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THE NON-STEROIDAL ANTI-INFLAMMATORY DRUG CARPROFEN NEGATIVELY IMPACTS NEW BONE FORMATION AND ANTIBIOTIC EFFICACY IN A RAT MODEL OF ORTHOPAEDIC-DEVICE-RELATED INFECTION

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used for pain management during recovery from orthopaedic surgery. NSAID use is associated with increased risk of bone healing complications but it is currently unknown whether NSAIDs increase the risk of developing an orthopaedic-device-related infection (ODRI) and/or affect its response to antibiotic therapy. The present study aimed to determine if administration of the NSAID carprofen [a preferential cyclooxygenase-2 (COX-2) inhibitor] negatively affected *Staphylococcus epidermidis* (*S. epidermidis*) bone infection, or its subsequent treatment with antibiotics, in a rodent ODRI model.

Sterile or *S. epidermidis*-contaminated screws (~ 1.5×10^6 CFU) were implanted into the proximal tibia of skeletally mature female Wistar rats, in the absence or presence of daily carprofen administration. A subset of infected animals received antibiotics (rifampicin plus cefazolin) from day 7 to 21, to determine if carprofen affected antibiotic efficacy. Bone changes were monitored using *in vivo* μCT scanning and histological analysis. The risk of developing an infection with carprofen administration was assessed in separate animals at day 9 using a screw contaminated with 10^2 CFU *S. epidermidis*. Quantitative bacteriological analysis assessed bacterial load at euthanasia.

In the 28 d antibiotic treatment study, carprofen reduced osteolysis but markedly diminished reparative bone formation, although total bacterial load was not affected at euthanasia. Antibiotic efficacy was negatively affected by carprofen (carprofen: 8 out of 8 infected; control: 2 out of 9 infected). Finally, carprofen increased bacterial load and diminished bone formation following reduced *S. epidermidis* inoculum (10^2 CFU) at day 9.

This study suggested that NSAIDs with COX-2 selectivity reduced antibiotic efficacy and diminished reparative responses to *S. epidermidis* ODRI.

Keywords: *Staphylococcus epidermidis*, osteomyelitis, *in vivo* μCT, non-steroidal anti-inflammatory drugs, carprofen, antibiotics.

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List of Abbreviations

AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
agr	accessory gene regulator
ANOVA	analysis of variance
BF	bone formation
BIC	bone-implant contact
BR	bone resorption
BV/TV	bone fraction
CCL11	C-C motif chemokine ligand 11
CFUs	colony-forming units
CoNS	coagulase-negative staphylococcus
COX	cyclooxygenase
CPP	Clinical Priority Program

EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
FRI	fracture-related infection
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GRO	growth-related oncogene
IFN-γ	interferon gamma
IL	interleukin
IP-10	IFN-γ-induced protein 10
KC	keratinocytes-derived chemokine
LIX	lipopolysaccharide-inducible CXC chemokine
MCP-1	monocyte chemoattractant protein-1
MIC	minimum inhibitory concentration
MIP	macrophage inflammatory protein
MMA	methylmethacrylate
NSAID	non-steroidal anti-inflammatory drug
ODRI	orthopaedic-device-related infection
PBS	phosphate-buffered saline
PEEK	polyetheretherketone
PJI	periprosthetic joint infection
RANTES	regulated upon activation, normal T cell expressed and secreted
RAPD	random amplification of polymorphic DNA
ROI	region of interest
S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*
SD	standard deviation
SEM	standard error of the mean
SPF	specific-pathogen free
TNF-α	tumour necrosis factor alpha
TSA	tryptic soy agar
TSB	tryptic soy broth
VEGF	vascular endothelial growth factor
μCT	micro-computed tomography

Introduction

NSAIDs are a drug class that are widely used in human clinical medicine due to their efficacy for reducing inflammation and pain (Jeffcoach *et al.*, 2014; Ong *et al.*, 2007). As inhibitors of the enzyme COX, which exists as a constitutive form (COX-1) and an inducible form (COX-2), these drugs reduce prostaglandin synthesis, thereby reducing systemic inflammation and pain. However, since inflammation and prostaglandin E2 play important roles in bone healing (Blackwell *et al.*, 2010; Einhorn and Gerstenfeld, 2015), NSAIDs may have a negative influence if taken during the early inflammation-driven phase of bone healing (Gerstenfeld and Einhorn, 2004; Zura *et al.*, 2016). The impact of NSAIDs on bone healing is a contentious topic in the literature, with a recent systematic review showing no clear trend toward either a positive or negative effect of NSAIDs on bone healing, but highlighting the paucity of high quality studies on the topic (Marquez-Lara *et al.*, 2016). This problem is further compounded by the issue that NSAIDs are generally available on an over-the-counter basis in numerous countries and, therefore, may be self-administered by the patient without the knowledge of the responsible physician.

ODRIs, including PJI and FRI, occur with an incidence ranging between less than 1 % [PJI after hip prosthesis (Li *et al.*, 2018)] and up to 30 % [FRI after open fracture (Kortram *et al.*, 2017; Puetzler *et al.*, 2019)]. An ODRI is a potentially devastating complication of orthopaedic surgery, both in terms of morbidity and mortality, as well as a financial burden for healthcare systems worldwide (Poultsides *et al.*, 2010). Although the majority of acute ODRIs are caused by *S. aureus*, a significant proportion of infections are caused by low virulence microorganisms such as *S. epidermidis*, a CoNS, which may be responsible for up to 40 % of ODRIs (Depypere *et al.*, 2020; Uckay *et al.*, 2009). These bacteria can develop a biofilm on inert materials and necrotic tissues and usually progress with a sub-clinical presentation, dramatically complicating a diagnosis.

Bone healing in the presence of an implant relies on a subtle balance of pro- and anti-inflammatory mediators and the coordinated activity of resident bone cells and immune cells to orchestrate the healing process. The presence of a bacterial infection dysregulates this process, with an excess of pro-inflammatory signals driving excessive bone destruction and also, depending on the causative pathogen, potentially driving diminished local BF or stimulating ectopic BF (Croes *et al.*, 2019). Therefore, the inhibition of COX by NSAIDs could serve to decrease inflammation-driven bone destruction. However, given the importance of prostaglandins in the bone healing process (Gerstenfeld and Einhorn, 2004; Kaneko *et al.*, 2007; Kawaguchi *et al.*, 1995), administration of NSAIDs may also inhibit reparative responses following infection. The issue is further complicated by the varying selectivity of different NSAIDs for inhibiting different isoforms of COX, with drugs such as ketoprofen displaying COX-1 selectivity, ibuprofen being relatively non-specific and celecoxib being COX-2 selective (Cryer and Feldman, 1998; Mandell, 1999).

In a paper aiming at investigating anti-virulence properties of diflunisal (a COX-1 selective NSAID) on *S. aureus*, Hendrix *et al.* (2016) demonstrated that diflunisal protected from osteolysis in a rodent model of *S. aureus*-induced osteomyelitis. Similar protective effects were also observed with aspirin (also a COX-1 selective NSAID) in a murine model of ODRI (Jiang *et al.*, 2019), thereby suggesting that COX inhibition may prevent infection-induced bone destruction. However, clinical evidence on the effect of NSAID use on fracture healing is limited, with relatively few high-quality studies available. Of particular interest, Jeffcoach *et al.* (2014), in a retrospective analysis of 1,901 patients with operative fixation of a long-bone fracture, demonstrated that perioperative NSAID use

Table 1. Overview of study design. ABs = antibiotics (rifampicin and cefazolin); QB = quantitative bacteriology.

Group	Treatment	Inoculum	Description	Duration (days)	Outcome	Group size
Treatment study						
1	Control	1.5×10^6 CFU	Sterile	28	µCT, QB	7
			S. epidermidis	28	µCT, QB	9
			S. epidermidis + ABs	28	µCT, QB (9), hstology (3)	12
2	NSAID	1.5×10^6 CFU	Sterile	28	µCT, QB	7
			S. epidermidis	28	µCT, QB	9
			S. epidermidis + ABs	28	µCT, QB (9), hstology (3)	12
Risk study						
3	Control	1×10^2 CFU	*S. epidermidis*	9	µCT, QB	10
4	NSAID	1×10^2 CFU	*S. epidermidis*	9	µCT, QB	10
					TOTAL	76

doubles the rate of complications (non-union or FRI), although the data were not subjected to sub-group analysis based on COX selectivity. In a separate study, the development of non-union after nailing of femoral diaphyseal fractures was correlated with NSAID use (Giannoudis *et al.*, 2000). Once again, no discrimination was made concerning COX-1 *vs.* COX-2 selectivity. A recent retrospective analysis of a large, long-bone fracture cohort provides the only large-scale study comparing NSAID use based on COX selectivity. George *et al.* (2020) concluded that COX-2 inhibitors, but not non-selective NSAIDs, are correlated with increased rate of non-union. However, whether NSAID treatment directly increases the risk of developing an ODRI or affects the course of an ODRI and/or its response to therapy has not been investigated in a pre-clinical model.

The excellent analgesic potential of NSAIDs underlies their widespread use in current clinical practice, managing pain and inflammation and ultimately permitting the prompt recovery of patients after trauma, but also reducing the necessity for opioid-based medications (of relevance in the context of a global rise of opioid prescription) (Metsemakers, 2020). The current clinical guidelines of many orthopaedic associations recommend the use of NSAIDs for pain management in the perioperative course of total joint arthroplasty or operative fracture treatment (Fillingham *et al.*, 2020), without mention of COX-selectivity. Of note, the 2018 guidelines of the Orthopedic Trauma Association discuss the potential impact of NSAIDs on fracture healing, underpinning the conflicting results evident in the scientific literature. Nevertheless, the guidelines support the use of NSAIDs regarding their proven analgesic potential (Hsu *et al.*, 2019) and lack of detrimental effects when used for short durations. However, to the best of the authors' knowledge, no clinical guidelines exist concerning the use of NSAIDs in the context of an ODRI.

The first aim of the present study was to investigate if treatment with carprofen, a NSAID with preferential COX-2 selectivity, influenced the nature of an *S. epidermidis* ODRI and the response of the infection to antibiotic treatment, using an established rat model of *S. epidermidis*-induced ODRI and longitudinal µCT scanning over a 28 d period (Stadelmann *et al.*, 2020; Thompson *et al.*, 2020). In addition, whether perioperative carprofen administration impacted early post-operative infection was investigated. To evaluate this, a markedly reduced inoculum of *S. epidermidis* was applied on the implanted screw and bacterial colonisation and bone responses were evaluated after a 9 d observation period.

Materials and Methods

Study outline

Consent to perform the study was granted by the ethical committee of the canton of Graubünden, Switzerland (approval numbers: 05/2015, 10/2017 and 04/2019), and was carried out in an AAALAC-accredited research institute. A previously reported model of ODRI in the rat proximal tibia was used (Freitag *et al.*, 2019; Stadelmann *et al.*, 2020; Thompson *et al.*, 2020), utilising custom PEEK screws. A total of 76 rats were included in the study (overview of grouping can be seen in Table 1). At 20-24 weeks of age, PEEK screws, either sterile or contaminated with *S. epidermidis*, were implanted into the left tibia, as described below. Animals in groups 1 and 2 (treatment study) received contaminated screws with a target inoculum of 1.5×10^6 CFUs, which is sufficient to guarantee infection in all animals, based on past experience (see preparation details below). Post-implantation, tibiae were monitored by *in vivo* µCT at 7 time points over a 28 d period. In addition, 2 further groups (groups 3 and 4; risk study) were included, to assess whether NSAID treatment

affected the risk of developing an infection to PEEK screws contaminated with a reduced *S. epidermidis* inoculum (1×10^2 CFUs) as a model of perioperative contamination. Animals in groups 3 and 4 had μCT scans performed immediately post-operatively and at day 9 (euthanasia).

Implant design and manufacturing

Custom-made screws (5 mm length, 1.5 mm diameter) were machined from medical grade PEEK containing 20 % (w/w) barium sulphate (material supplied by Invibio Biomaterials Ltd.) by RISystem AG, Davos, Switzerland.

Bacterial inoculum preparation

S. epidermidis (strain 103.1; a clinical isolate from a patient with a chronic ODRI and available from the Culture Collection of Switzerland, strain number CCOS 1152) was recovered from frozen stocks and cultured on TSA (Oxoid, Basel, Switzerland) or in TSB (Oxoid) at 37 °C. The *S. epidermidis* 103.1 strain is sensitive to both antibiotics used in this study, rifampicin and cefazolin, with MIC values of 0.015 mg/mL and < 1 mg/mL, respectively.

The bacterial inoculum was introduced to the rats on pre-contaminated screws prepared immediately prior to each surgery. On the day of surgery, *S. epidermidis* overnight cultures were centrifuged (2,500 ×*g* for 10 min), washed in PBS twice and then adjusted to an optical density of 0.50 (± 0.01) at 600 nm. The threaded portion of the screw was submerged and incubated statically at room temperature for 25 min. Test screws were inoculated in parallel within each series of experiments. A quantitative assessment of *S. epidermidis* adhesion to the test screw was performed by sonication in PBS (3 min) followed by serial dilution, plating on 5 % horse blood agar (Oxoid) and incubation overnight at 37 °C. The target inoculum for each screw was 1.5×10^6 CFUs/screw (range: 0.9-2 × 10^6 CFUs/screw). All screws were implanted within 30 min of preparation.

Animal welfare, observation and euthanasia

Skeletally mature, adult female, SPF Wistar rats, purchased from Charles River (Germany), were used and housed until skeletal maturity (20-24 weeks). Animal monitoring, care and potential exclusion criteria were as previously described (Freitag *et al.*, 2019; Stadelmann *et al.*, 2020; Thompson *et al.*, 2020). Animals were randomly allocated to their group (Table 1). For the 28 d antibiotic treatment study (groups 1 and 2), all animals were scanned by μCT immediately following surgery (day 0 – to confirm appropriate positioning of screw) and at 6 further time points post-surgery (see details below). For the perioperative risk study (groups 3 and 4), all animals were scanned immediately following surgery (day 0) and at day 9 (euthanasia). Animals were weighed at the same time points as μCT scans were taken. Blood samples (using EDTA as an anticoagulant) were also collected from the lateral tail-vein pre-operatively and on days 9, 20 and 28, prior to μCT scans. Plasma was isolated following centrifugation (400 ×*g*, 5 min) and subsequently stored at – 20 °C until analysis. On day 28 (groups 1 and 2) or day 9 (groups 3 and 4), animals were euthanised using an overdose of pentobarbital through intracardiac injection, under isoflurane anaesthesia.

Surgery, anaesthesia and medication administration

Anaesthesia and surgery were performed as previously described (Freitag *et al.*, 2019; Stadelmann *et al.*, 2020; Thompson *et al.*, 2020). In brief, screw insertion surgery was performed at 20-24 weeks, using the previously described protocol (Stadelmann *et al.*, 2015). The mean weight (± SD) of the animals at surgery was 336.0 ± 26.4 g. Under isoflurane anaesthesia, a sterile or a contaminated screw (for the control and the infected groups, respectively) was inserted in the proximal tibia 2 mm distal to the growth plate. NSAID groups received a daily carprofen injection [5 mg/kg, subcutaneously (*s.c.*)]. Further subgroups of animals infected with *S. epidermidis* were treated with a combination antibiotic regimen (25 mg/kg rifampicin plus 30 mg/kg cefazolin, *s.c.*) twice daily from day 7 after screw implantation, for a period of 14 d, followed by a 7 d washout period, to prevent false negative results in the quantitative bacteriological analysis. Subcutaneous injections of carprofen and the combination antibiotic regimen (rifampicin and cefazolin) were performed at separate sites in the scruff of the neck, using minimal volumes (≤ 100 mL/injection).

***In vivo* μCT and image processing**

The evolution of peri-prosthetic bone structure following screw implantation surgery was assessed in groups 1 and 2 using time-lapsed *in vivo* μCT immediately post-operatively and at 3, 6, 9, 14, 20 and 28 d as previously described (Stadelmann *et al.*, 2020). Groups 3 and 4 were scanned immediately post-operatively and at day 9. Briefly, animals were scanned under isoflurane anaesthesia and a 10 mm long ROI, with a diameter of 25.6 mm field of view and centred on the implanted screw was scanned at a nominal resolution of 25 μm. Time-lapse μCT image processing was performed to compute BIC, BV/TV, BF and BR rates in a ROI within 700 μm from the screw surface. Periosteal reaction was quantified within the medial periosteal region 2 mm distal and proximal from the screw head. All image processing and analysis were performed using EasyIPL (Web ref. 1), a high-level library of macros using a scanner software (SCANCO Image Processing Language, IPL and OpenVMS Digital Command Language, DCL).

Bacteriology

Following euthanasia, the respective tibiae were dissected and the screws and bones collected in separate, sterile containers with sterile PBS. Any soft fibrous tissue overlying the protruding head of the screw was also collected in separate sterile containers

containing PBS. The number of bacteria adhering to the *S. epidermidis*-contaminated screws was determined by sonicating the screws for 3 min and vortex mixing for 10 s, before performing serial dilutions and viable bacteria counts on blood agar. Then, the entire tibia from each animal was mechanically homogenised (Omni Tissue Homogeniser and Hard Tissue Homogenising tips; Omni International, Kennesaw, GA, USA) and the quantity of bacteria associated with bone was similarly quantified by serial dilution and viable bacteria counts on blood agar. Soft tissue samples were processed in the same manner. All agar plates were incubated for 24 h at 37°C and checked for contamination or signs of co-infection. Animals were considered as infected when at least one sample (bone, soft tissue or screw) was culture positive. Identification of *S. epidermidis* 103.1 in culture-positive samples was performed for at least one colony from each culture positive animal using RAPD PCR (Versalovic *et al.*, 1991) following comparison with the original *S. epidermidis* 103.1 strain.

Histological processing

A total of 6 randomly chosen animals [3 infected animals treated with antibiotics (group 1) and 3 infected animals treated with NSAIDs and antibiotics (group 2)] were allocated for histological analysis. Following euthanasia, the lower limb was dissected and the overlying skin removed. Then, all samples were fixed for a minimum of 2 weeks in 70 % methanol. After fixation, samples were dehydrated through an ascending ethanol series (70 %, 96 %, absolute ethanol), with two changes for each step, every 2-5 d. Then, samples were transferred to xylene and finally to MMA (Sigma-Aldrich) for embedding. The polymerised samples were sectioned using a Leica 1600 annular blade saw (Leica Biosystems Nussloch GmbH). At least two serial sections in the frontal plane through the centre of the screw were made from each sample. One section was used for Giemsa-eosin staining and the remaining section for Brown and Brenn staining, to detect Gram-positive bacteria.

Histopathological analysis (semi-quantitative)

The slides were analysed by a certified veterinary pathologist (DN) using a semi-quantitative method (grade 0-5) comparing parameters for implant integration, inflammation and infection in both groups. Due to the limited sample number (n = 3), histological analysis was performed to give a general morphological overview of the two compared groups and no statistical analysis was performed.

Histomorphometric analysis (quantitative)

Additionally, a quantitative histomorphometric method was used to measure implant integration. Using Adobe Photoshop 2020 (including analysis plug-in), the bone area was measured in a sleeve of 300 μm around the threaded part of the PEEK screw on each individual digital image, with a calibration of 0.452 pixels/μm. The ROI was defined by using the image of the first control sample animal (an identical ROI was applied for analysis of all other samples) and resulted in a polygon around the implant with an area of ~ 6.6 mm^2 (exactly 6,654,197 μm^2). Its width was defined by the maximum width of the screw head at its neck (~ 2,090 μm), its height by the length of the screw from the neck to the tapered tip (~ 3,443 μm). The bone area inside the ROI was marked by Photoshop's magic wand tool interactively to avoid marking non-bone areas with similar grey values [settings: point sample, tolerance 50, continuous area, grey value min (mean): 21.1, grey value max (mean): 154.4].

Cytokine measurements

Plasma samples collected during the 28 d study [day 0 (pre-operative), 9, 20 and 28] were analysed for inflammatory cytokines using a 27-plex Milliplex xMAP Rat Cytokine/Chemokine Magnetic Bead Panel (EMD Millipore) according to manufacturer's instructions. Plasma (25 μL) was used to analyse the following cytokines/chemokines/growth factors in the Luminex immunoassay: EGF, eotaxin/CCL11, fractalkine, G-CSF, GM-CSF, GRO/KC, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p70), IL-13, IL-17A, IL-18, IP-10, leptin, LIX, MCP-1, MIP-1α, MIP-2, RANTES, TNF-α and VEGF. Based on instrument settings and standard curves, the assay limit of detection was set at 10 pg/mL. Among the 27 proteins measured in the immunoassay, the following cytokines were expressed at undetectable concentrations (< 10 pg/mL): G-CSF, GM-CSF, MIP-1α, IL-2, EGF and TNF-α and the corresponding data are, therefore, not presented.

Statistical analysis

μCT time series curves are shown as mean ± SEM for the measured data points. Two-way ANOVA with a Tukey's *post-hoc* test for multiple comparisons was used to analyse changes in bone parameters over time and cytokine measurements. Kruskall-Wallis tests were used to analyse quantitative bacteriology data. The Fisher Exact Test was used to check for differences in proportions of infected animals between groups. Threshold for statistical significance was set as $p < 0.05$. All analyses were performed using GraphPad Prism software (GraphPad Software, Inc.).

Results

Animal welfare

All animals recovered well from surgery and anaesthesia. In groups 1 and 2 (28 d antibiotic treatment study), mean weight loss following surgery was ~ 4.7 % of initial body weight at 3 d. All animals recovered to pre-operative body weight by 28 d. The recovery of body weight following surgery was not significantly affected by *S. epidermidis* infection,

antibiotic treatment or NSAID administration (data not shown). One animal from group 1 (infected + antibiotics) was found dead in the cage during the study and was not replaced. Data pertaining to this animal were excluded from the subsequent analysis.

Bone changes in vicinity of implant

Rats receiving screws contaminated with *S. epidermidis* developed marked osteolysis in the vicinity of the screw by day 6, with maximal decreases in BIC and BV/TV observed at day 14 (Fig. 1 and Fig. 2). Consistent with this, there was a significant reduction in BF and a marked increase in BR evident at day 6 (Fig. 3). The osteolytic response to infection was also associated with a subsequent reparative response, which became evident at day 14. Specifically, an extensive periosteal reaction was observed from day 14 onwards, which was also associated with the deposition of further mineralised tissue within the vicinity of the implant (Fig. 1,3).

Daily administration of carprofen to non-infected animals had limited effects on bone parameters. However, carprofen administration resulted in pronounced changes in the response of bone tissue to a *S. epidermidis* infection. Osteolytic responses in the vicinity of the screw were markedly reduced following carprofen administration, with a trend for increased BV/TV relative to infected control animals observed at day 6 ($p = 0.059$) and day 9 ($p = 0.070$) (Fig. 2). Of particular note, the rate of BR was significantly diminished at day 6 with carprofen administration ($p = 0.021$). However, the most pronounced effects of carprofen concerned BF, with significantly reduced periosteal reaction on days 9-28 (day 9, $p = 0.002$; day 14, $p < 0.001$; day 20, $p = 0.026$; day 28, $p = 0.012$) and also a trend for reduced rates of BF at days 20

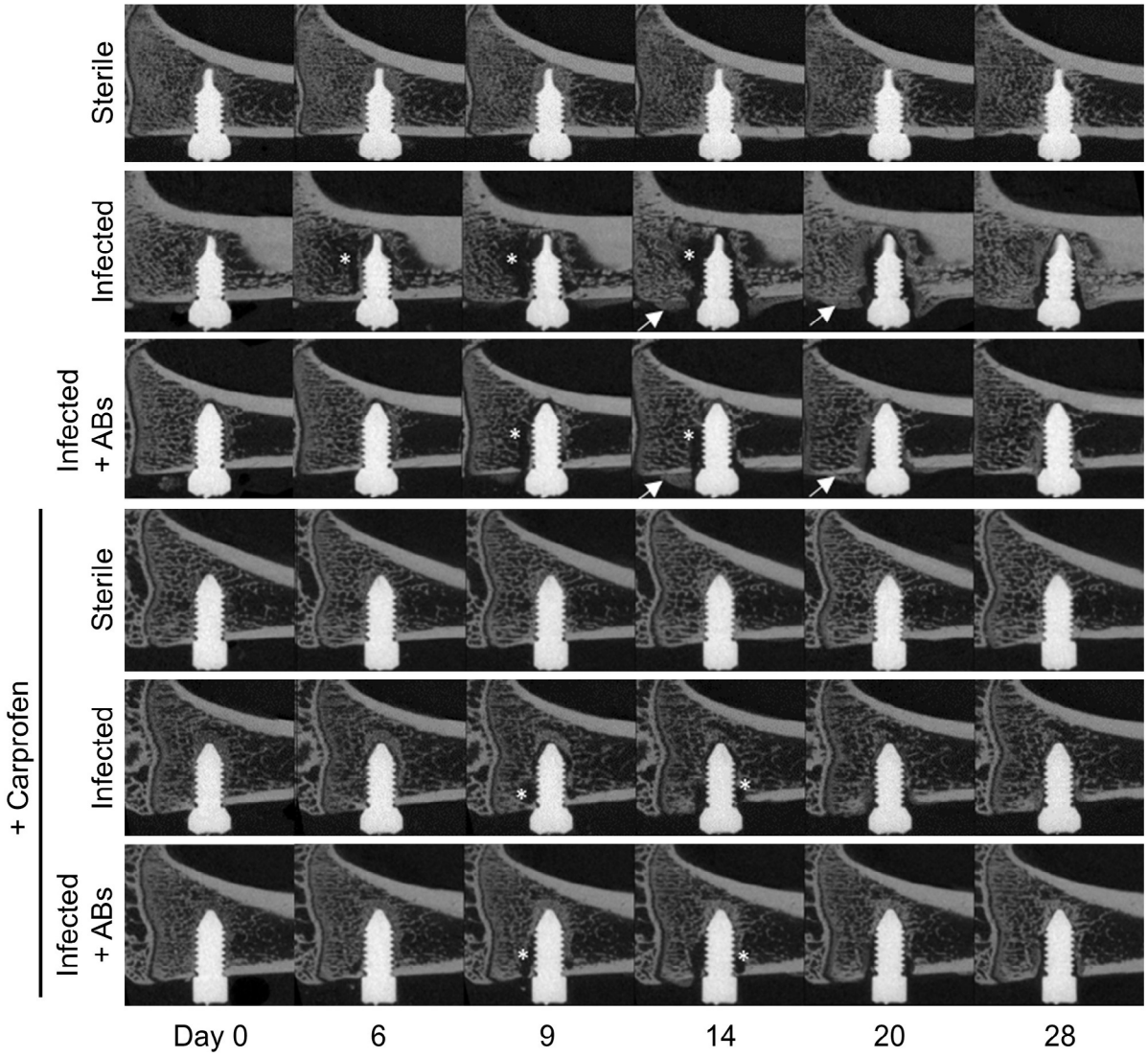

Fig. 1. Representative time series of longitudinal *in vivo* μCT scans from *S. epidermidis*-infected animals and following carprofen administration, in comparison to non-infected animals (sterile). The effect of a combination antibiotic regimen (+ ABs), when administered from days 7-21, is also shown. Representative images are from the same animal and aligned to the baseline (day 0) scan, chosen based on median BV/TV values from each experimental group. Asterisks indicate regions of osteolysis and white arrows indicate periosteal BF. Note the limited efficacy of antibiotic treatment for preventing bone changes in response to infection and the reduced osteolytic response and absence of periosteal BF in the carprofen-treated groups.

(p = 0.061) and 28 (p = 0.108) (Fig. 3). No changes in BIC were observed between infected control and NSAID-treated animals.

Bone changes in response to antibiotic therapy

The efficacy of a combination antibiotic therapy on the bone response to *S. epidermidis* infection was assessed. Administration of rifampicin and cefazolin on day 7 did not significantly affect *S. epidermidis*-induced changes in BIC or BV/TV relative to infected animals or infected animals receiving carprofen (Fig. 1 and Fig. 2). Similarly, antibiotic therapy did not change periosteal reaction or rates of BF or resorption (Fig. 3) in infected animals in the absence or presence of carprofen.

Histomorphometric analysis

The amount of bone tissue near the implant was assessed in a ROI of 300 µm around the threaded part of the PEEK screw. Due to previous observations, whereby antibiotic administration did not alter *S. epidermidis*-induced bone parameters (Fig. 2, 3), and for increased clinical relevance, all animals chosen for this aspect of the study had received antibiotic therapy. In 3 randomly chosen animals (receiving *S. epidermidis*-inoculated screws and antibiotics), carprofen administration reduced the amount of bone tissue present in the vicinity of the screw by ~ 38 % compared to control animals (mean bone area ± SD: control = 1.098 ± 0.166 mm^2; NSAID = 0.680 ± 0.110 mm^2) (Fig. 4**a**). This reduction in bone tissue was more pronounced in the medullary region compared to the cortical region, which was consistent with µCT-based observations.

Histopathological analysis

None of the infected control or carprofen-treated animals that received antibiotic therapy displayed evidence of focal or diffuse inflammation in the bone marrow (*i.e.* no myelitis with/without formation of micro-abscesses), but did exhibit a low grade, diffuse activation, predominantly involving the myelopoietic lineage. Limited evidence of Giemsa-positive coccoid bacteria was observed in the Giemsa-eosin-stained

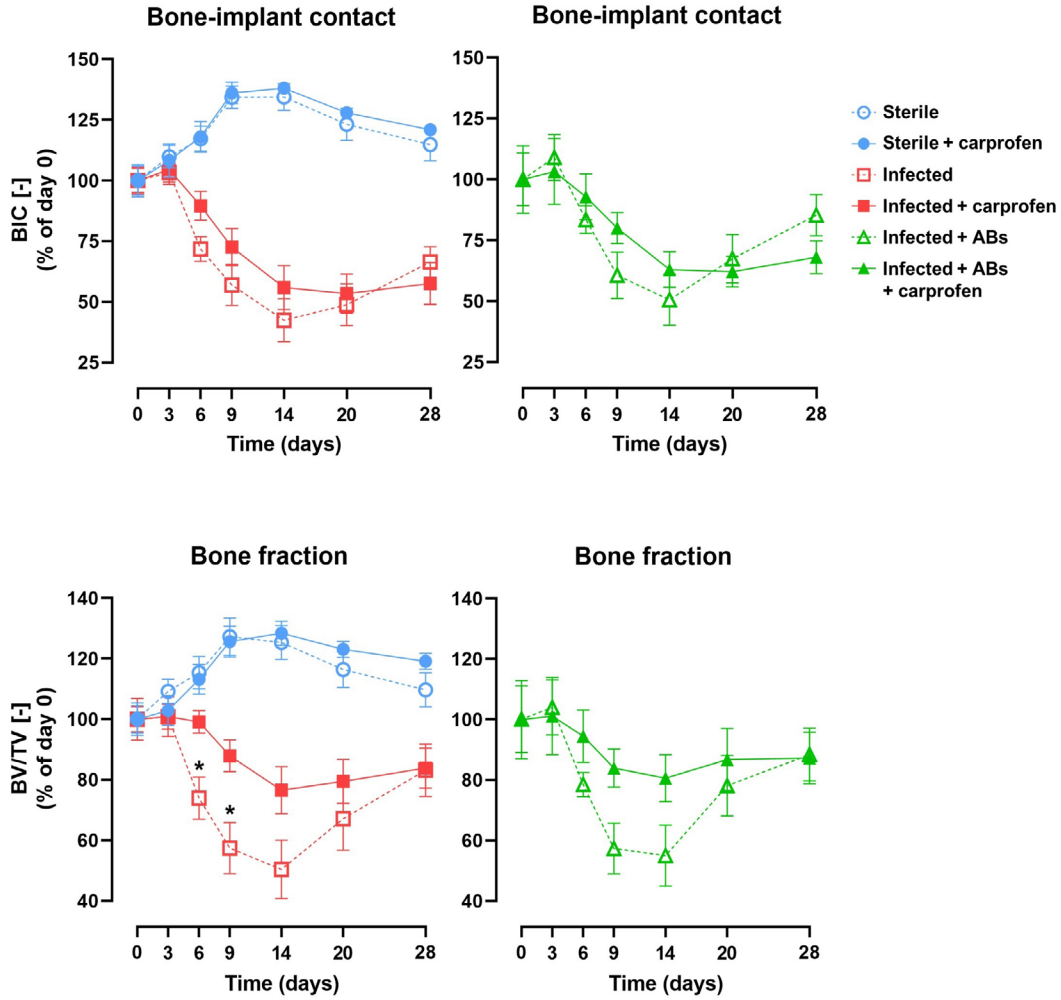

Fig. 2. *S. epidermidis* infection led to changes in normalised BIC (top row) and BV/TV (bottom row), over time. Responses in non-infected animals (sterile), infected animals and impact of carprofen administration are shown in the left panels. The influence of antibiotic therapy on bone changes in the absence or presence of carprofen is shown in the right panels. Data shown are the mean ± SEM. Two-way ANOVA with a Tukey's *post-hoc* test for multiple comparisons was performed to assess significant differences between non-carprofen- and carprofen-treated infected animals, in the absence or presence of antibiotic therapy. * $p < 0.05$.

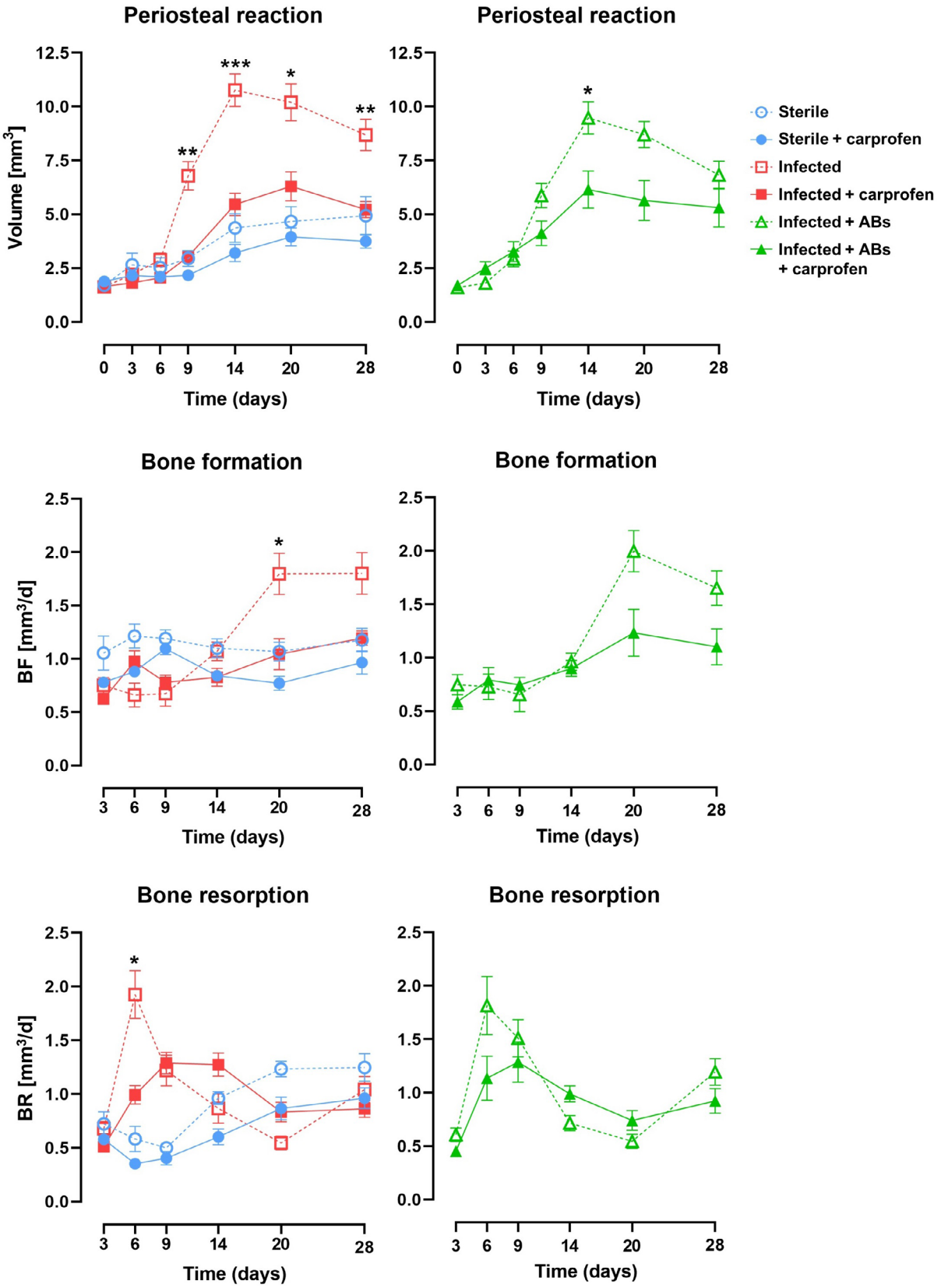

Fig. 3. *S. epidermidis* infection led to changes in periosteal BF (top row) as well as rates of BF (middle row) and BR (bottom row) over time. Responses in non-infected animals (sterile), infected animals and the impact of carprofen administration on each parameter are shown in the left panels. The influence of antibiotic treatment in the absence or presence of carprofen is shown in the right panels. Data shown are the mean ± SEM. Two-way ANOVA with a Tukey's *post-hoc* test for multiple comparisons was performed to assess significant differences between non-carprofen- and carprofen-treated infected animals, in the absence or presence of antibiotic therapy. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

section, which was also confirmed by Brown-Brenn staining (Fig. 4**b**), in 1 out of 3 animals in the carprofen-treated group, whereas no bacteria were detected in the control animals. These bacteria were found on sequestered and necrotic bone particles near the implant thread (data not shown).

Effect of carprofen on bacterial burden

The viable bacteria resident in the proximity of the implanted screw were quantified to determine the impact of carprofen administration and the efficacy of the antibiotic regimen. All *S. epidermidis*-infected animals that were not treated with antibiotics remained infected at euthanasia. In addition, all animals receiving sterile screws were culture-negative at euthanasia (data not shown). Despite the decreased osteolysis evident with carprofen administration, there was no difference between mean CFU counts in the infected animals as compared to the NSAID-treated infected animals (mean CFU count ± SEM: control = 53,686 ± 22,698 CFUs; NSAID = 45,617 ± 12,019 CFUs, $p > 0.999$) (Fig. 5). Furthermore, whilst the antibiotic therapy had no detectable effect on any infection-induced changes in the assessed bone parameters, the antibiotic regimen was markedly effective at reducing bacterial burden in infected animals (mean CFU count = 2'506 ± 1,776; $p = 0.016$), with the successful clearance of the infection in 7 out of 9 animals. However, antibiotic efficacy was dramatically reduced in carprofen-

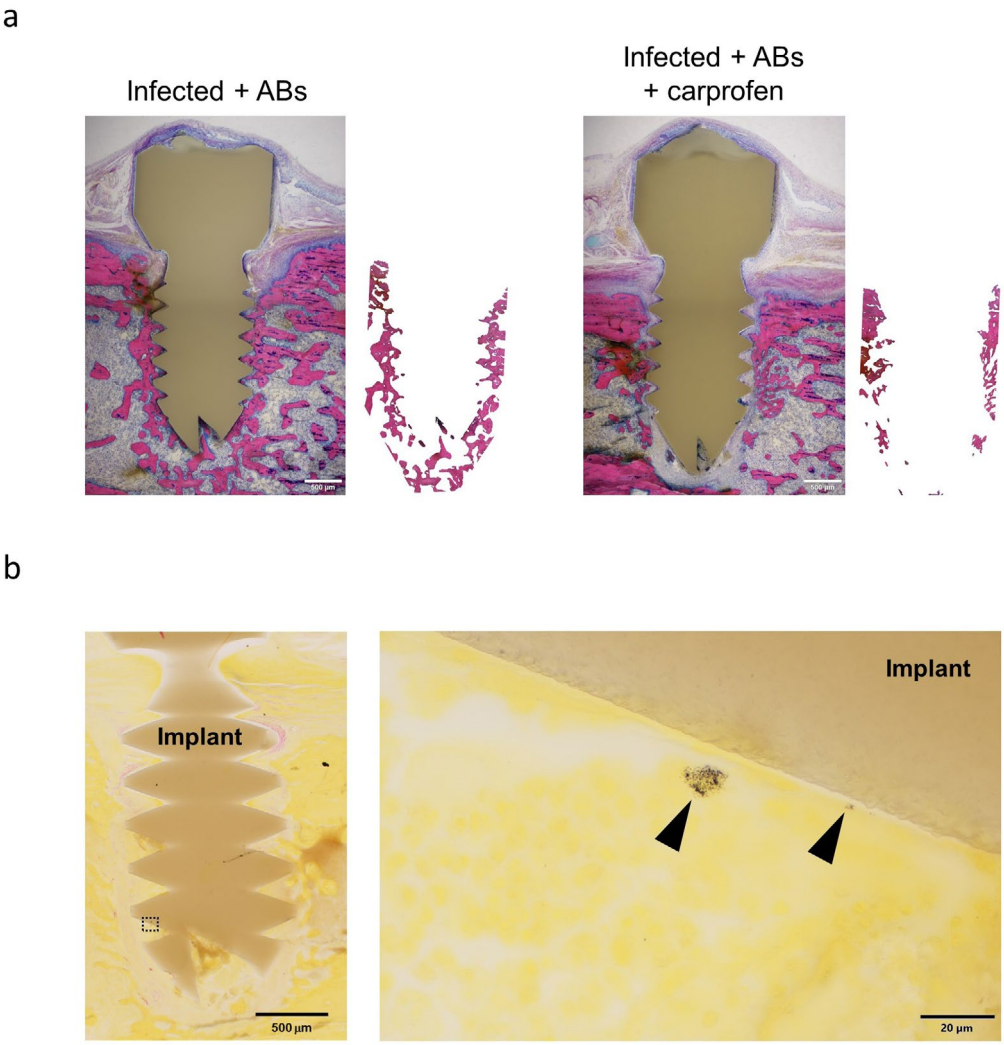

Fig. 4. Histological and histopathological assessment of bone changes resulting from implantation of *S. epidermidis* contaminated screws in the absence or presence of carprofen administration after 28 d. MMA-embedded thick sections, cut in the frontal plane through the centre of the PEEK implant, were stained with (**a**) Giemsa-eosin, and the proportion of trabecular bone resident in a defined ROI was then quantified, or (**b**) Brown-Brenn stain to detect the presence of bacteria. (**a**) Representative images of the implanted screw, with the bone content of the selected ROI used for quantification shown to the right of the overview image, in the absence (left) or presence (right) of carprofen. Magnification: 4× objective; scale bar = 500 µm. (**b**) Left panel: overview image from 1 carprofen-treated animal showing the presence of the implant and highlighting the region of the higher magnification view in the right panel (dashed box). Magnification: 4× objective; scale bar = 500 µm. Right panel: higher magnification view (dashed box in the left panel) displaying evidence of *S. epidermidis* microcolonies (indicated by black triangles) in the vicinity of the implanted screw. Magnification: 100× objective (oil); scale bar = 20 µm.

treated animals, with only a minor reduction in the mean CFU count (16,438 ± 6,426) and a complete failure to clear the infection in all (8 out of 8) animals ($p = 0.002$, Fisher's exact test). Bacterial colonies taken from animals found to be culture-positive despite antibiotic therapy were also assessed for resistance to rifampicin but were found to be equally susceptible to rifampicin as the parental strain (data not shown).

Effect of carprofen on inflammatory responses due to *S. epidermidis* infection

Whether the differences in bacterial CFU counts after antibiotic therapy were due to disparities in inflammatory responses induced by carprofen administration was examined. Plasma samples collected over time were analysed for cytokine/chemokine secretion using multiplex immunoassay. Trending upregulation of proinflammatory cytokines such as IL-6, IFN-γ, IL-12p70 and MCP-1 was observed in all rats at day 9 post-inoculation (Fig. 6). However, no statistically significant differences between the carprofen-treated and the control group were found. All other cytokines/chemokines measured showed no significant differences between groups (data not shown).

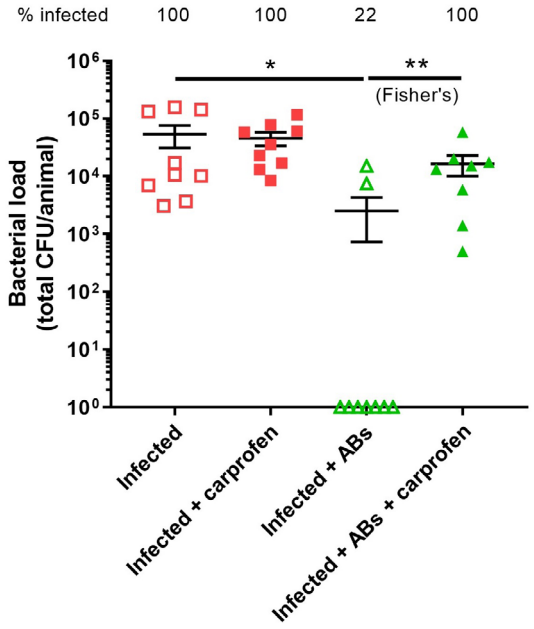

Fig. 5. Quantitative bacteriological assessment indicating total bacterial load per animal and the percentage of infected animals per group after 28 d. Culture-negative samples were arbitrarily assigned a value of 1 CFU to permit display on a logarithmic axis. Data shown are the total CFU counts from each animal with mean ± SEM indicated. 9 animals were included per group, with the exception of the infected + ABs + carprofen group, where 1 animal was excluded due to the detection of a polymicrobial infection following dissection. A Kruskall-Wallis test with Dunn's *post-hoc* test for multiple comparisons was performed to compare differences in CFU counts between groups. * $p < 0.05$. A Fisher's exact test (indicated) was performed to compare proportions of infected animals between antibiotic-treated groups in the absence or presence of carprofen. ** $p < 0.01$.

Impact of carprofen administration on risk of developing an infection

Following the characterisation of the bone changes and inflammatory responses induced by the standard inoculum of *S. epidermidis* on the implanted screw (~ 1.5×10^6 CFUs), it was determined whether carprofen administration would influence the risk of developing, or the severity of, an infection following implantation of a screw inoculated with a markedly reduced amount of *S. epidermidis* (1×10^2 CFUs) within the early post-operative period. Most control animals (9 out of 10) and all carprofen-treated animals (10 out of 10), receiving the reduced inoculum of *S. epidermidis* (*i.e.* 4 orders of magnitude less than the standard CFU inoculum) were infected at day 9 (Fig. 7). Interestingly, in contrast to earlier findings at day 28, carprofen administration significantly increased mean bacterial load by ~ 16-fold over control animals at day 9 post-inoculation (mean CFU count ± SEM: control = 3,030 ± 2,491; NSAID = 47,495 ± 37,731; $p = 0.018$).

The next step was to characterise the bone changes occurring in response to implantation of a screw with the low *S. epidermidis* inoculum and the impact of carprofen administration on these responses at day 9. Conversely to the findings with the standard inoculum, 1×10^2 CFUs *S. epidermidis* did not decrease BIC or BV/TV values, with an overall trend for increased BF in control animals at 9 d (Fig. 8). Of particular note, despite these observed differences in bone responses between the low and standard *S. epidermidis* inoculum, 1×10^2 CFUs *S. epidermidis* induced a comparable periosteal reaction volume to that observed with the standard inoculum at 9 d (Fig. 3, 8). Carprofen administration did not significantly affect bone responses in terms of BIC, BV/TV or rates of bone formation/resorption at 9 d, compared to control animals. However, carprofen administration significantly reduced periosteal reaction at 9 d (Fig. 8).

Discussion

Pro-inflammatory cytokine production in response to ODRI stimulates bone destruction, which may be diminished by anti-inflammatory drugs such as NSAIDs. However, since NSAIDs inhibit the production of prostaglandins, which are important mediators of the bone healing cascade, administration of NSAIDs may also be associated with inhibitory effects on reparative responses of bone following injury or infection. The findings of the present study indicated that administration of carprofen, a preferential COX-2 selective NSAID, reduced *S. epidermidis*-induced osteolysis but also markedly reduced reparative bone-forming responses. In addition, carprofen exacerbated bacterial burden at

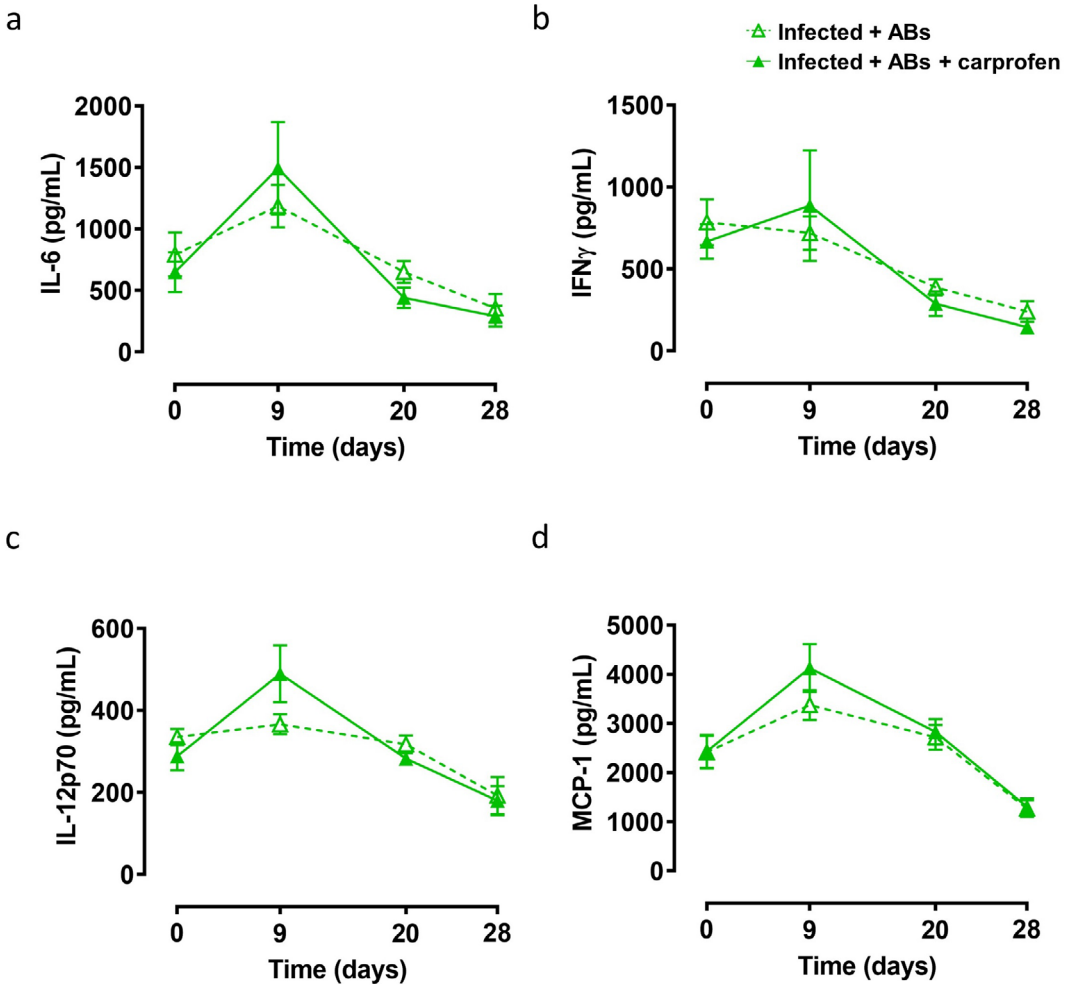

Fig. 6. Influence of carprofen administration on inflammatory cytokine secretion in *S. epidermidis* infected animals. Plasma samples collected over time [day 0 (pre-operatively), 9, 20, 28] from infected animals treated with antibiotics, with or without carprofen, were subjected to inflammatory cytokine secretion analyses using a bead-based multiplexed Luminex immunoassay (n = 11-12 animals in each cohort).

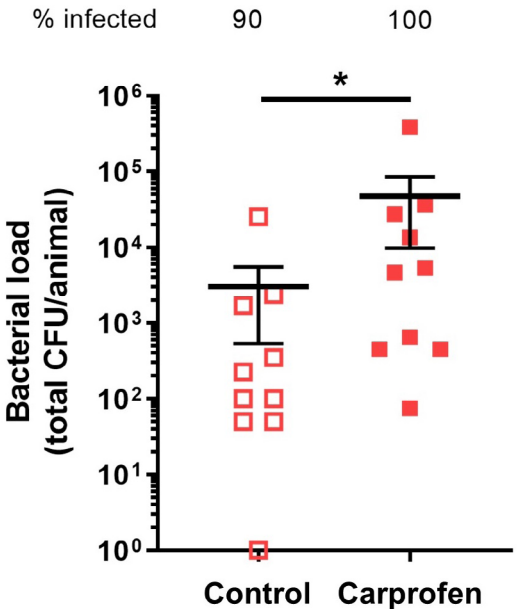

Fig. 7. Quantitative bacteriological assessment of total bacterial load per animal after 9 d following implantation of a screw contaminated with 10^2 CFU of *S. epidermidis*. Culture-negative samples were arbitrarily assigned a value of 1 CFU to permit display on a logarithmic axis. Data shown are the total CFU counts from individual animals with mean ± SEM indicated. 10 animals were included per group. A Mann-Whitney test was performed to compare differences in CFU counts between groups. * $p < 0.05$.

an early phase of the infection and negatively affected antibiotic efficacy for clearing *S. epidermidis* infections in the presence of an implant.

Using an established μCT-based model for monitoring bone infection, complex effects of carprofen were observed on the response of bone to *S. epidermidis* infection. Perhaps the most striking finding concerned the decrease in antibiotic efficacy in carprofen-treated infected animals. This is consistent with a previous study reporting that non-selective NSAIDs decrease antibiotic efficacy in group-A streptococci-induced necrotising fasciitis in mice (Hamilton *et al.*, 2014). Although the specific mechanism for such an interaction of NSAIDs with antibiotics is currently unknown, NSAID administration may be contributing to bacterial survival in the presence of antibiotics by increasing multidrug efflux pump expression in bacteria and/or decreasing expression of antibiotic target proteins, as reported for the active metabolite of aspirin, salicylate (Hartog *et al.*, 2010; Riordan *et al.*, 2007; Shen *et al.*, 2011; Zimmermann and Curtis, 2018). Conversely, it is purported that some NSAIDs could be substrates for the efflux pump in Gram-negative rods (Laudy *et al.*, 2016) and also in *S. aureus* (Price *et al.*, 2002), leading to saturation of the efflux pump, thereby increasing the intracellular antibiotic concentration and suggesting a synergistic effect of NSAIDs with antibiotics. Other reported examples of potential synergistic interactions of NSAIDs with antibiotics includes diclofenac, which may act indirectly by increasing the pharmacodynamics of ciprofloxacin when both are co-administered (Iqbal *et al.*, 2009), and celecoxib, which may sensitise *S. aureus* to antibiotics by altering membrane potential and increasing membrane permeability (Annamanedi and Kalle, 2014; Varma *et al.*, 2019). Plasma cytokine data suggested that carprofen administration did not seem to influence systemic pro-inflammatory responses due to *S. epidermidis* infection up to 28 d post-infection. However, it cannot be discounted that long-term NSAID administration may impair the host immune response to *S. epidermidis* infection, thereby allowing persistence of the infection despite antibiotic

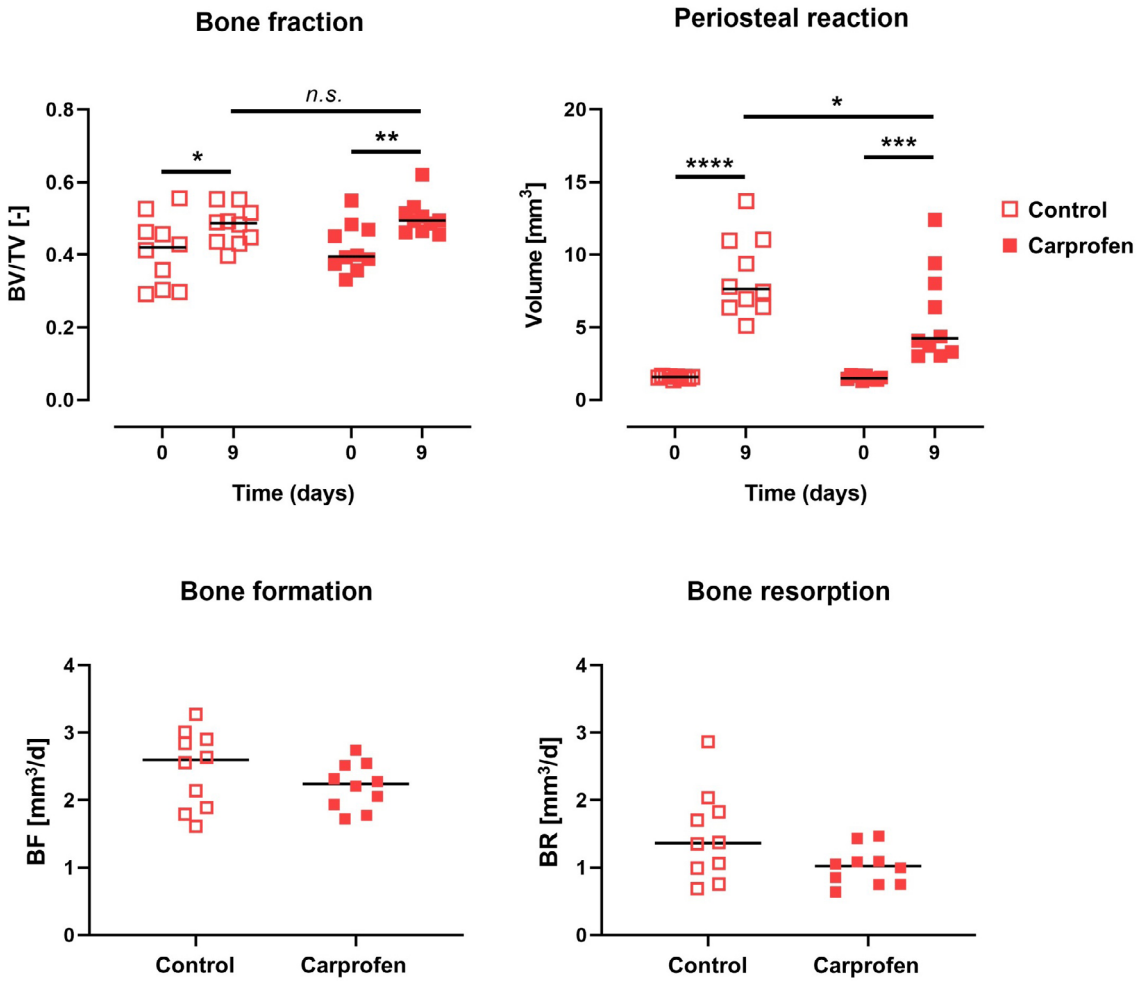

Fig. 8. Bone changes induced by a low inoculum of *S. epidermidis* in the absence (Control) or presence of carprofen administration (Carprofen) after 9 d. Bone fraction (BV/TV; top row, left panel), periosteal reaction volume (top raw, right panel) and rates of new bone formation (bottom row, left panel) and bone resorption (bottom row, right panel) are shown. Data shown are from individual animals with the mean indicated by the horizontal bar. One way-ANOVA with Tukey's *post-hoc* test was performed to determine significant differences between > 2 groups. Paired *t*-tests were performed to assess changes in rates of BF and resorption at 9 d. n.s.: not significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

treatment. Nevertheless, given the findings of the study, chronic NSAID administration in combination with antibiotic therapy is not without risk and, therefore, should be carefully considered. Further studies are necessary to elucidate the underlying mechanism for this phenomenon, given the potential for complex pharmacokinetic and pharmacodynamic interactions with such combination drug regimens.

Carprofen displayed limited effects on osseointegration of the non-inoculated implant. However, carprofen administration in the presence of *S. epidermidis* resulted in diminished osteolysis in the vicinity of the implant. This observation was consistent with the bone protective effects of NSAID treatment previously observed in rodent models of *S. aureus* infection in the absence of an implant, including the non-selective NSAID ibuprofen (Rissing and Buxton, 1986) and the COX-1 selective NSAID diflunisal (Ford *et al.*, 2020; Hendrix *et al.*, 2016), which was interestingly shown to exhibit anti-virulence properties unrelated to its inhibitory effects on COX. Although the underlying mechanism for these bone protective effects in *S. epidermidis* osteomyelitis is currently unknown, previous studies have identified that diflunisal inhibits the quorum-sensing *agr* pathway in *S. aureus*, thereby limiting virulence factor production (Khodaverdian *et al.*, 2013) and protecting osteoblasts from cell death (Hendrix *et al.*, 2016). Since *S. epidermidis* lacks the cytolytic virulence factors of *S. aureus*, the bone protective effect of NSAID administration that was observed suggested that prostaglandin E2 is likely generated in response to *S. epidermidis* infection and drives osteoclast-mediated bone destruction, since prostaglandin E2 is a known stimulator of osteoclast formation (Kaneko *et al.*, 2007). However, longitudinal μCT analysis also revealed profound changes in the capacity of new bone to form (defined as deposition of periosteal woven bone and medullary cancellous BF) following infection in animals treated with carprofen. Such inhibitory effects of NSAIDs (both selective and non-selective) on bone repair following stress fracture have previously been reported in rodent models (Kidd *et al.*, 2013) and in human patients (Hughes *et al.*, 2019). As far as it can be ascertained, this is the first demonstration that a NSAID inhibited reparative responses to bone infection with a low virulence organism such as *S. epidermidis* in the presence of an implant. The pronounced periosteal-derived BF in control animals in response to *S. epidermidis* infection was consistent with previous reports demonstrating that the presence of either live or dead *S. aureus* bacteria on an implant generates both osteolysis and new BF (Croes *et al.*, 2017; Croes *et al.*, 2019). The present study data indicated that such inflammation-induced BF in response to bacterial infection was sensitive to carprofen, suggesting that prostaglandin E2 may play a role in the reparative bone-forming response to bacterial antigens.

The failure of carprofen to affect osseointegration (as assessed using BIC measurements as a proxy) of a sterile implant was in contrast to other reports. Trancik *et al.* (1989) described a reduction in bone ingrowth of porous coated cobalt-chrome implants in rabbits after treatment with the non-selective NSAIDs indomethacin or ibuprofen; Cook *et al.* (1995) reported retarded osseointegration of porous-coated titanium implants in an indomethacin-treated canine model at early time-points (3 weeks), although this difference was not observed at later time-points (24 weeks). Studies from clinical dentistry have also suggested a negative effect of NSAIDs on implant osseointegration (Etikala *et al.*, 2019; Winnett *et al.*, 2016). Interestingly, some authors have suggested a detrimental effect in the case of selective COX-2 inhibition (Gomes *et al.*, 2015). Indeed, a model of COX-2-deficient mice showed dramatically reduced osseointegration of dental implant (Chikazu *et al.*, 2007). Clinical data in orthopaedic surgery offer conflicting results, perhaps reflecting the variability of COX-isoform selectivity of the evaluated NSAIDs. A prospective study on 80 patients receiving indomethacin (which displays COX-1 selectivity) for preventing heterotopic ossification after total hip arthroplasty did not reveal any trend for decreased osseointegration or increased implant-loosening after a 6 year follow-up (Wurnig *et al.*, 1999). In contrast, a randomised trial on total hip arthroplasty patients receiving ibuprofen for prophylaxis of heterotopic ossification revealed a significantly higher risk of revision for aseptic loosening at 10 years in the NSAID-treated group (Persson *et al.*, 2005). The specific NSAID used in the present study, carprofen, is a reversible COX inhibitor, with a preference for COX-2 in a variety of different species (Brideau *et al.*, 2001; Radi, 2009), although this COX-2 selectivity is markedly lower than that of highly COX-2 selective drugs such as celecoxib. Thus, specific NSAIDs with differing selectivity for COX-1 *vs.* COX-2 may be associated with increased risk of aseptic loosening, although further large-scale studies are necessary to clarify this issue.

While the study findings demonstrated that carprofen administration did not increase bacterial load at late time points in response to infection – which was consistent with an earlier study by Rissing and Buxton (1986) – an increased bacterial load was observed at the point of peak osteolysis (day 9) when administering a markedly reduced inoculum of *S. epidermidis*. Although these low inoculum studies were designed to determine whether carprofen treatment promoted the risk of developing an implant-related infection, it was found that even 10^2 CFUs of *S. epidermidis* (*i.e* 4 orders of magnitude less than used in the routine model) could reliably establish an infection in ≥ 90 % of experimental animals. This lower inoculum was also more realistic in terms of the number of bacteria that may enter a wound in the early peri-operative or post-operative period. This highlights the fundamental importance of an implant for establishing an infection where profoundly reduced inoculum doses can reliably

trigger an infection in host animals, as previously reported by Zimmerli *et al*. (1982): 10^2 CFUs of *S. aureus* could establish an infection in 95 % of implants in a preclinical guinea pig model, while 10^8 CFUs were unable to form abscesses in the absence of an implant. However, such potential effects of NSAIDs for exacerbating bacterial load in ODRI patients are poorly understood. Although some authors have suggested a relationship between NSAID administration and more severe course of bacterial infection, no clinical trial to date has confirmed this. No increased risk for septic shock was reported in NSAID-treated bacteriemia patients in a multi-centre case control study (Legras *et al.*, 2009). More recently, a randomised double blind clinical trial could not establish a link between aspirin (a relatively COX-1 selective NSAID) and death associated with sepsis (Eisen *et al.*, 2020).

A specific limitation of the study concerned the chronic nature of the NSAID dosing regimen administered. However, NSAID administration is typically viewed as relatively benign in terms of side effects, aside from the known potential of NSAIDs to elicit gastrointestinal issues and/or affect kidney function with long-term dosing regimens. Thus, given the widespread availability and routine use of NSAIDs in patients, such a dosing regimen was considered justified. The concerning nature of the findings regarding carprofen co-administration with antibiotic therapy, itself a fundamental mainstay of ODRI treatment, suggested that further studies are warranted to elucidate the mechanisms underlying this interaction (specifically, the impact of selective COX-1 *versus* COX-2 inhibitors on bone responses following infection) to minimise the risk of impaired healing responses with NSAID use in ODRI patients.

Conclusion

The study characterised the impact of administration of a preferential COX-2 inhibitory NSAID, carprofen, on the course of a *S. epidermidis* implant-related infection and the subsequent response to antibiotic therapy. A marked inhibitory effect of carprofen on bone-forming reparative responses to *S. epidermidis* infection was confirmed and a marked reduction in antibiotic efficacy was revealed when co-administered with preferential COX-2 inhibitory NSAID regimens. Given the widespread use of NSAIDs in the general population, confirmatory studies are necessary to determine whether NSAID use is contraindicated in ODRI patients and the role of COX-1 *vs.* COX-2 selectivity in this process.

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No conflicts of interest are noted in relation to the study.

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Discussion with Reviewers

William T. Obremskey: Will those finding be duplicated in another animal model?

Authors: It would be of interest to investigate the impact of selective COX-1 and COX-2 inhibitors in this model, to confirm which specific COX isoform underlies the mechanistic effects observed with *S. epidermidis*. Subsequent studies to investigate whether the same observations hold true with other common

but under-studied ODRI bacterial species, such as *Cuteibacterium acnes* and *Escherichia coli*, could also reveal important insights into the role of chronic NSAID administration in the context of ODRI. Applying these findings in the context of a fracture model could also yield important insights into ODRI and how different mechanical fixation environments may impact on the process. Concerning the use of other animal models, we feel that, given its low cost, the rat model is useful to investigate the mechanistic aspects (role of COX-1 *vs.* COX-2 selectivity) and to optimise dosing and duration. However, testing in a larger-animal model (pigs or sheep) would ideally be performed, *e.g.* with a clinically-relevant plate fixation, to confirm the relevance of the findings from the rodent model.

Reviewer 1: What would be a follow-up study that would further improve our knowledge on this topic?

Authors: Given the clinical literature suggesting an impact of COX-2 inhibition on bone healing, it would be of interest to use the same animal model and compare bone responses after treatment with selective COX-1 *vs.* COX-2 inhibitors. These initial studies, performed in the absence *vs.* presence of bacterial infection, could confirm the role of COX selectivity, which could then be further evaluated in the context of a fracture/osteosynthesis model in the absence or presence of infection. In addition, such an approach could reveal appropriate durations of NSAID treatment capable of preserving the beneficial aspect of a NSAID treatment (*e.g.* reduced osteolysis) whilst limiting its detrimental effects (*e.g.* inhibitory effects on reparative bone formation).

Editor's note: The Guest Editor responsible for this paper was Willem-Jan Metsemakers.