and 100 μL of PBS per well were added. Then, the 96-well plate was sealed with parafilm and placed inside a plastic bag, and sonicated for 5 minutes floating in an ultrasonic water bath (Elma Transsonic T460, Germany). The sonicate in each well was subsequently serially diluted and plated on blood agar plates in order to quantify the numbers of CFUs.

RESULTS

Kirby–Bauer Assay

The vancomycin-loaded PALACOS LV + V showed a ZOI until 20 days and none thereafter (Fig. 5B). The gentamicin- and vancomycin-loaded COPAL G+V showed a ZOI until the end of the experiment at 28 days (Fig. 5B). Consequently, the activity might have lasted longer. As expected, the non-loaded cement did not form any ZOI.
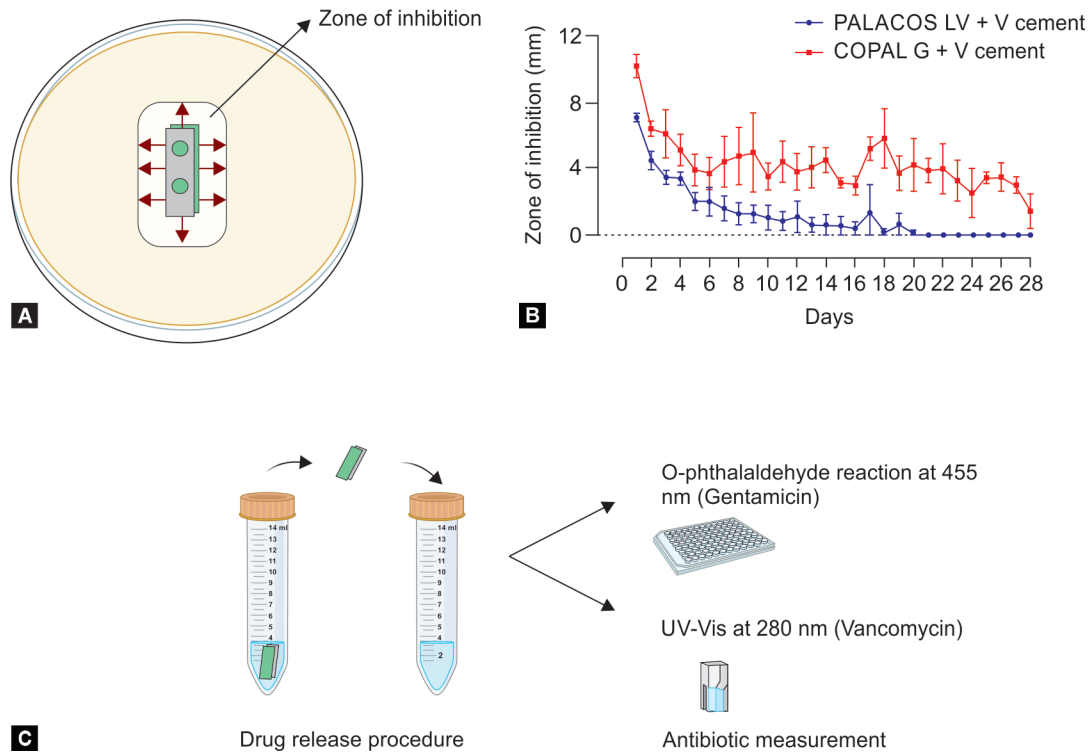
Figs 5A to C: (A) Schematical presentation of the zone of inhibition measurement; (B) Average of the zones of inhibition (in mm) on agar plates seeded with the MRSA clinical strain around the plate segments with either PALACOS LV + V cement or COPAL G+V cement. The plate segments were transferred to fresh inoculated plates each day for 28 consecutive days ($n = 5$ per antibiotic formulation); (C) Schematical presentation of the drug release assay. Eluates were collected and stored at –20ºC for further analysis. The amount of gentamicin was measured with the O-phthalaldehyde reagent, and the amount of released vancomycin was quantified with quartz cuvettes by a UV–Vis spectrometer based on UV absorption at 280 nm

In Vitro Drug Release Assay

Release of antibiotic from the ACPs with the two cements formulations was studied in a setup where the ACPs were submerged in a volume of 3 mL PBS (Figs 4 and 5C). Vancomycin release from PALACOS LV + V showed a burst release of 754 µg (251 µg/mL) in the first hour, followed by a release of 768 µg (256 µg/mL) at 6 hours and 599 µg (200 µg/mL) at 24 hours (Fig. 5D). Then, vancomycin release decreased from 225 µg (75 µg/mL) at 48 hours to 21 µg (4 µg/mL) at day 29. At 50 days, the release was 34 µg (11 µg/mL). Vancomycin was not detected at 44 and 47 days. Finally, 3252 µg (12%) of the total amount of vancomycin was released (Fig. 5E).

The release of vancomycin from COPAL G+V cement showed a burst of 694 µg (231 µg/mL) at 1 hour, followed by 273 µg (91 µg/mL) and 211 µg (70 µg/mL) at 6 and 24 hours, respectively (Fig. 5F). Vancomycin release decreased from 157 µg (52 µg/mL) at 48 hours to 34 µg (11 µg/mL) at day 44. At day 50, the release was 63 µg (21 µg/mL). Finally, 2574 µg (9%) of the incorporated vancomycin was released (Fig. 5G).

Gentamicin from COPAL G+V cement showed a burst release in the first 48 hours ranging from 322 to 353 µg (108–118 µg/mL) at 1, 6, 24, and 48 hours and a gradually decreasing release thereafter (Fig. 5H). There was a second smaller peak at 19 days of 135 µg (45 µg/mL). The minimal gentamicin release was 30 µg (10 µg/mL) at day 44. At day 50, the release was 66 µg (22 µg/mL). An average of 3063 µg (22%) of the incorporated gentamicin was released (Fig. 5I).

The VITEK analysis indicated that the MRSA strain had an MIC of 0.5 µg/mL for gentamicin and 1 µg/mL for vancomycin.

### Checkboard Biofilm-killing Assay

The checkboard biofilm-killing assay showed that vancomycin alone, even at 2048 µg/mL, did not reduce MRSA biofilm CFU. Gentamicin alone did provide some reduction of the biofilm CFU numbers at 256 µg/mL. The combination of both antibiotics had a synergistic effect. For biofilm CFU reduction, a combination of 32 µg/mL vancomycin and 64 µg/mL gentamicin resulted in 4-log numbers of CFU reduction. Complete biofilm eradication occurred for vancomycin concentrations of 256 –2048 µg/mL when combined with 512 µg/mL of gentamicin (Fig. 6).

DISCUSSION

There is no standard treatment for patients with an infected non-union of the femur. The choice of treatment depends on the location of the non-union, the type of fixation used previously, the identified bacteria and their antibiotic susceptibility, the general condition of the patient, and the expertise and preference of the treating surgeon. The mainstay of treatment is surgical debridement during which necrotic and infected bone and soft tissues are removed and complemented by local and systemic antibiotic therapies.[4]

Over the past two decades, the use of ACP has been popularised to provide (temporary) stability and local antibiotics in the setting of an infected bone defect.[12,14,15,22] Compared with intramedullary
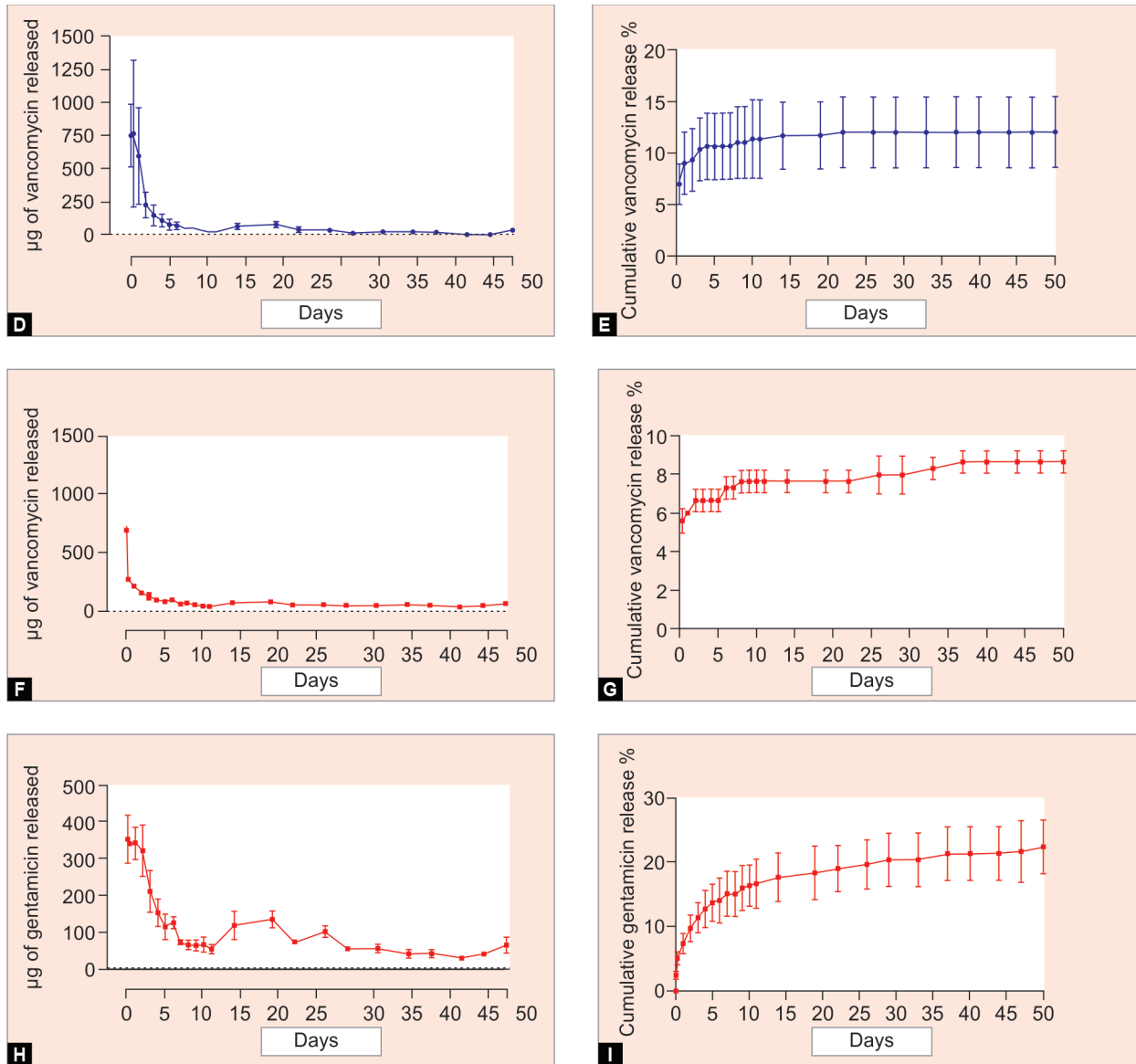
Figs 5D to I: (D) Amount of vancomycin released (in μg) PALACOS LV + V; (E) Cumulative release (in w/w% of the original drug loading) over time up to 50 days from the PALACOS LV + V (n = 3); (F) Amount of vancomycin released (in μg) COPAL G + V; (G) Cumulative release (in w/w% of the original drug loading) over time up to 50 days from the COPAL G + V cement (n = 3); (H) Amount of gentamicin released (in μg); (I) Cumulative release (in w/w% of the original drug loading) over time up to 50 days from the COPAL G+V cement (n = 3)

nails that are coated with antibiotic-impregnated cement, plates can be used for periprosthetic non-unions. Furthermore, removal of nails may be difficult, and detachment of PMMA in the medullary canal is common.[23] The antibiotics that are incorporated in PMMA need to be heat-stable as PMMA polymerises with an exothermic reaction up to 80°C. Gentamicin, vancomycin and tobramycin are frequently incorporated in bone cement because of their thermostability and high local antimicrobial activity. The elution of antibiotics from PMMA has extensively been studied.[24] It is a process of diffusion, with the antibiotic then being transported to the surrounding tissue by a concentration gradient. Elution profiles differ based on the antibiotics used, the surface area and the mixing process.[25] Cements to which antibiotics are added manually show higher peak concentrations.[26,27] The release is also

more irregular compared with commercially available antibiotic cement, as hand-mixing may cause 'clumps' of antibiotics in the cement that, when released, cause spikes in the release. Hand-mixing allows customising the PMMA with one or more antibiotics, based on antibiotic susceptibly of the infecting bacteria, and is a more economical strategy. Antibiotics may be added in powder or in liquid form. Adding liquid antibiotics may increase elution properties, although it negatively affects mechanical properties of the cement.[28] The choice, therefore, depends on the location and purpose of the ACP and may also be affected by local availability.

Local administration of antibiotics in biomaterials like PMMA provides a higher concentration of antibiotics at the fracture site than when the antibiotics are given systemically with minimal toxic effects.[29]
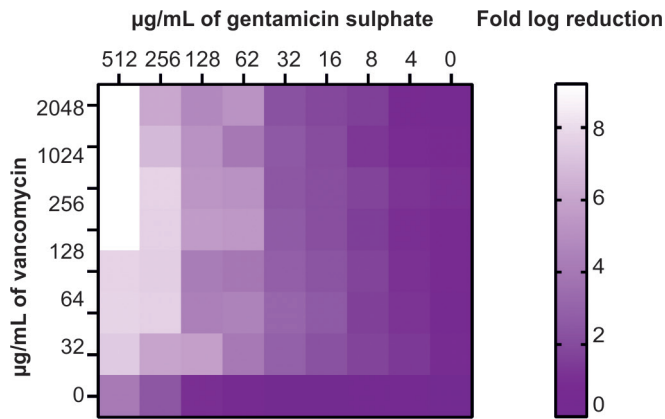
Fig. 6: Results from the checkboard biofilm-killing assay of gentamicin sulphate (512, 256, 128, 64, 32, 16, 8 and 4 μg/mL) and vancomycin (2048, 1024, 512, 256, 128, 64 and 32 μg/mL) against the MRSA strain. The data show the logarithmic reduction on CFU counts compared to the non-treated well in a colour pattern from a logarithmic reduction of 9–0

The use of ACP has been associated with good clinical results. The largest series in the setting of an infected non-union has been described by Wang et al.[14] They treated 548 patients, mainly with an infected non-union of the tibia or femur, with a two-stage revision in which temporary stability was provided with an LCP coated with PMMA with gentamicin and vancomycin. If there were no signs of infection after at least 6 months, definitive osteosynthesis was attempted. At 12-months follow-up, the infection control rate of the complete cohort was 97.6%. Nevertheless, there were 92 (17%) patients that required a second debridement and repeat 'first stage' procedure after 6 months. Patients with sinuses and patients with tibial non-union had a significantly higher chance of recurrence. Jia et al. treated 183 patients with infected lower extremity bone defects in a similar procedure.[14] After the first stage, 8.7% of patients had recurrence of infection. Finally, bone healing was achieved in 95.9% of patients. In this study, recurrence of infection was more common for patients with systemic compromise, a finding that was not substantiated in the study of Wang et al.[13]

In addition, successful infection clearance may in part be determined by the capacity of ACP in treating residual biofilm after debridement and prevention of novel recolonisation of the plate. To provide more insight into the mechanisms underlying the reported clinical outcomes, we assessed the capacity of ACPs in treating residual biofilm and preventing bacterial recolonisation. Using Kirby–Bauer zone inhibition tests with the MRSA isolate from our patient, we saw inhibition zones until 20 days for PALACOS LV + V and until the end of the experiment at 28 days for commercially prepared COPAL G + V. This indicates that the antibiotics released from these cements would also have exerted an antibiotic effect against the MRSA strain present in the infected bone for at least these periods. However, ZOIs are not an adequate measure of antimicrobial capacity over time. For that, the release profiles in liquid are more relevant. For our ACPs, antibiotic release in liquid was characterised by an early peak followed by a long, sustained release in vitro above the MIC.

Regarding the early peak, with the COPAL G+V cement, a total of 1179 μg (393 μg/mL) of vancomycin and 1037 μg (346 μg/mL) of gentamicin were released in the first 24 hours. These concentrations released in our in vitro experiment would result in

a 7.7-fold log reduction of viable bacteria (counted as CFU) in the biofilm. In our patient, the COPAL G+V cement was used to fill the segmental bone defect that is created by debridement (which is the site where one can expect residual biofilm to be present) and to cover the screw holes of the ACP. Possibly, the concentrations achieved with the combination of local and systemic antibiotics will be sufficient to strongly reduce biofilm that may have remained after debridement in the patient. In addition, the local antibiotics also provide protection against possible bacterial recolonisation during surgery and for the next 7 days while the wound has not completely healed yet.[30] Therefore, filling segmental defects and coating fixation plates after debridement of an infected non-union with COPAL G + V may favour the clinical success of this treatment strategy. The concentrations released from PALACOS LV + V were not as effective in reducing CFU in the biofilm. With regard to sustained release of antibiotics, the vancomycin release from PALACOS LV + V was 11 μg/mL at day 50, which is still 11 times above the MIC for the MRSA strain, although no ZOI was observed any more after 20 days, which is likely due to the poor diffusion of vancomycin through agar at lower concentrations.[31] For COPAL G+V, both gentamicin and vancomycin may have been responsible for the ZOI until and including at 28 days, as gentamicin release was 19 μg/mL at this time point and vancomycin release was 14 μg/mL, which both are well above the MICs for the MRSA strain (gentamicin 0.5 μg/mL, vancomycin 1.0 μg/mL). At day 50, the minimal concentrations released were 22 μg/mL and 21 μg/mL for gentamicin and vancomycin, respectively. After prolonged exposure to low concentrations of antibiotics, there are concerns regarding the development of antibiotic resistance due to exposure of the bacteria to sub-therapeutic levels.[32,33] Furthermore, as concentrations released ultimately fall below the MIC, the antibiotic-coated plate itself might become a colonised surface. As the concentrations released in vitro were still well above the MIC at 50 days, it is to expect that the antibiotic is able to prevent bacterial recolonisation of the ACP for this time period.

There are several limitations to these results. First, the antibiotics from the ACPs in vitro are released into 3 mL of PBS, whereas in vivo the antibiotics are released into a volume that might have a different antibiotic-releasing surface-to-volume ratio. Also, the in vivo volume has a different composition (patient plasma, wound fluid, and actual tissue) than the PBS that was used in vitro. Second, the antibiotic cement was present in the patient much longer (10 months) than the duration of the in vitro release assay. We do not know if and for how long the antibiotic release will have remained above the MIC beyond 50 days. Lastly, the biofilm for the checkboard killing assay was inoculated over a 24-hour period. The biofilm in an in vivo non-union is older and more mature and might therefore require higher antibiotic concentrations for reduction and eradication.

In conclusion, we have found that the in vitro release is characterised by an early burst followed by a prolonged sustained release above the MIC until 50 days for both hand-mixed PALACOS LV + V and commercially prepared COPAL G + V. The initial burst of COPAL G + V is likely to be sufficient to reduce biofilm and prevent early recolonisation at the non-union site in the early postoperative period. This underlines the importance of thorough and meticulous debridement. The antibiotic released from the cement was only a relatively small proportion of the amount incorporated. However, the total amount released is still considerable, allowing antimicrobial activity in the 50-day release period to prevent bacterial recolonisation of the ACP. The combined results provide an in-depth insight into the mechanisms of antimicrobial effectiveness that contribute to the clinical results

of this approach and confirm the usefulness of coating fixation plates with antibiotic-impregnated cement in the treatment of an infected femoral non-union.

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