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Peri- and Post-operative Influence of Tourniquet Application on Cefuroxime Tissue Concentrations and Tissue Ischemia

PhD Thesis

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Preface

During medical school, I came in contact with a small group who worked with the pharmacokinetic tool, microdialysis, which I, at that time, only understood to a very limited extend. However, with an ambition in the group to optimise current empirical-based antimicrobial usage, I was captivated. In 2014, Professor Kjeld Søballe, Mats Bue and Mikkel Tøttrup offered me a research year. Doing research exited me, and the potential of the microdialysis method was now obvious. At that time, it was very clear to me that I had to pursue a PhD within this field. Luckily, I was offered an integrated PhD by Professor Maiken Stilling, Professor Kjeld Søballe and Mats Bue in 2017. Now, seven years later, I can honestly say that my application for the research year within this research group has been the most important and definable document for my medical career. I have enjoyed every year in this research group and genuinely hope to continue my research career within this research group.

Pelle Hanberg

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This PhD thesis consists of four papers and a review dealing with the effect the tourniquet application has on both the peri- and post-operative cefuroxime and ischemic metabolite concentrations in orthopedically relevant tissues. The review describes the existing knowledge and clinical relevance, methodological strengths and limitations, and discuss the findings.

Within this PhD thesis, re-use and copy of my own work could occur (Studies I–IV).

The four studies were conducted at the following locations:

Studies I and II: Institute of Clinical Medicine, Aarhus University Hospital, Denmark

Studies III and IV: Department of Orthopaedic Surgery, Horsens Regional Hospital, Denmark.

All chemical analyses were performed at the Department of Clinical Biochemistry, Aarhus University Hospital, Denmark

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List of Papers

This thesis is based on the following papers:

Paper I:

Hanberg P, Bue M, Öbrink-Hansen K, Kabel J, Thomassen M, Tøttrup M, Søballe K, Stilling M. Simultaneous Retrodialysis by Drug for Cefuroxime Using Meropenem as an Internal Standard-A Microdialysis Validation Study. J Pharm Sci. 2020 Mar;109(3):1373–1379. doi: 10.1016/j.xphs.2019.11.014. Epub 2019 Nov 20. [1]

Paper II:

Hanberg P, Bue M, Öbrink-Hansen K, Thomassen M, Søballe K, Stilling M.
Timing of Antimicrobial Prophylaxis and Tourniquet inflation - A Randomized Controlled Microdialysis Study. J Bone Joint Surg Am. 2020 Nov 4;102(21):1857-1864. doi: 10.2106/JBJS.20.00076. [2]

Paper III:

Hanberg P, Bue M, Kabel J, Jørgensen AR, Jessen C, Søballe K, Stilling M.
Effects of tourniquet inflation on peri- and post operative cefuroxime concentrations in bone and tissue. Acta Orthop. Jan 2021. Submitted. [3]

Paper IV:

Hanberg P, Bue M, Kabel J, Jørgensen AR, Søballe K, Stilling M.
Tourniquet Induced Ischemia and Reperfusion in Subcutaneous Tissue, Skeletal Muscle, and Calcaneal Cancellous Bone. Apmis. Jan 2021. Accepted for publication. [4]

Abbreviations

AUC: Area under the concentration-time curve

C_{max}: Peak drug concentration

MIC: Minimal inhibitory concentration

PK/PD: Pharmacokinetic/pharmacodynamic

T_{max}: Time to C_{max}

T>MIC: The time for which the free drug concentration is maintained above the MIC

$T_{1/2}$: Half-life

UHPLC: Ultra-high performance liquid chromatography

Table of contents

1 English summary

Tourniquets are widely used in orthopaedic surgery due to their ability to reduce intra-operative bleeding and improve visualisation. However, as the blood supply to the operating field is occluded during surgery, correct timing of antimicrobial prophylaxis administration and tourniquet inflation is essential in order to ensure therapeutic tissue concentrations at the surgical site. Currently, the guidelines are ambiguous.

Tourniquet use has been associated with multiple adverse effects, including longer recovery time, reduced muscle strength, soft tissue damage and slow wound healing. Although many of these adverse effects may be related to tourniquet-induced ischemia, only a few studies have investigated local tissue metabolite changes during and after tourniquet application.

The overall objective of this PhD project was to validate and apply microdialysis in order to evaluate the effects of tourniquet application on both peri- and post-operative cefuroxime and ischemic metabolite concentrations in orthopedically-relevant tissues. These objectives were evaluated in a three-step approach: (1) *in vitro* and *in vivo* evaluation of the microdialysis calibration method used, (2) an experimental *in vivo* study evaluating different timepoints for cefuroxime administration and tourniquet inflation, and lastly, (3) a clinical study evaluating the effects of tourniquet application on cefuroxime and ischemic metabolite tissue concentrations peri- and post-operatively over two dosing intervals. The design of each individual step in this three-step approach was reliant on the preceding findings.

Meropenem was validated as a suitable internal standard for cefuroxime, and microdialysis was successfully applied to evaluate cefuroxime and ischemic metabolite tissue concentrations before, during and after tourniquet application. Administering 1.5 g cefuroxime 15–45 min prior to tourniquet inflation was found to be a safe window in order to achieve bone and soft tissue concentrations above 4 µg/mL, and a tourniquet application time of approximately 60 min did not affect the cefuroxime tissue concentrations in the following dosing interval. Furthermore, a tourniquet-application time of approximately 60–90 min was found to induce only limited tissue ischemia and cell damage in cancellous bone and soft tissues.

2 Danish summary

Blodtomhed er et hyppigt anvendt ortopædkirurgisk hjælpemiddel til at mindske den perioperative blødning og bedre det kirurgisk overblik. For at sikre perioperative terapeutiske antibiotika-koncentrationer i det opererede væv er det essentielt, at den perioperative antibiotika profylakse administreres i tilstrækkelig god tid inden anvendelse af blodtomheden. I øjeblikket er der dog ingen klare retningslinjer for brugen af blodtomhed og timingen af antibiotikaadministration.

Blodtomhed er associeret med multiple bivirkninger, fx forlænget genoptrænings-periode, reduceret muskelstyrke, bløddelsskader og langsommere sårheling. Mange af disse bivirkninger kan være relateret til vævsiskæmi induceret af blodtomhed. Trods denne viden, har kun få studier undersøgt udviklingen i de lokale iskæmiske metabolitter før, under og efter anvendelsen af blodtomhed.

Der overordnet formål med dette PhD projekt, var at validere og anvende mikrodialysemetoden til at undersøge koncentrationerne af cefuroxim og iskæmiske metabolitter før, under og efter blodtomhed i ortopædkirurgisk relevante væv. Dette blev undersøgt med en 3-trins fremgangsmåde, bestående af (1) en *in vitro* og *in vivo* evaluering af den anvendte mikrodialyse kalibreringsmetode, (2) et eksperimentelt *in vivo* studie hvor forskellige tidsintervaller fra cefuroxim administration til anvendelse af blodtomhed blev undersøgt og slutteligt (3) et klinisk studie hvor blodtomhedens effekt på både de peri- og postoperative vævskoncentrationer af cefuroxim og iskæmiske metabolitter over to doseringsintervaller blev undersøgt. Studiedesignet af hvert trin i denne 3-trins fremgangsmåde var afhængigt af resultaterne fra foregående trin.

Meropenem blev valideret som en egnet intern kalibrator for cefuroxim, og mikrodialysemetoden blev anvendt til at evaluere lokale vævskoncentrationer af cefuroxim og iskæmiske metabolitter før, under og efter anvendelsen af blodtomhed. Administration af 1,5 g cefuroxim 15–45 min før anvendelsen af blodtomhed viste sig som et sikkert tidsinterval til at sikre knogle og bløddelskoncentrationer over 4 µg/mL, og anvendelse af ca. 60 min blodtomhed påvirkede ikke cefuroxim koncentrationerne i det efterfølgende doseringsinterval. Derud-over var et blodtomhedsinterval på 60–90 min kun forbundet med begrænset vævs-iskæmi og celleskade i knogle og bløddele.

3 Introduction

3.1 Surgical site infections

Millions of surgical procedures are performed throughout the world every day. As an example, in the United States alone, 27 million surgical procedures are performed each year[5]. In terms of all surgical procedures, postoperative surgical site infections have been reported in the range of 1–26% and are a major cause of postoperative morbidity and mortality[5-7]. For patients who contract a surgical site infection, the mortality risk is twice as high, the need for intensive care is 60% higher, and the risk of being readmitted to the hospital is four times higher compared with patients without a surgical site infection[8]. Consequently, surgical site infections have high costs for both the patient and the healthcare system.

Bacterial wound contamination during surgery is unavoidable, with the skin flora being the most common source[5]. However, hematogenous and exogenous environment disseminations are also frequent sources of surgical site infections [5]. A surgical site infection develops when the contamination load in the wound suppresses the immune defence of the host and perioperative antimicrobial prophylaxis. As such, the development of surgical site infections is a dynamic process and depends on factors like the virulence of the bacteria, wound condition (e.g. tissue ischemia and necrosis), presence of a foreign body (e.g. prosthesis), immunocompetence and condition of the host (e.g. age, obesity, smoking, diabetes mellitus), etc.[5, 6].

A surgical site infection can be difficult to diagnose. The National Nosocomial Infections Surveillance System (NNIS) has developed criteria for defining a surgical site infection (Table 1)[7]. In these guidelines, the surgical site infection is divided according to the tissue depth: superficial (involving skin or subcutaneous tissue), deep (involving fascia or muscle), or organ/space (involving any other spaces than the incision layers of the body wall)[5]. A bone infection is considered an organ/space surgical site infection[5].

The most important preventive measures of surgical site infections are proper perioperative antimicrobial prophylaxis, aseptic surgical preparation and techniques, and postponing an elective surgery when a patient suffers from a systemic or local infection[6, 7, 9]. These preventive measures have all been supported by high

Table 1. National nosocomial infections surveillance system definition criteria for surgical site infections. This table has been modified from the original table published by Prokuski et al. 2008[6].

Superficial incisional surgical site infection Infection occurs within 30 days after the operation *and* the infection involves only skin or subcutaneous tissue of the incision *and* at least one of the following findings: 1. Purulent drainage, with or without laboratory confirmation, from the superficial incision 2. Organisms isolated from an aseptically obtained culture or fluid or tissue from the superficial incision 3. At least one of the following signs or symptoms of infection: pain or tenderness, localized swelling, redness, or heat *and* superficial incision is deliberately opened by surgeon, *unless* the incision is culture-negative 4. Diagnosis of superficial incisional surgical site infection by the surgeon or attending physician
Deep incisional surgical site infection Infection occurs within 30 days after the operation when no implant is left in place, or within 1 year when the implant is in place and the infection appears to be related to the operation *and* infection involves deep soft tissues e.g. fascial and muscle layers of the incision *and* at least one of the following: 1. Purulent drainage from the deep incision but not from the organ/space component of the surgical site 2. A deep incision spontaneously dehisces or is deliberately opened by a surgeon when the patient has at least one of the following signs or symptoms: fever (>38°C), localized pain, or tenderness, unless the site is culture-negative 3. An abscess or other evidence of infection involving the deep incision is found on direct examination, during revision, or by histopathologic or radiologic examination 4. Diagnosis of a deep incisional surgical site infection by a surgeon or attending physician
Organ/Space Surgical site infection Infection occurs within 30 days after the operation if no implant is left in place, or within 1 year if the implant is left in place and the infection appears to be related to the operation *and* infection involves any part of the anatomy e.g. organs and spaces, other than the incision, that was opened or manipulated during an operation *and* at least one of the following: 1. Purulent drainage from a drain that is placed through a stab wound into the organ/space 2. Organisms isolated from an aseptically obtained culture of fluid or tissue in the organ/space 3. An abscess or other evidence of infection involving the organ/space that is found on direct examination, during revision, or by histopathologic or radiologic examination 4. Diagnosis of an organ/space SSI by a surgeon or attending physician

levels of evidence[6, 7, 9]. However, other measures to prevent surgical site infection, e.g. normal blood glucose in patients with diabetes, sufficient oxygen supply, avoidance of perioperative hypothermic conditions, and use of postoperative occlusive dressings, etc., have also been associated with a positive preventative effect against surgical site infection[7, 9].

3.2 Perioperative antimicrobial prophylaxis in orthopaedics

Current recommendations for the use of perioperative antimicrobial prophylaxis is primarily based on a study performed by Burke in 1961[10]. Burke investigated the effect of different antimicrobial administration timepoints in a *Staphylococcus aureus*-contaminated guinea pig model. He found that if the perioperative antimicrobial prophylaxis was

administered 1 hour prior to incision, the surgical wound presented with only slight oedema and white-cell infiltration and was comparable with controls that received dead bacteria[10]. However, when the perioperative antimicrobial prophylaxis was administered 3 hours post incision, the surgical wound presented with oedema, extensive necrosis and white-cell infiltration, with considerable infiltration into the deeper tissue layers, and was very similar to the group that received *S. aureus* without additional perioperative antimicrobial prophylaxis[10]. As such, Burke concluded that the perioperative antimicrobial prophylaxis displayed the highest efficacy against bacteria when presented in the tissue before bacterial contamination[10]. The beneficial effect of perioperative antimicrobial prophylaxis has later been acknowledged in several clinical studies[11-15].

No definitive target for the ideal perioperative antimicrobial prophylaxis has been defined. However, it is acknowledged that therapeutic antimicrobial concentrations should be achieved in both plasma and relevant tissues from the time of incision until, as a minimum, the wound is closed[5, 7]. The basic rules of thumb for applying perioperative antimicrobial prophylaxes are to choose an antimicrobial agent which covers the most likely contaminant bacteria, administer the antimicrobial agent within 30–60 min prior to skin incision in order to achieve therapeutic plasma and tissue concentrations at the time of surgery, and intraoperatively repeat the dose after 3–4 hours for prolonged surgeries or when the blood loss is greater than 2000 mL[5-7, 9].

The most common aetiology of surgical wound contamination in orthopaedic surgery is *S. aureus*[16]. However, *Escherichia coli*, coagulase-negative staphylococci (most common pathogen in prosthetic joint infections), and *Streptococcus* species are also commonly found[16, 17]. Given its susceptibility range, cephalosporines are often the globally preferred antimicrobial agent group[5-7, 9, 10]. Cefazolin (first-generation) and cefuroxime (second-generation) are among the most commonly used cephalosporines as they cover most staphylococci[18]. Moreover, they provide coverage, cefuroxime more than cefazolin, against some gram-negative bacteria[18]. In Denmark, cefuroxime has until now been one of the preferred drugs for perioperative antimicrobial prophylaxis.

3.3 Cefuroxime

Cefuroxime was approved for medical use in 1977 and was later listed in the *World Health Organization Model List of Essential Medicines*[19], which counts the most effective and safe medicines needed in the healthcare system[19, 20]. Cefuroxime is a second-generation cephalosporin exerting its bactericidal effect by interfering with peptidoglycan synthesis and thereby hindering bacterial cell wall synthesis[21]. Cefuroxime is primarily cleared by renal excretion and is, therefore, dosed according to creatinine clearance[20, 22]. The usual adult dosage is 750–1,500 mg 3–4 times daily[21]. However, for creatinine clearances less than 20 mL/min, dosing should be reduced[20, 22]. The protein binding is reported in the range from 33–50% and the half-life in plasma is described to range between 60–90 min[20, 22-24].

Cefuroxime is available as oral, intramuscular and intravenous administrations. In this PhD project, we only evaluated tissue and plasma pharmacokinetics of intravenous administered cefuroxime, which is also the most commonly applied administration route of cefuroxime. Cefuroxime for intravenous use is delivered as sodium. Prior to administration, the cefuroxime sodium is dissolved in saline water[22]. Cefuroxime has long stability (up to 24 hours) in its dissolved form and in plasma[25, 26]. It is generally administered as a bolus infusion over 5–15 min in a peripheral vein. However, studies have also investigated the benefits of continuous infusions[27, 28]. The concentration-dependent toxicity of cefuroxime is very limited[20, 29]. Administration of high doses to achieve therapeutic plasma and tissue concentrations is, therefore, not limited by drug toxicity as for other antimicrobials (e.g. vancomycin and gentamycin).

Various methodological approaches have been used to quantify cefuroxime concentrations, including high performance liquid chromatography with tandem mass spectrometry and high performance liquid chromatography with ultraviolet detection[25, 30]. For pharmacokinetic studies, where dense sampling with possible limited sampling volumes is applied, it is important to use a very sensitive, accurate and precise method with limited volume demands and a short analysis time. Both high performance liquid chromatography with tandem mass spectrometry and high performance liquid chromatography with UV detection meet these demands [25, 30].

3.4 Antimicrobial pharmacokinetics and pharmacodynamics

Pharmacokinetics and pharmacodynamics are the two main sub-branches of pharmacology (the study of drugs). Pharmacokinetics is the study of the effect the body has on drugs, which can be subdivided into absorption, distribution, metabolism and excretion[31, 32]. The following pharmacokinetic metrics were evaluated in the present PhD project for each investigated compartment: the peak drug concentration (C_{max}), the time to C_{max} (T_{max}), the half-life ($T_{1/2}$) defined as the time required for the drug concentration to reach half its concentration, and the area under the concentration-time curve (AUC).

Pharmacodynamics, on the other hand, is the study of the effect the drugs have on the body[31, 32]. For most drugs, the effect is often exerted through different receptors and enzyme activations or inhibitions[31, 32]. Thus, the description of drug pharmacodynamics can be rather complex. However, for antimicrobials, the pharmacodynamics are relatively simple as the amount of drug exposure is associated with the clinical and microbiological effects observed[31, 33]. The association between drug exposure and efficacy is most often determined *in vitro* in relation to the minimal inhibitory concentration (MIC), defined as the lowest drug concentration that prevents visible bacterial growth, or minimal bactericidal concentration, defined as the lowest drug concentration required for bacterial killing[31]. *In vivo* antibacterial effects are, as for *in vitro*, determined by sufficient antimicrobial tissue (target site) concentrations.

The pharmacokinetic/pharmacodynamic (PK/PD) index refers to the quantitative correlation between pharmacokinetic parameters, which should always be based on the steady-state concentration of the antimicrobial agent's unbound fraction and a microbiological parameter, e.g. MIC[34]. Antimicrobial PK/PD indices are used to describe therapeutic efficacy. However, defining a specific PK/PD index target for optimal clinical effect is challenged, due to the lack of studies correlating these two measures, and should be taken with some reservations. Antimicrobial effect is divided into two main categories[31, 35-38]:

Concentration-dependent antimicrobials display a linear relationship between the concentration and bacterial killing. The PK/PD index that best correlates with efficacy is AUC/MIC and C_{max}/MIC[31, 33, 35, 39-42]. Aminoglycosides and

fluoroquinolones are examples of antimicrobials belonging to this group.

Time-dependent antimicrobials present a limited relationship between the concentration and bacterial killing. Instead, bacterial killing is determined by the time of exposure. The time in a dosing interval, for which the free drug concentration is maintained above the MIC (T>MIC) is considered the best predictor of efficacy[31, 43-47]. Interestingly, the definition of adequate T>MIC is a matter of dispute and varies depending on the beta-lactam subgroup. For cephalosporins, 70% T>MIC has been correlated with therapeutic efficacy in some treatment settings[31, 47]. However, in recent years, more aggressive targets of 100% T>MIC, or even 100% T>4–5xMIC, have been suggested for certain infections and patient populations[44-46].

Despite the fact that most bacterial infections reside in the interstitial space of solid tissues, the current PK/PD index targets are merely based on plasma and not tissue concentrations[31, 48]. Whether the targets based on plasma concentrations are applicable to tissues needs further investigations.

No definitive PK/PD index targets have been validated, neither for perioperative prophylactic settings nor therapeutic settings, as definitive *in vivo* targets are difficult to determine. Nonetheless, in perioperative antimicrobial prophylactic settings, it is generally recommended that the antimicrobial plasma and tissue concentration exceed relevant MIC values from the time of incision until, as a minimum, the wound is closed[5, 7]. However, these recommendations are primarily based on expert opinions.

3.5 Cefuroxime tissue concentrations

The optimal cefuroxime treatment is characterised by achieving therapeutic target site concentration, i.e. where the infection is to be prevented or at the site of infection. This makes the understanding of the target site concentration of cefuroxime in both the perioperative antimicrobial prophylactic setting and the therapeutic setting fundamental.

As mentioned in the previous section, current antimicrobial PK/PD index targets are based on plasma concentrations, as plasma concentrations were earlier believed to reflect tissue concentrations[48]. In recent decades, multiple studies have investigated cefuroxime tissue

concentrations by means of the pharmacological tool, microdialysis[1, 27, 28, 49-60]. Most studies found heterogeneous and incomplete tissue penetrations of cefuroxime. Accordingly, it can be speculated that treatment targets based on plasma concentration measurements may account for some treatment failures. As a result, there is a current need for investigating cefuroxime tissue concentrations under different conditions in order to optimise current prophylaxis and treatment regimes.

3.6 Tourniquet

In 1718, a French surgeon, Jean-Louis Petit, invented the first tourniquet device, which consisted of a screw device that could occlude the blood flow to the extremities during amputation surgery[61]. The tourniquet device has developed over the years, and in 1903, the first pneumatic tourniquet was invented by Harvey Cushing, which is still the preferred type of tourniquet device today[62]. The original pneumatic tourniquet was superior to the original models, as it was easier and faster to use and reduced the risk of nerve paralysis[62].

Nowadays, tourniquets are widely used in surgery of the extremities in order to improve the visualisation of the surgical area and reduce intraoperative bleeding[63, 64]. However, tourniquet use has been associated with multiple adverse events, e.g. increased postoperative pain, soft tissue damage, nerve paralysis, thromboembolism, slow wound healing, wound infection, reduced muscle strength, compartment syndrome, rhabdomyolysis, and longer recovery times[64-66]. Clear evidence-based guidelines for the use of tourniquets are sparse and call for further investigation.

Wakai et al. highlighted four important measures in order to minimise the risk of tourniquet-related complications[66]:

1. Careful selection of patients. Peripheral vascular disease, prosthetic vascular graft presence beneath the tourniquet cuff, extensive soft tissue damage, and patients with sickle cell disease are all associated with an increased risk of complications if surgery is performed with the use of a tourniquet[66].
2. Use of a wide low-pressure tourniquet cuff. The use of a wide low-pressure tourniquet cuff is more effective and thereby decreases the risk of complications[67, 68]. A pressure of 50–75 mmHg and 100–150 mmHg above the systolic blood pressure is generally

recommended for upper and lower limb surgery, respectively[66].

3. Correct timing of perioperative antimicrobial prophylaxis administration before tourniquet inflation. Due to the occlusion, correct timing of perioperative antimicrobial prophylaxis is essential to ensure therapeutic intraoperative antimicrobial tissue concentrations[5, 9]. However, only a few studies have investigated these matters, and the guidelines are ambiguous[69-71]. Currently, three different studies recommend three different antimicrobial administration scenarios; 10 min prior to tourniquet inflation[70], 30–60 min prior to tourniquet inflation[69], and at tourniquet release[71].

4. The tourniquet time should not exceed 2 hours, as tourniquet-induced ischemia is the primary cause of most tourniquet-related complications[64-66]. A tourniquet time of 2 hours or less is considered safe[65, 66]. This recommendation is primarily based on studies evaluating skeletal muscle, which is also considered the most vulnerable tissue[72-74]. However, the basic cellular and ischemic changes in tourniquet-affected bone and soft tissues have been poorly investigated.

Peri- and post-operative influences of tourniquet application on cefuroxime tissue concentrations have never been investigated for orthopaedically-relevant tissues.

3.7 Tissue ischemia

Tissue ischemia is a consequence of reduced oxygen supply to the cells, which is an unavoidable feature of tourniquet inflation[75]. In order to maintain energy production under ischemic conditions, the ischemic cells are forced to change from oxidative phosphorylation (aerobic cellular respiration) to anaerobic glycolysis (anaerobic cellular respiration). This leads to an increase in lactate concentrations while glucose and pyruvate concentrations decrease, resulting in increased lactate/pyruvate ratios, which is considered an accurate ischemic marker[75]. A lactate/pyruvate ratio above 25 is considered to signify ischemia[76]. Most cell membranes consist of two fatty acid chains, glycerol and a phosphate group, which together forms a glycerophospholipid[75]. As these cell membrane components are released when the cell membrane is damaged, glycerol can be used as a marker of cell death/damage[75].

Two clinical studies have previously investigated skeletal muscle glucose, lactate, pyruvate and glycerol changes both

during and after tourniquet inflation[77, 78]. Based on the individual ischemic markers, the ischemic changes were suggested to be maintained for approximately 2.5 hours after tourniquet release. However, based on the more precise ischemic marker, the lactate/pyruvate ratio, the recovery time from tourniquet release was found to be only 30 min in the skeletal muscle[77]. The ischemic changes during and after tourniquet inflation have never been investigated for other orthopaedically-relevant tissues.

4 Aim of the thesis

The overall objective of this PhD project was to validate and apply microdialysis in order to evaluate the effects of tourniquet application on both peri- and post-operative cefuroxime and ischemic metabolite concentrations in orthopedically-relevant tissues. Microdialysis allows for continuous sampling of the free concentrations of antimicrobials and ischemic metabolites from the interstitial space of various tissues, permitting estimation of e.g. T>MIC and lactate/pyruvate ratios. Moreover, T>MIC can be compared for different time windows between cefuroxime administration and tourniquet inflation, for following dosing intervals after tourniquet release, and simultaneously allows for the evaluation of the lactate/pyruvate ratio peri- and post-operatively. These objectives were evaluated in a three-step approach: (1) *in vitro* and *in vivo* evaluation of the microdialysis calibration method used, (2) an experimental *in vivo* study evaluating the different timepoints of cefuroxime administration and tourniquet inflation, and lastly, (3) a clinical study evaluating the effects of tourniquet application on cefuroxime and ischemic metabolite tissue concentrations peri- and post-operatively over two dosing intervals. The design of each step in this three-step approach was reliant on the preceding findings.

4.1 Hypotheses for studies I–IV

4.1.1 Study I

Primary hypothesis

- Meropenem can be used as an internal standard for cefuroxime, and cefuroxime relative recovery, thereby, resembles meropenem relative recovery both *in vitro* and *in vivo.*

Secondary hypotheses

- Relative recovery of cefuroxime is concentration-independent.
- Presence of meropenem does not affect cefuroxime relative recovery.
- Neither meropenem nor cefuroxime adheres to the microdialysis catheters.
- The relative recovery is, as a minimum, constant over a 6-hour period.

4.1.2 Study II

Primary hypothesis

- A cefuroxime concentration of 4 µg/mL is maintained throughout a tourniquet-time duration of 90 min

in plasma, subcutaneous tissue, and calcaneal cancellous bone when administered 45 min prior to tourniquet inflation and not maintained when administered 15 min prior to tourniquet inflation.

Secondary hypotheses

- Administration of cefuroxime at tourniquet release results in prolonged T>MIC (4 µg/mL) in the tourniquet-exposed subcutaneous tissue and calcaneal cancellous bone due to a hyperaemic effect.
- Tourniquet induces ischemia in both subcutaneous tissue and calcaneal cancellous bone.

4.1.3 Study III

Primary hypothesis

- A cefuroxime concentration of 4 µg/mL is maintained throughout surgery in tourniquet-exposed subcutaneous tissue, skeletal muscle and calcaneal cancellous bone when administered 15 min prior to tourniquet inflation.

Secondary hypothesis

- The use of a tourniquet does not affect the T>MIC (4 µg/mL) of cefuroxime in tourniquet-exposed subcutaneous tissue, skeletal muscle and calcaneal cancellous bone in the following dosing interval.

4.1.4 Study IV

Primary hypothesis

- The lactate/pyruvate ratio is peri- and post-operatively increased in tourniquet-exposed subcutaneous tissue, skeletal muscle and calcaneal cancellous bone.

5 Materials and methods

In this PhD project, the pharmacological tool, microdialysis, was used for dense sampling of cefuroxime and the metabolites' glucose, lactate, pyruvate and glycerol concentrations in subcutaneous tissue, skeletal muscle and cancellous bone. The cefuroxime concentration in the collected samples was quantified using ultra-high performance liquid chromatography (UHPLC) with UV detection. The ischemic metabolites were quantified using a CMA 600 Microdialysis Analyzer. The following chapters will summarise the basic principles of the applied methods, including the *in vitro*, porcine and clinical models as well as the following ethical and statistical considerations.

5.1 Microdialysis

Microdialysis is a catheter-based technique that allows for continuous sampling of water-soluble molecules in the interstitial space in the tissue of interest (Figure 1)[79-82]. The microdialysis catheter consists of an inlet tube wherein a perfusate, a physiological fluid, is lead to a semipermeable membrane at the tip of the catheter. The diffusion (sampling) of molecules from the interstitial space occurs

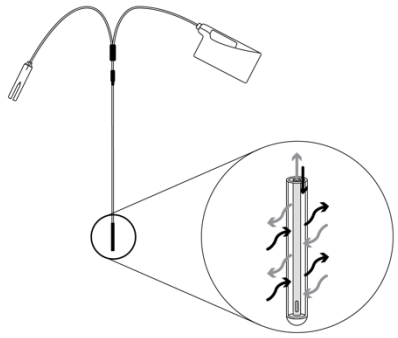

Figure 1. Illustrative drawing of the microdialysis system with an enlargement of the membrane. This figure was published by Hanberg et al. 2021[4].

across the semipermeable membrane according to the concentration gradient, given that the size of the molecule allows it to pass through the pores in the membrane. The pores in the membrane come in different sizes. From the membrane, the fluid with the sampled molecules, called dialysate, is then lead through an outlet tube and finally collected in small containers (microvials). The whole microdialysis system is driven by a precision pump producing a low and constant flow through the microdialysis catheter. As the catheter is continuously perfused equilibrium across the semipermeable membrane never occurs[79-82]. Consequently, the molecules in the dialysate will only represent a fraction of the actual tissue concentration. This fraction is referred to as the relative recovery, which can be determined by various calibration methods, see section *5.1.1 Calibration techniques*. When absolute

tissue concentrations are of interest as in pharmacokinetic studies, catheter calibration is imperative[79-82]. However, when changes in the concentration ratios and variation between interventions or compartments are of interest, as they are when comparing ischemic metabolite concentrations between a tourniquet and non-tourniquet-exposed legs and for ratios between metabolites (e.g. lactate/pyruvate), this is not essential[79].

The microdialysis technique has gained a great foothold in the pharmacological research area since the concept was presented in the early 1960s[83, 84]. Since the first studies, the microdialysis technique has developed through the years to the more sophisticated needle catheter which is currently used. Today more than 10,000 studies have been performed using the microdialysis technique[79].

Nowadays, a typical microdialysis system consists of a microdialysis catheter, a microdialysis pump and the perfusate (perfusion fluid). Microdialysis catheters differ with respect of the catheter dimension, length, the membrane cutoff (pore size), and material of the membrane. The microdialysis pump produces a continuous, low (typical range: 0.1–5 µL/min), and precise flow of the perfusate through the microdialysis catheter. The perfusate is generally recommended to mirror the isotonic composition of the interstitial space fluid of the investigated tissue and can, therefore, be varied according to the object[79, 80]. In the present studies, microdialysis equipment from M Dialysis AB (Stockholm, Sweden) was used. The catheters used were CMA 63 (membrane length 10 and 30 mm with a 20 kilo Dalton cutoff), and a CMA 107 precision pump produced a flow rate of 2 µL/min with a perfusate consisting of 0.9% NaCl holding 5 µg/mL.

5.1.1 Calibration techniques

Calibration and thereby determination of the relative recovery are imperative in a pharmacokinetic setting. Relative recovery depends on several factors; flow rate, membrane size and permeability, temperature, the diffusivity of the substances in the tissue, physiochemical properties of the analyte being analysed, etc.[79-82]. Importantly, the relative recovery of a specific analyte should be independent of the concentration gradient across the membrane[79-82]. A number of well-described calibration methods can be used to determine the relative recovery[79-82]. Common for all calibration methods is

the assumption that the relative recovery by gain equals the relative recovery by loss[79-82]. The relative recovery by gain is an expression of how much of the drug in the media surrounding the membrane that passes from outside to inside the membrane, whereas the relative recovery by loss is an expression of how much of the drug within the microdialysis system that passes from inside to outside the membrane. The relative recovery by gain and relative recovery by loss can be calculated by the following equations:

$$RR_{gain} = \frac{C_{dialysate}}{C_{media}} \quad (1)$$

$$RR_{loss} = 1 - \frac{C_{dialysate}}{C_{perfusate}} \quad (2)$$

where the RR_{gain} is the relative recovery by gain, the RR_{loss} is the relative recovery by loss, the $C_{dialysate}$ is the concentration in the dialysate, the C_{meadia} is the concentration in the media surrounding membrane, and the $C_{perfusate}$ is the concentration in the perfusate. While equation 1 relies on the assumption that the concentration in the perfusate ($C_{perfusate}$) is 0, equation 2 relies on the assumption that the concentration in the media surrounding the membrane (C_{media}) is 0. When the relative recovery has been determined, the absolute tissue concentration can be calculated using equation 3:

$$C_{tissue} = \frac{C_{dialysate}}{RR} \quad (3)$$

where the C_{tissue} is the concentration in the tissue surrounding the membrane.

The most commonly used calibration methods are the no-net-flux method, the low-flow-rate method, and the retrodialysis by drug or by an internal standard method[79-82]. In antimicrobial pharmacokinetic studies, retrodialysis by drug method is the most commonly used calibration method[82].

Calibration with the retrodialysis by drug method can be performed either in the beginning or at the end of the experiment. The calibration is performed by adding a known concentration of the analyte of interest to the perfusate. By quantifying the concentration in the dialysate ($C_{dialysate}$), relative recovery by loss can be calculated using equation 2. The retrodialysis by drug method was originally proposed by Stahle et al. in 1991[85]. In studies investigating, e.g. steady-state concentrations, calibrations by the retrodialysis by drug method is not

applicable, if the tissue concentration of the investigated drug is not 0 prior to the next dosing interval[79-82]. Moreover, if calibration is performed at the beginning of the study, this calibration method requires a washout period to prevent spillover in the tissues[79-82]. Thus, calibration by the retrodialysis by drug method can be time-consuming or impractical.

Alternatively, the relative recovery can be determined by retrodialysis by drug with an internal standard. Calibration with an internal standard requires an internal standard with physiochemical similarities to the analyte of interest so that the diffusion properties over the microdialysis membrane are similar in both directions[82]. Furthermore, there should be no interference between the internal standard and analyte of interest[82]. Calibration with the retrodialysis by drug with an internal standard method is based on the assumption that the diffusion of the analyte of interest from the interstitial space over the membrane (relative recovery by gain) equals the diffusion of the internal standard from the perfusate (inside the membrane) to the interstitial space (relative recovery by loss)[82]. The internal standard method is advantageous due to continuous calibration throughout the study period, which is both timesaving and allows for calibration in settings where the patients or experimental animals are being pre-treated with the drug of interest. Although the internal standard method is associated with considerable advantages, a thorough validation of the internal standard is compulsory, which can be time-consuming. Besides finding an internal standard with the same physiochemical properties as the analyte of interest, interactions and diffusion properties between the internal standard and the analyte of interest need to be thoroughly investigated. If an internal standard is not thoroughly validated, the calibration method may be inaccurate, providing the investigator with incorrect and uncertain results. Furthermore, it is important to notice that *in vitro* relative recovery does not necessarily reflect *in vivo* relative recovery[86]. Consequently, an *in vitro* validation of an internal standard should not stand alone[82]. As the first part of this PhD project (Study I), the retrodialysis by drug method using meropenem as an internal standard was validated and applied as the calibration method in the following studies (Studies II–III).

5.1.2 Advantages and limitations

Microdialysis is, in contrast to other methods used for determination of

antimicrobial tissue pharmacokinetics, advantaged by the serial sampling of the extracellular and unbound fraction of drug from multiple tissues, which for antimicrobial is known to be pharmaceutically active. Sampling of only the pharmaceutically active drug with a relatively high time resolution allows the results to be compared directly to relevant PK/PD index targets. This provides more solid data and thereby reduces the needed number of patients or animals for a given study.

For all tissues, the placement of the microdialysis catheter will inevitably traumatise the tissue to some extent, which can influence the subsequent analysis, especially if the aim of the study is to investigate ischemic or inflammatory metabolites. For a variety of tissues, studies have investigated the changes in tissue trauma markers and alterations in local blood flow[79-81, 87, 88]. The biochemical trauma-related changes after catheter placement differ from tissue to tissue and have been reported to typically return to baseline within 0.5–5 hours[79-81, 87]. However, for glycerol, the trauma-related changes associated with the insertion of microdialysis catheters in bone tissue have been reported to sustain for up to 8 hours[88]. These issues have not been investigated in this PhD project as the primary endpoint in the ischemic metabolite investigations were to compare intervention vs non-intervention, which does not necessitate a tissue recovery period.

It is important to recognise that a compromise between the ideal setup and experimental requirements is unavoidable in microdialysis studies. Relative recovery is one of the most important factors that may be compromised by the experimental needs. Adjustable experimental factors are the size of the microdialysis membrane and the flow rate. A short membrane and a high flow rate will contribute to a low relative recovery and vice versa[79-82]. In pharmacokinetic studies, the obligatory correction of the measured concentrations for the relative recovery will lead to a magnification of the variations associated with the preanalytical sample handling and the chemical assay. This magnification increases exponentially with decreasing relative recoveries. Accordingly, measures should be taken to ensure relative recovery as high as possible, and it is, therefore, generally recommended to exceed 20%[79]. Thus, the longest membrane and lowest flow rate allowed by the experimental setup should be used in order to increase the relative recovery.

However, this can be challenged by tissues with limited space and by measuring on short half-lived drugs, which needs a relatively high temporal resolution and thereby a high flow rate to produce sufficient volume of dialysates for the subsequent chemical analysis.

Dialysates are serially sampled over a defined time interval, and the measured dialysate concentration represents an average of the sampling interval. Commonly, the measured tissue concentration is, ascribed to the midpoint of each sampling interval. Thus, this is a necessary simplification of the true concentration.

Microdialysis is almost limited to water-soluble molecules of relatively small sizes[79-81]. It is therefore important to take the molecule size of the analyte of interest into account when designing a microdialysis study, to ensure that the analyte can diffuse across the membrane. Lately, high cutoff membranes with large pore sizes have been developed, so that even larger molecules can be collected.

It is important to recognise that microdialysis remains a sampling technique that must be linked to an appropriate analytical assay in order to determine the investigated drug concentrations. The magnification of the variations associated with the preanalytical sample handling and the chemical assay calls for a precise and accurate analytical assay. Furthermore, the analytical assay is required to overcome the challenges of low dialysate volumes and low concentrations. In order to achieve the most feasible microdialysis setup, it is important to integrate information about the analytical assay quality into both the adjustment of the experimental study design and in the evaluation of the resulting findings to achieve the most feasible methodological microdialysis setup[81]. In the following sections, the UHPLC and CMA 600 Microdialysis Analyzer assays, and the following methodological considerations will be presented along.

5.2 Ultra-high performance liquid chromatography

Cefuroxime and meropenem concentrations were quantified using a UHPLC (Agilent 1290 Infinity; Agilent Technologies, USA) with UV detection at 275 nm and 304 nm, respectively (Figure 2). The method was validated with respect to the selectivity, linearity, precision, accuracy, lower limit of quantification, stability and recovery and in accordance with Clinical and Laboratory Standards Institute

Figure 2. An example of an UHPLC system.

recommendations[Hardlei, TF – unpublished data]. Inter-run imprecisions (percent coefficients of variation) were 4.7% at 2.5 µg/mL for quantification of cefuroxime and 3.0% at 2.0 µg/mL for quantification of meropenem. The lower limits of quantification, defined as the lowest concentration with an intra-run CV of less than 20%, were 0.06 µg/mL for cefuroxime and 0.5 µg/mL for meropenem[89]. The needed volume for quantification of cefuroxime and meropenem was 15 µL and the total time of analysis was 4 min per sample[89]. The stability of cefuroxime and meropenem is adequate for the present assay. Calculation of the cefuroxime and meropenem concentrations was conducted with ChemStation software (Agilent Technologies) and were based on the peak areas of both drugs. A representative chromatogram for the quantification of both the cefuroxime and meropenem concentrations can be found in Figure 3. A detailed description of the practical procedures for quantification of cefuroxime and meropenem can be found elsewhere[89].

All cefuroxime and meropenem analyses were performed at the Department of Clinical Biochemistry, Aarhus University Hospital, Denmark.

5.2.1 Advantages and limitations

UHPLC has been validated as a specific, sensitive, accurate method for quantifying cefuroxime and meropenem concentrations in both plasma and dialysates. Most bacteria involved in orthopaedic settings exhibit MICs for cefuroxime in the range of 0.5–4 µg/mL. UHPLC, therefore, allows for the opportunity to quantify significantly lower concentrations than the clinically relevant concentrations.

In the present study, the dialysate volumes ranged between 30–120 µL. With a standard volume demand of 15 µL for quantification of cefuroxime and meropenem, the number

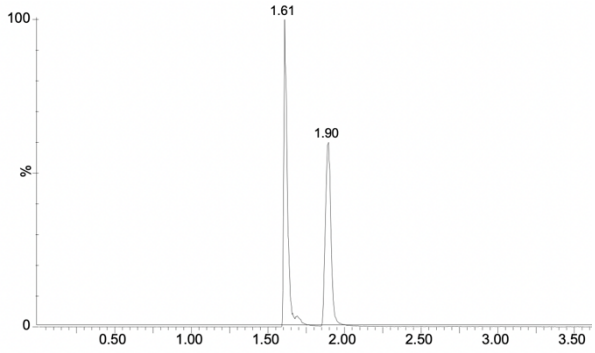

Figure 3. A representative chromatogram for the quantification of both the cefuroxime and meropenem concentrations.

of analyses was limited to only 1–2 runs for the dialysates with the lowest volume. Furthermore, when pipetting such small volumes, even small fluctuations of the samples can affect the results.

5.3 CMA 600 Microdialysis Analyzer

Glucose, lactate, pyruvate, and glycerol were quantified using the CMA 600 Microdialysis Analyzer with Reagent Set A (M Dialysis AB, Sweden, Stockholm). The CMA 600 Microdialysis Analyzer uses colourimetric measurements with enzymatic reagents[90]. The used reagents enzymatically oxidise the sampled substrates, leading to hydrogen peroxide formation. The peroxidase then catalyses a reaction between hydrogen peroxide and 4-amino-antipyrine, and phenol or N-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine to form a red-violet quinoneimine or quinonediimine (colourimetric indicators)[90]. The amount of formatted, coloured substance is proportional to the substrate concentration and is measured photometrically as the change of absorbance at 546 nm wavelengths. The needed volume for quantification of each analyte (glucose, lactate, pyruvate and glycerol) is 1 µL and the total time of analysis of all four metabolites is approximately 6 min per sample[90]. Inter-run imprecisions (percent coefficients of variation) were 3.3% at 1 mmol/L for quantification of glucose, 6.9% at 2.2 mmol/L for quantification of lactate, 3.5% at 198 µmol/L for quantification of glycerol, and 5.0% at 173 µmol/L for quantification of pyruvate[90]. The lower limits of quantification, defined as the lowest concentration with an intra-run CV of less than 20%, were 0.1 mmol/L for glucose and lactate and 10 µmol/L for glycerol and pyruvate[90].

5.4 *In vitro* model

In order to apply microdialysis for use in clinical pharmacokinetic studies, a number of *in vitro* and *in vivo* studies must be conducted to evaluate its use for the sampling of the analyte of interest. In the present PhD project, microdialysis was used to evaluate cefuroxime tissue concentrations, and meropenem was used as an internal standard for cefuroxime in the

calibration process. When calibrating with the retrodialysis by drug with an internal standard method, following issues should be investigated: (1) Does the relative recovery by gain of the analyte of interest resemble the relative recovery by loss of the internal standard and vice versa at different concentrations; (2) does the relative recovery by gain resemble the relative recovery by loss for both the analyte of interest and the internal standard at different concentrations; (3) does the analyte of interest relative recovery depends on the analyte concentration; (4) does the presence of the internal standard affect analyte of interest relative recovery; (5) does the analyte of interest and internal standard adhere to the microdialysis catheter. These matters are prerequisites for performing pharmacokinetic studies. In the present PhD project, these matters were investigated in a series of *in vitro* studies in Study I[1].

5.4.1 Advantages and limitations

When performing *in vitro* studies, the environment on both sides of the membrane can be controlled. As such, relative recovery by gain and loss can be compared for different analytes and internal standards. *In vivo* studies only allow the control of concentrations in the perfusate (inside the membrane).

As previously mentioned, the diffusion coefficient in the tissue or medium surrounding the catheter is partly decisive for the relative recovery. As such, the *in vivo* physiochemical effects on the relative recovery can only be investigated in *in vivo* studies. *In vitro* validation of an internal standard should therefore not stand alone.

In Study I, we evaluated meropenem as an internal standard for cefuroxime both *in vitro* and *in vivo*. However, Study I does not clarify all aspects of the microdialysis calibration technique as the influence of the temperature and flow rate was not investigated in the *in vitro* study[1]. However, we applied the same flow rate throughout Studies I–IV, and the relative recovery was investigated at physiological temperatures in the *in vivo* part of Study I[1].

5.5 The porcine models

Study I:

In the *in vivo* part of Study I, the porcine model was used to investigate the following in issues: Does relative recovery remained constant over time and is the meropenem relative recovery by loss equal to cefuroxime relative recovery by loss. With the *in vitro* study (Study I) a safe methodological setup

was ensured, which was a premise for the further progress of this PhD project.

Study II:

With Study II the following issues were investigated in a porcine model: how do the timing of antimicrobial (cefuroxime) perioperative prophylaxis and tourniquet inflation affect the tissue concentrations and how does tourniquet inflation affect the tissue ischemia. The influence of different time intervals between cefuroxime administration and tourniquet inflation was evaluated in a randomised setting in order to evaluate whether insufficient antimicrobial prophylaxis could be a consequence of an incorrect time interval between antimicrobial administration and tourniquet inflation. The following cefuroxime administration timepoints were investigated: 15 min prior to tourniquet inflation (Group A), 45 min prior to tourniquet inflation (Group B), and at tourniquet release (Group C)[2]. The investigated time intervals were based on the current but sparse literature. In 1987, Johnson stated that the administration of antimicrobial prophylaxis 10 min prior to tourniquet inflation was sufficient in order to maintain therapeutic concentrations throughout the operation[70]. However, in 1996 Deacon et al. advocated for a 30 to 60 min interval[69]. Finally, in a more recent study, Soriano et al. suggested a beneficial effect of administrating antimicrobial prophylaxis at tourniquet release[71].

In Study II, the cancellous bone catheter was placed in the calcaneus bone, as the tourniquet cuff could only be placed at the lower hind legs due to the anatomy of the pig[2]. As the lower hind leg is cone-shaped, a tourniquet pressure of 400 mmHg was chosen to ensure occlusion.

Study II was randomised in terms of tourniquet cuff side and study group. After the surgical procedure was performed and all the catheters were placed, the tourniquet cuff was placed on a randomly picked leg by drawing a note from an opaque envelope containing a total of 24 notes (12 marked right leg and 12 marked left leg). Each pig was then randomised to either Group A, B, or C by drawing a note from an opaque envelope containing a total of 24 notes (8 marked Group A, 8 marked Group B and 8 marked Group C).

5.5.1 Surgical procedure

Study I:

After induction of anaesthesia, surgery was initiated. With the pig in a supine position, the right calcaneus was exposed via a

longitudinal plantar incision. Using fluoroscopic guidance, a drill hole (diameter: 2 mm; length: 4 mm) was made from the inferior part of the calcaneocuboid joint to the proximal part of the calcaneus bone. A catheter was placed in the drill hole and fixed with a single skin suture. Subsequently, a subcutaneous tissue catheter was placed in the right thigh in accordance with the guidelines of the manufacture. Correct bone catheter placements were documented using fluoroscopy.

Study II:

The surgical procedure for Study II was very similar to Study I. However, in Study II, the subcutaneous tissue catheter was placed in the plantar side of the hindfoot. Furthermore, the surgical procedure was performed on both hind legs so that catheters could be placed on both sides. After placement of the catheters, the tourniquet cuff was placed in a randomly picked leg. Correct bone catheter placements were, as in Study I, documented using fluoroscopy.

5.5.2 Ethical considerations

The experimental studies were conducted at the Institute of Clinical Medicine, Aarhus University Hospital, Denmark. The studies were carried out according to existing laws and approved by the Danish Animal Experiments Inspectorate (license no.: 2017/15-0201-01184).

5.5.3 Advantages and limitations

Pigs were chosen as experimental models because they resemble humans in terms of their anatomy and physiology[91]. Moreover, the composition, size, density and quality of the porcine bone is to a great extent comparable to that of man[92]. To minimise the number of groups and thereby patients in the clinical study (Study III), it appeared most ethically to explore how the timing of cefuroxime perioperative prophylaxis and tourniquet inflation affect the cefuroxime tissue concentrations in an animal model. Based on the results from the porcine study (Study II), a reasonable time interval from cefuroxime prophylaxis administration to tourniquet inflation could be chosen for the clinical study (Study III). Furthermore, results from the porcine study (Study II) could support a sample size calculation for the clinical study (Study III). Finally, a long tradition of conducting porcine experiments at the Institute of Clinical Medicine, Aarhus University Hospital, Denmark, has resulted in excellent local facilities for this purpose.

Despite the fact that pigs resemble humans in terms of anatomy and physiology, important interspecies differences have to be taken into account[91]. In Studies I and II, juvenile pigs (aged 5 months) were used as the animal weight at that age resemble that of an average human being. It could therefore be speculated that the cefuroxime tissue penetration and the ischemic metabolites after tourniquet release would be greater and faster, respectively, compared to mid-aged to old humans due to better tissue perfusions. The weight-bearing impact and stress of the calcaneal bone differ between pigs and humans, and the impact of this on the cefuroxime tissue penetration and ischemic metabolites are unknown. Furthermore, the applied tourniquet cuff pressure (400 mmHg) was substantially higher in the porcine model than in normal clinical use, which could have impacted the amount of cell damage and ischemia during and after tourniquet application.

In order to prevent displacement of the microdialysis catheters, the pigs were kept under general anaesthesia during the entire study period. Anaesthesia is known to cause physiological changes[93], which may affect drug pharmacokinetics. However, this may also represent the true antimicrobial perioperative prophylactic setting, as most patients are kept in general anaesthesia during surgery.

5.6 The clinical model

In the clinical study (Studies III and IV) we evaluated the effects of the tourniquet application on both the peri- and post-operative cefuroxime and ischemic metabolite concentrations in subcutaneous tissue, skeletal muscle and calcaneal cancellous bone. Based on the results from Study II, a time interval of 15 min from cefuroxime administration to tourniquet inflation was chosen, and a total of 10 patients were included. A second dose of cefuroxime was administered 6 hours after the first administration to compare the two dosing intervals.

Different patient categories were considered for the clinical setup. Firstly, we had to decide whether the setup should be performed on the upper or lower extremity, as the surgery should be tourniquet-aided. The lower extremity was chosen to reflect the porcine model. Secondly, it was important to ascertain that enough patient from the chosen patient group could be included within a predictable period of time. Thirdly, subcutaneous tissue, skeletal muscle and cancellous bone should be

accessible distal for the tourniquet cuff. We identified and choose to include a study population receiving forefoot operation, which allowed us to measure in the calcaneal cancellous bone, which was untouched on both sides. As the subcutaneous tissue and skeletal muscle catheters were placed at the lower leg, the tourniquet cuff was placed on the thigh of the operating leg. The clinical study was conducted at the Department of Orthopaedic Surgery, Horsens Regional Hospital, Denmark, who have a large volume of forefoot operation.

The inclusion and exclusion criteria and patient characteristics are listed in the manuscript for Study III and IV[3, 4].

5.6.1 Surgical procedure

After spinal anaesthesia and before the planned surgical procedure, microdialysis catheters were placed similarly in both legs: in the subcutaneous tissue at the posterior part of the mid-lower leg, in the gastrocnemius muscle of the medial head, and in the calcaneal cancellous bone via drill holes (diameter: 2 mm; depth 30 mm). The drill holes were made by a posterolateral approach aiming for the anteromedial side of the calcaneal bone. The subcutaneous tissue and skeletal muscle catheters were placed in accordance with the guidelines of the manufacture. The calcaneal bone was entered via a 5 mm stab-incision, and the microdialysis catheter was placed using a splitable introducer and the Seldinger technique.

5.6.2 Ethical considerations

The clinical study was approved by the Danish Medicines Agency (EudraCT number 2018-000217-21), the Central Denmark Region Committees on Health Research Ethics (Registration number 1-10-72-47-18), and the Danish Data Protection Agency (Registration number 1-16-02-88-18). The study was registered at www.clinicaltrialsregister.eu (number 2018-000217-21) and conducted in accordance with the Declaration of Helsinki and the ICH Harmonized Tripartite Guideline for Good Clinical Practice. The Good Clinical Practice Unit at Aalborg and Aarhus University Hospitals (Denmark) conducted the mandatory monitoring procedures.

5.6.2 Advantages and limitations

In the clinical study, we conducted a setup which allowed us to sample cefuroxime and ischemic metabolites concentrations pre-, peri- and post-operatively. Accordingly, the

generalisation to the prophylactic setting is credible.

In order to attain generalisable data, we included both genders. However, as most patients with hallux valgus and hallux rigidus are females, this gender is over-presented in Study III and IV (7 females/3 males), this makes the generalisation to the average population questionable. Moreover, neither of the measuring sites (tissues) were subjects to surgery as they were not implicated in the surgical procedure. The generalisation of the measured tissue concentrations of both cefuroxime and ischemic metabolites to the surgical site is therefore dubious. However, as the investigated compartments have been subjects to the same trauma, the comparison of the tourniquet and non-tourniquet-exposed compartment seems valid.

5.7 Statistical considerations

The resulting pharmacokinetic data can be calculated and analysed by different approaches. As we had no intention of describing the pharmacokinetic variability due to intrinsic and extrinsic factors and simulating dosage adjustments based upon these factors, we applied the noncompartmental analysis. In the following sections, the principle of the noncompartmental analysis will be outlined, followed by the considerations on the sample size calculation.

5.7.1 Statistical analysis

In a noncompartmental analysis, pharmacokinetic parameters are individually calculated from the concentration-time profiles for each compartment separately. Subsequently, comparative and descriptive statistics can be conducted, and pharmacokinetic measures such as T>MIC, tissue penetration ratios, AUC, C_{max}, half-life etc. can be calculated. The noncompartmental analysis is advantaged by its simplicity as it requires fewer assumptions than compartmental models[94]. The major disadvantage of the noncompartmental analysis is that it is limited to the actual data, and pharmacokinetic parameters for other dosing regimens cannot be predicted[94].

The pharmacokinetic parameters were determined for each compartment in all pigs/patients using noncompartmental analysis in Stata (v. 15.1, StataCorp, College Station, TX, United States). The AUC was calculated using the trapezoidal rule. However, this approach is not exact, as it is limited to the widths of the trapezoids (i.e.

the sampling interval) and not the true concentration-time profile form. In the case of first-order kinetics, AUC during the infusion phase may be underestimated when using the trapezoidal method and the AUC during the elimination phase may be overestimated[94]. Accordingly, the relationship between the half-life and sampling interval is the key determinant for the size of the estimation error, which can be reduced by decreasing the sampling interval.

The maximum of all the recorded concentrations was defined as C_{max}, enabling calculation of the T_{max}, as the time to C_{max}. The $T_{1/2}$ was calculated as $\ln(2)/\lambda_{eq}$, where λ_{eq} is the terminal elimination rate constant estimated by linear regression of the log concentration on time. The $AUC_{tissue}/AUC_{plasma}$ ratio was calculated as a measure of tissue penetration.

Microsoft Excel (v. 16.16.11, Microsoft Corporation, Redmond, Washington) was used to estimate the T>MIC (4 µg/mL) using linear interpolation. Linear interpolation approach relies on the assumption that the increase or decrease between the coordinates surrounding the point of interest is linear. This assumption is, as for the determination of the AUC, somewhat violated and may lead to under- or over-estimation of selected timepoints. The magnitude of this error is again determined by the temporal resolution of the sampling intervals.

A general comparison of the pharmacokinetic parameters and T>MIC was conducted using a repeated measurements analysis of variance followed by pairwise comparisons made by linear regression. The Kenward-Roger approximation method was used for degrees of freedom correction due to the small sample size. The model assumptions were tested using the visual diagnosis of residuals, fitted values and estimates of random effects. A significance level of 5% was used.

5.7.2 Sample size

As no previous studies have investigated and compared the T>MIC in both tourniquet-exposed and non-tourniquet-exposed tissues for different time intervals between cefuroxime administration and tourniquet inflation, it was difficult to establish relevant assumptions for a proper a sample size calculation for the porcine study (Study II). The porcine study was, therefore, primarily characterised as an explorative study and used as a stepping stone for performing a relevant sample size calculation for the

clinical study (Study III). Even so, in a post-study sample size calculation of two independent means, with a significance level of 5% and a power of 90%, comparing T>MIC (4 µg/mL) in tourniquet-exposed subcutaneous tissue for Group A (mean 198 min, SD 37 min) vs Group B (mean 204 min, SD 39 min) and in tourniquet-exposed calcaneal cancellous bone for Group A (mean 208 min, SD 43 min) vs Group B (mean 245 min, SD 37 min), a sample size of 845 and 26 pigs in each group was calculated for subcutaneous tissue and calcaneal cancellous bone, respectively. This indicated that the T>MIC differences for the group receiving cefuroxime 15 min (Group A) and 45 min (Group B) prior to tourniquet inflation were small and may lack clinical significance.

As administering cefuroxime 15 and 45 min prior to tourniquet inflation was found equal in regards to T>MIC in the porcine study, cefuroxime was administered 15 min prior to tourniquet inflation in the clinical study. The sample size calculation for the clinical study (Study III) was based on the clinical target for perioperative antimicrobial prophylaxis: maintaining therapeutic cefuroxime plasma and tissue concentrations throughout surgery[5, 7]. We defined therapeutic concentrations to be cefuroxime concentrations above 4 µg/mL. With a significance level of 5% and a power of 90%, a sample size calculation comparing one mean to a reference value of 105 min (pre tourniquet time (15 min) + expected tourniquet time (90 min)) was performed for the T>MIC (4 µg/mL) of plasma (Study II results: mean 145 min, SD 28 min), tourniquet-exposed subcutaneous tissue (Study II results: mean 198 min, SD 37 min), and calcaneal cancellous bone (Study II results: mean 208 min, SD 43 min). Based on these estimates, a sample size of eight, four and five patients were needed to demonstrate that our target of maintaining target tissue concentrations above 4 ug/ml for a minimum of 105 min was achieved in plasma, tourniquet-exposed subcutaneous tissue and calcaneal cancellous bone, respectively. Thus, it was decided to include 10 patients in the clinical study in order to accommodate the drop-out of patients and/or microdialysis probes.

6 Summary of studies

6.1 Study I

Simultaneous Retrodialysis by Drug for Cefuroxime Using Meropenem as an Internal Standard - A Microdialysis Validation Study[1]

Primary hypothesis: Meropenem can be used as an internal standard for cefuroxime, and cefuroxime relative recovery, thereby, resembles meropenem relative recovery both *in vitro* and *in vivo.*

Hypothesis disproved: No

6.1.1 Comments

Study I was separated into an *in vitro* and an *in vivo* part. The *in vitro* part was designed to investigate the basic prerequisites for determining cefuroxime concentrations within a relevant concentration range by means of microdialysis and for calibrating with the retrodialysis by drug method using meropenem as an internal standard, which was further evaluated in the *in vivo* part of the study.

A setup overview for the *in vitro* study exemplified by a forest plot comparing relative recovery values for the following issues can be found in Figure 4: the relative recovery for cefuroxime and meropenem was similar for all tested concentrations ($p > 0.20$), indicating that meropenem relative recovery by loss and relative recovery by gain is representative of cefuroxime relative recovery by gain and relative recovery by loss, respectively (Figure 4, Issue 1). No differences were found between relative recovery by gain and relative recovery by loss for either cefuroxime or meropenem ($p > 0.09$) (Figure 4, Issue 2). The concentration of cefuroxime, ranging from 1–30 µg/mL, had no impact on the cefuroxime relative recovery ($p > 0.10$) (Figure 4, Issue 3). The cefuroxime relative recovery by gain and relative recovery by loss were not affected by the presence of meropenem ($p > 0.90$) (Figure 4, Issue 4). No drug adherence problems to the microdialysis catheter of either cefuroxime and meropenem were found.

In the *in vivo* part of the study the following results were found: the mean relative recovery (95% CI) in subcutaneous tissue was 0.28 (0.23; 0.35) when calibrating with the retrodialysis by drug method using cefuroxime and 0.29 (0.22; 0.37) when calibration with the internal standard method using meropenem ($p = 0.84$). For

calcaneal cancellous bone, the mean relative recovery (95% CI) was 0.30 (0.25; 0.36) for the retrodialysis by drug method using cefuroxime and 0.32 (0.23; 0.40) for the internal standard method using meropenem ($p = 0.38$). When calculating the pharmacokinetic parameters for subcutaneous tissue and calcaneal cancellous bone using both calibration methods, similar pharmacokinetic results were found ($p > 0.70$). Furthermore, a stable relative recovery was found for a minimum of 6 hours, with a relative recovery/mean relative recovery range for meropenem of 0.92–1.12 for subcutaneous tissue and 0.93–1.09 for calcaneal cancellous bone (Figure 5). No distinct patterns were found.

In summary, we found meropenem suitable as an internal standard for cefuroxime in the subcutaneous tissue and calcaneal cancellous bone under the investigated experimental conditions. This was observed in both the *in vitro* and *in vivo* part of the study. Furthermore, the *in vitro* cefuroxime relative recovery was not affected by either the cefuroxime concentration or the presence of meropenem. *In vivo*, the meropenem relative recovery remained constant for a minimum of 6 hours.

A

	Setup 1	Setup 2	Setup 3	Setup 4	Setup 5	Setup 6	Setup 7	Setup 8
RRgain	Cef 15 µg/mL	Cef 1 µg/mL	Cef 15 µg/mL	Cef 30 µg/mL	–	Mero 5 µg/mL	Mero 5 µg/mL	Mero 5 µg/mL
RRloss	–	Mero 5 µg/mL	Mero 5 µg/mL	Mero 5 µg/mL	Cef 15 µg/mL	Cef 1 µg/mL	Cef 15 µg/mL	Cef 30 µg/mL

B

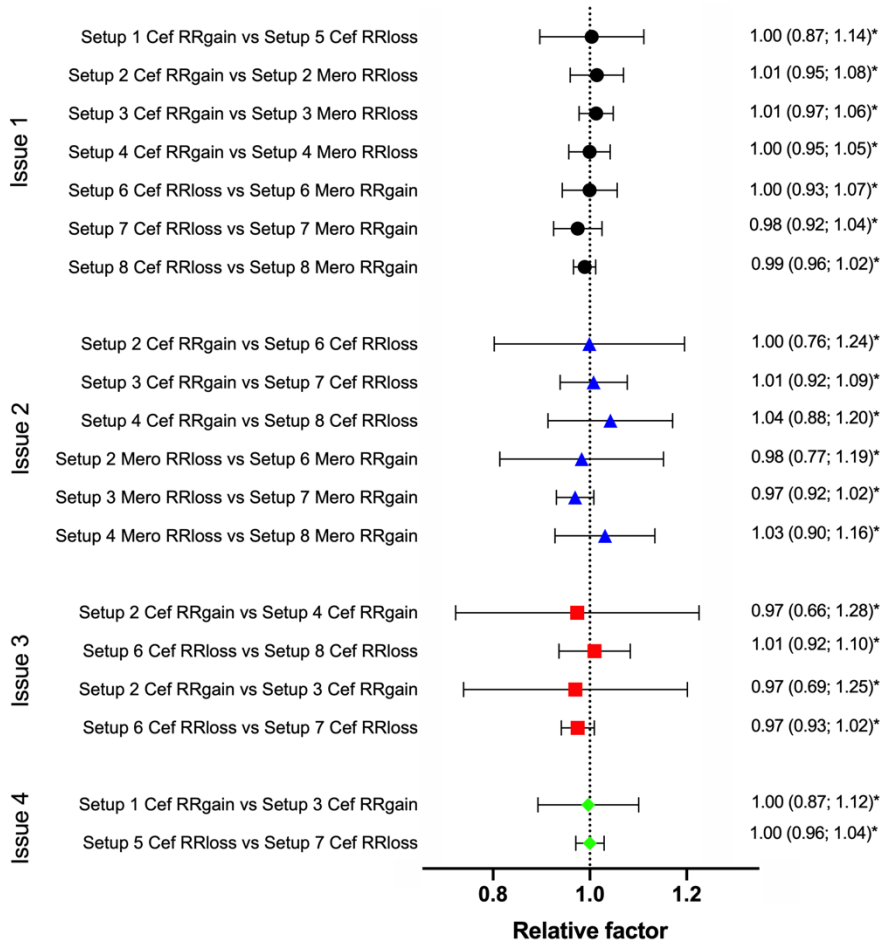

Figure 4. A: Setup overview for the *in vitro* study. B: Forest plot comparing relative recovery values for the following four issues in the *in vitro* study: 1) Does cefuroxime relative recovery resemble meropenem relative recovery; 2) Does relative recovery by gain resemble relative recovery by loss for both cefuroxime and meropenem; 3) Does cefuroxime relative recovery depend on the cefuroxime concentration; and 4) Does the presence of meropenem affect cefuroxime relative recovery.

Estimated mean relative factors are represented with 95% CI as bars. The mean values (95% CI) are given to the right. * $p > 0.05$

Abbreviations: Cef, cefuroxime; Mero, meropenem; RR, relative recovery; RRgain, relative recovery by gain; RRloss, relative recovery by loss. This figure was published by Hanberg et al. 2020[1].

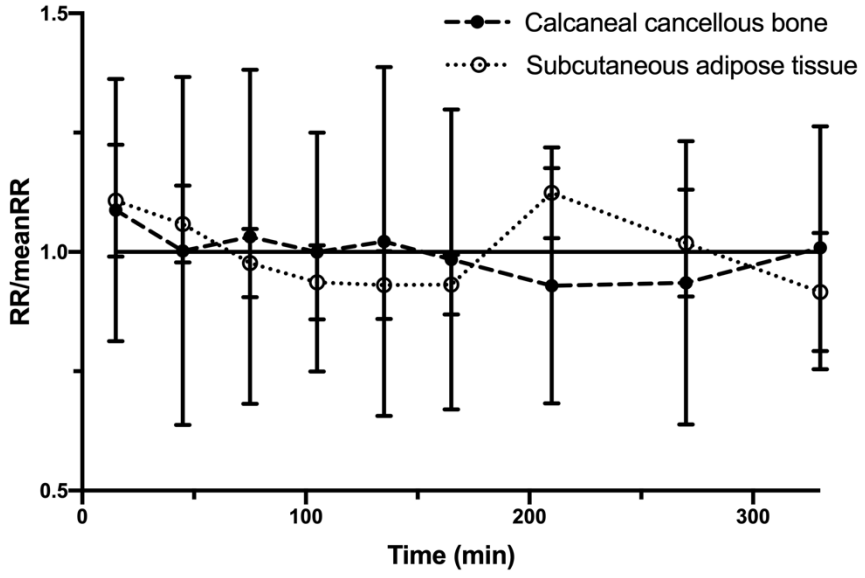

Figure 5. The relationship of the Relative recovery/mean relative recovery for meropenem over 6 hours *in vivo* for subcutaneous tissue and calcaneal cancellous bone. Bars represent 95% CI. No distinct patterns were found.

Abbreviations: RR, relative recovery. This figure was published by Hanberg et al. 2020[1].

6.2 Study II

Timing of Antimicrobial Prophylaxis and Tourniquet inflation - A Randomized Controlled Microdialysis Study[2]

Primary hypothesis: A cefuroxime concentration of 4 µg/mL is maintained throughout a tourniquet-time duration of 90 min in plasma, subcutaneous tissue, and calcaneal cancellous bone when administered 45 min prior to tourniquet inflation and not maintained when administered 15 min prior to tourniquet inflation.

Hypothesis disproved: Yes

6.2.1 Comments

In a porcine model we evaluated the subcutaneous tissue and calcaneal cancellous T>MIC at different timepoints of cefuroxime administration and tourniquet inflation. Three tourniquet application scenarios were evaluated: cefuroxime (1.5 g) administered intravenously 15 min prior to tourniquet inflation (Group A), 45 min prior to tourniquet inflation (Group B), and at tourniquet release (Group C). In the same study setup subcutaneous tissue and calcaneal cancellous bone ischemic metabolites were evaluated in relation to tourniquet application (before, during and after).

To evaluate T>MIC, the cefuroxime clinical breakpoint MIC for *S. aureus* (4 µg/mL) was applied. The T>MIC (4 µg/mL) is depicted in Table 2. In Groups A and B, the cefuroxime concentrations were above 4 µg/mL throughout the 90 min tourniquet duration time and for approximately 1 hour after tourniquet release in both subcutaneous tissue and calcaneal cancellous bone. In Group C, the cefuroxime concentrations in tourniquet-exposed subcutaneous tissue and calcaneal cancellous bone were above 4 µg/mL for approximately 3.5 hours after tourniquet release. Between groups no significant differences were found for subcutaneous tissue or calcaneal cancellous bone T>MIC (4 µg/mL). However, tourniquet-exposed calcaneal cancellous bone T>MIC tended to be shorter in Group A compared with Group C ($p = 0.08$). In all groups, plasma T>MIC was lower compared with the investigated tissues.

For subcutaneous tissue and calcaneal cancellous bone, the mean ischemic metabolite concentration differences (in percentages) between the tourniquet-exposed and nonexposed leg are depicted in

Figure 6. Immediately after tourniquet inflation, the lactate/pyruvate ratio was subject to a three-fold increase in both subcutaneous tissue and calcaneal cancellous bone. While the subcutaneous tissue lactate/pyruvate ratio decreased to baseline directly after tourniquet release, the calcaneal cancellous bone lactate/pyruvate ratio normalised after 2.5 hours. Additionally, the glucose and glycerol ratio decreased and increased, respectively, in calcaneal cancellous bone during tourniquet application.

In summary, this study suggests that administering cefuroxime 15–45 min prior to tourniquet inflation seems as a safe window. However, if the target is to maintain cefuroxime concentrations above relevant MIC values postoperatively, this study suggests to administer a second dose of cefuroxime at tourniquet release. Furthermore, this study shows that the tourniquet application induces tissue ischemia and cell damage in subcutaneous tissue and calcaneal cancellous bone, which was resolved within 2.5 hours from tourniquet release.

Table 2. The mean time with concentrations above the minimal inhibitory concentration (T>MIC) of 4 µg/mL given in min for plasma, subcutaneous tissue and calcaneal cancellous bone on both the tourniquet and non-tourniquet sides. This table was published by Hanberg et al. 2020[2].

Parameter	Group A	Group B	Group C
Plasma	145 (116; 174)[a]	147 (118; 175)[a]	142 (123; 171)[a]
Non-tq subcutaneous adipose tissue	198 (169; 227)	207 (178; 236)	204 (175; 233)
Tq subcutaneous adipose tissue	198 (169; 226)	204 (175; 233)	226 (197; 255)
Non-tq calcaneal cancellous bone	187 (158; 216)	213 (184; 242)	206 (177; 235)
Tq calcaneal cancellous bone	208 (179; 237)	245 (216; 273)	240 (211; 269)

Values are given as means (95% CI).

Comparisons within the group:

[a] $p < 0.05$ for comparison with all compartments

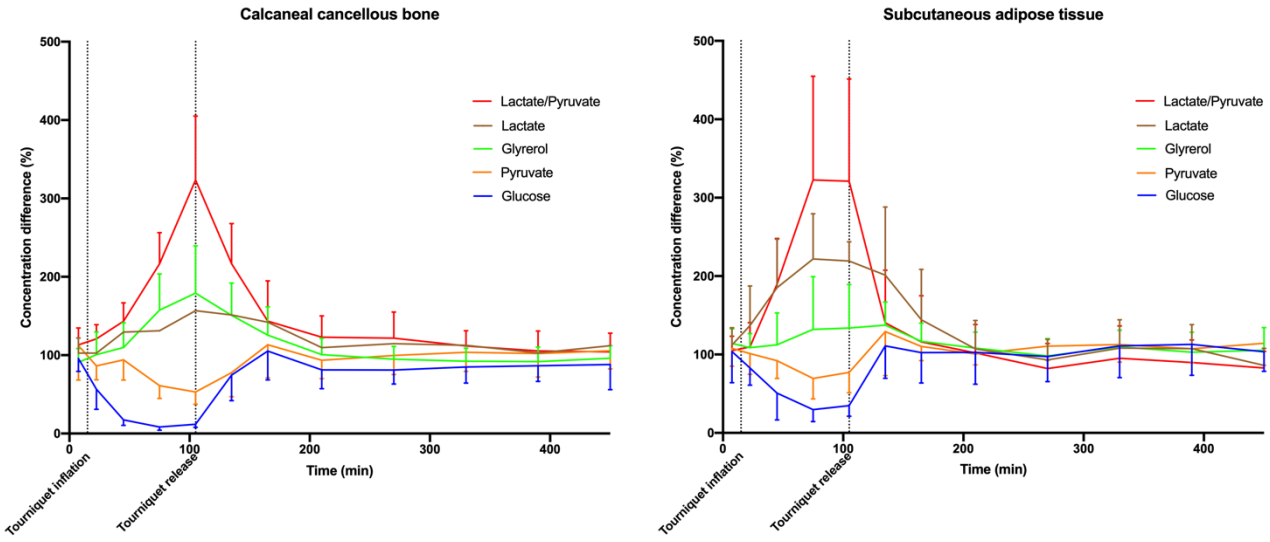

Figure 6. The mean ischemic marker concentration differences (%) between the tourniquet and non-tourniquet-exposed legs for both calcaneal cancellous bone and subcutaneous tissue. Bars represent the 95%-CI. This figure was published by Hanberg et al. 2020[2].

6.3 Study III

Effects of tourniquet inflation on peri- and post operative cefuroxime concentrations in bone and tissue[3]

Primary hypothesis: A cefuroxime concentration of 4 µg/mL is maintained throughout surgery in tourniquet-exposed subcutaneous tissue, skeletal muscle and calcaneal cancellous bone when administered 15 min prior to tourniquet inflation.

Hypothesis disproved: No.

6.3.1 Comments

The objective of this study was to dynamically evaluate the effects of tourniquet application on both peri- and post-operative *in situ* cefuroxime concentrations in subcutaneous tissue, skeletal muscle, calcaneal cancellous bone and plasma. Cefuroxime (1.5 g) was administered intravenously as a bolus 15 min prior to tourniquet inflation and followed by a subsequent dose 6 hours later. The mean tourniquet duration time (range) was 65 (58–77) min.

Similar results were observed for T>MIC (4 µg/mL) between the first and second dosing intervals. The T>MIC results for the first dosing interval can be found in Table 3. A cefuroxime concentration of 4 µg/mL was reached within 22.5 min in all compartments and patients. The T>MIC (4 µg/mL) ranged between 4.8–5.4 hours across compartments, with similar results for the tourniquet and non-tourniquet-exposed leg. When comparing tourniquet and non-tourniquet-exposed legs separately, lower T>MIC values were found for calcaneal cancellous bone compared to the remaining compartments in the tourniquet leg, including plasma ($p < 0.05$). No differences were found between the compartments in the non-tourniquet-exposed leg. Similar tissue penetrations were observed when comparing both the tourniquet and non-tourniquet-exposed legs and the first and second dosing interval. The concentration-time profiles of plasma and the investigated tissues for both the first and second dosing interval can be found in Figure 7.

In summary, this study suggests that administering cefuroxime (1.5 g) 15 min prior to tourniquet inflation is safe in order to achieve tissue concentrations above 4 µg/mL throughout surgery and that a tourniquet application time of approximately 1 hour does not affect the cefuroxime tissue penetration in the following dosing interval.

Table 3. The time with concentrations above the minimal inhibitory concentration (T>MIC) (4 µg/mL) in min for plasma, subcutaneous tissue, skeletal muscle and calcaneal cancellous bone on both the tourniquet and non-tourniquet-exposed legs from the first dosing interval (unpublished data).

Compartment	Time (min) Non-tourniquet leg	Time (min) Tourniquet leg	*P* values
Plasma	318 (297; 338)	-	-
Subcutaneous tissue	312 (292; 333)	322 (302; 343)	0.40
Skeletal muscle	320 (300; 341)	316 (295; 336)	0.73
Calcaneal cancellous bone	306 (285; 326)	289 (269; 310)[a]	0.18

Time given as mean (95% CI)

[a] $P < 0.05$ for comparison with all compartments in the tourniquet side and with plasma.

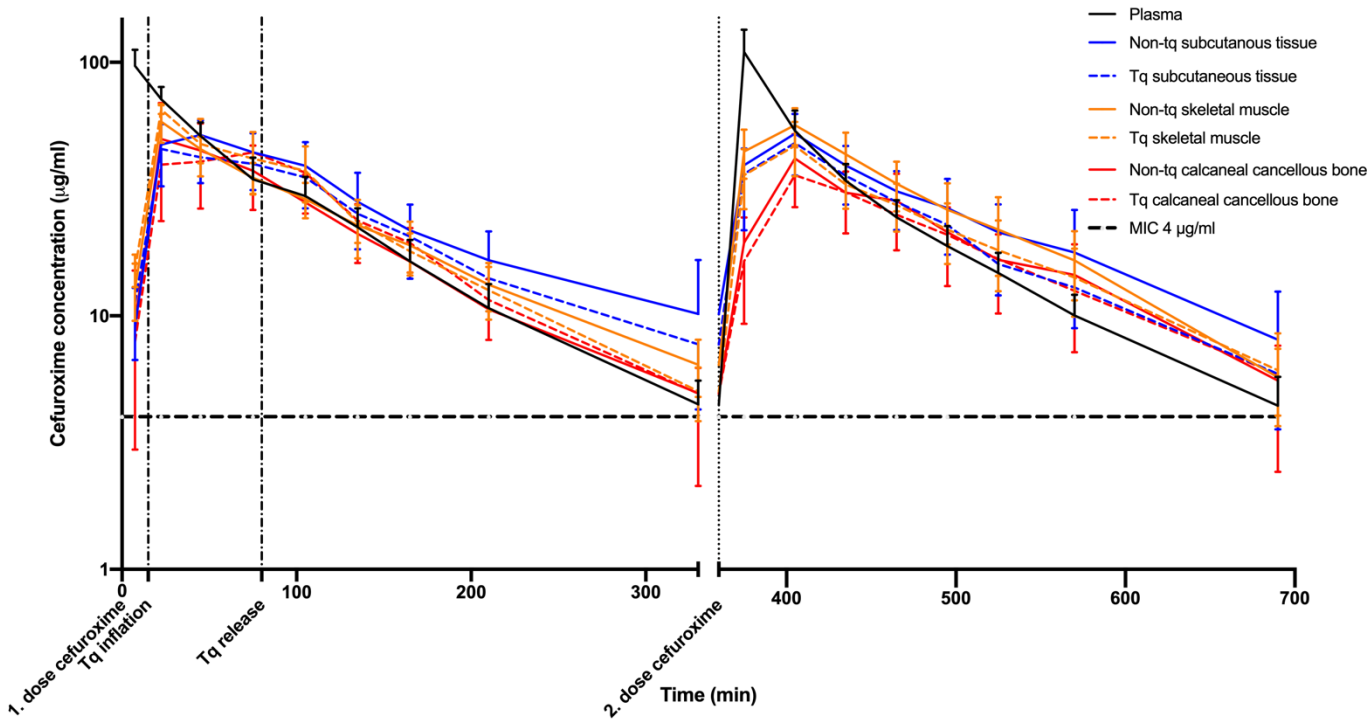

Figure 7. Mean concentration-time profiles of cefuroxime for plasma, subcutaneous tissue, skeletal muscle and calcaneal cancellous bone on both the tourniquet and non-tourniquet-exposed legs. Bars represent 95% CI. The y-axis is in log scale. The first and second dose of 1.5 g cefuroxime was administered at time 0 and 6 hours, respectively. Tourniquet inflation and mean release times were 15 and 80 min, respectively.
Abbreviations: Tq, Tourniquet; MIC, minimal inhibitory concentration (unpublished data).

6.4 Study IV

Tourniquet Induced Ischemia and Reperfusion in Subcutaneous Tissue, Skeletal Muscle, and Calcaneal Cancellous Bone [4]

Primary hypothesis: The lactate/pyruvate ratio is peri- and post-operatively increased in tourniquet-exposed subcutaneous tissue, skeletal muscle and calcaneal cancellous bone.

Hypothesis disproved: Yes, at least to some extent.

6.4.1 Comments

The objective of Study IV was to evaluate the glucose, lactate, pyruvate, glycerol and the lactate/pyruvate ratio in subcutaneous tissue, skeletal muscle and calcaneal cancellous bone in relation to tourniquet application (before, during and after) in a paired comparison of the tourniquet- and non-tourniquet-exposed legs.

For all investigated tissues, we found a two-to-three-fold increase of the mean lactate/pyruvate ratio during tourniquet inflation on the tourniquet-exposed leg in comparison to the nonexposed leg (Figure 8). The lactate/pyruvate recovery time from tourniquet release was within 30, 60 and 130 min for skeletal muscle, subcutaneous tissue and calcaneal cancellous bone, respectively. Evaluating the tissue lactate/pyruvate ratio in relation to the ischemic cutoff level of 25, only skeletal muscle was found ischemic during tourniquet inflation; however, the tissue ischemia resolved immediately after tourniquet release. The glycerol ratio, which is a marker of cell damage, increased immediately after tourniquet inflation on the tourniquet-exposed leg in subcutaneous tissue and skeletal muscle and recovered within 130 and 60 min from tourniquet release, respectively (Figure 9).

In summary, this study found limited subcutaneous tissue, skeletal muscle and calcaneal cancellous bone ischemia and cell damage following a tourniquet application time of approximately 1 hour; all investigated tissue ischemic metabolites were recovered within 130 min from tourniquet release.

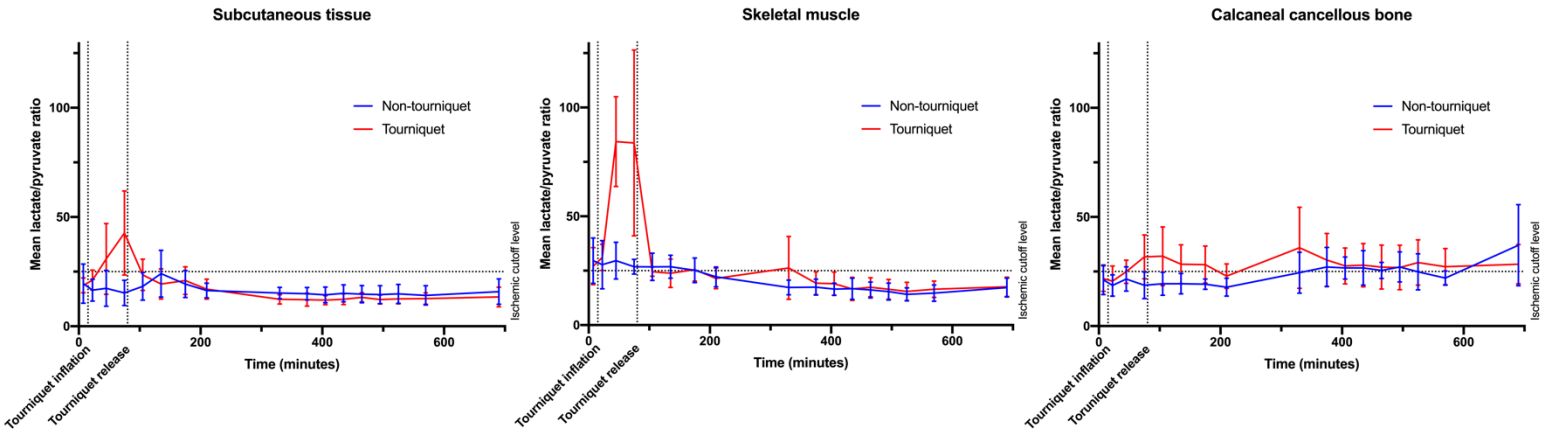

Figure 8. The mean lactate/pyruvate ratios for subcutaneous tissue, skeletal muscle and calcaneal cancellous bone for both the tourniquet and non-tourniquet-exposed legs. Tourniquet inflation time: 15 min, mean (range) tourniquet release time: 80 (73; 92) min (both are marked with vertical dotted lines). The ischemic cut off level of 25 is marked with horizontal dotted lines. Bars represent the 95% CI. This figure was published by Hanberg et al. 2021[4].

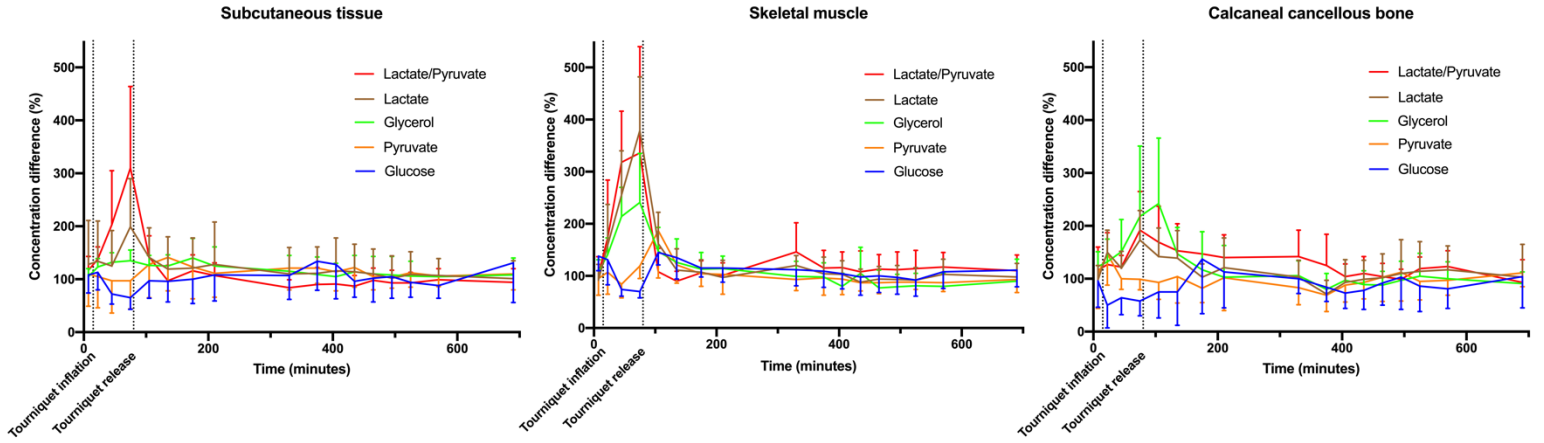

Figure 9. The mean ischemic marker concentration differences (%) between the tourniquet and non-tourniquet-exposed legs for subcutaneous tissue, skeletal muscle and calcaneal cancellous bone. Tourniquet inflation time: 15 min, mean (range) tourniquet release time: 80 (73; 92) min (both are marked with vertical dotted lines). Bars represent the 95% CI. This figure was published by Hanberg et al. 2021[4].

7 Discussion

7.1 Antimicrobial tissue pharmacokinetics

Traditionally, antimicrobial tissue concentrations have been considered to reflect plasma concentrations, which evaluates the antimicrobial body distribution as one homogeneous compartment[95, 96]. Over the last decades, this assumption has been challenged by repeated findings showing heterogeneous tissue distributions of different antimicrobials[1, 18, 27, 28, 50, 51, 57, 58, 88, 89, 97-113]. In order to achieve a successful therapeutic outcome, it is increasingly pertinent that an adequate antimicrobial concentration at the target site is maintained. The lack of knowledge regarding antimicrobial tissue distribution may explain why the incidence of surgical site infections remains rather high in some settings despite the recommended administration of antimicrobial prophylaxis[114, 115].

An increasing focus on tissue distribution of various antimicrobials under different conditions has emerged. Even so, for bone tissue, the current literature regarding antimicrobial tissue distributions remains sparse. So far, most studies investigating these matters have been performed by means of bone specimens[116]. However, this method suffers from a number of important methodological limitations. When quantifying the antimicrobial concentration by means of tissue specimens, it does not distinguish between the free (unbound) and bound antimicrobial concentration; what's more, the concentration is given by weight rather than volume. As it is only the unbound fraction of antimicrobials that are considered pharmaceutically active, antimicrobial tissue concentrations quantified by means of tissue specimens can lead to an overestimation of the pharmaceutically-active fraction of the antimicrobial concentration[31]. Additionally, the tissue specimen method includes a homogenisation procedure, which ignores the fact that tissues comprise of multiple compartments (e.g. cells, interstitial space and lingered blood). Finally, only a rather limited number of specimens can be harvested during surgery due to the invasiveness of the method, resulting in a poor temporal resolution. This makes the investigation of the pre- and post-operative tissue concentrations inaccessible. Consequently, tissue concentrations obtained by means of tissue specimens are arduously relatable to relevant PK/PD index targets. Hence, it has been argued that

tissue pharmacokinetics obtained by means of tissue specimens may be misleading and, at worst, harmful to patients[117, 118]. In contrast, microdialysis has the ability to overcome most of these limitations[79-82]. An in-depth description of the advantages and limitations of microdialysis can be found in section *5.1.2 Advantages and limitations*.

Indeed, it is acknowledged that the present attempt to quantify cefuroxime bone and soft tissue concentrations suffers from the lack of a validated reference method. This is important to remember when interpreting the current results.

7.2 Microdialysis sampling from drill holes in bone

Due to the compact nature of bone, a drill hole has to be made for placement of the microdialysis catheter. This raises the obvious question: "Does the analyte sampled from a drill hole reflect bone concentrations or is it a mixture of bone concentrations, adjacent tissue concentrations, and/or a blood clot filling the dead space?". In the case of concentration differences between bone and the adjacent tissue, this would lead to an over- or under-estimation of the actual bone concentrations. However, as the basic law of diffusion states that the diffusion time increases proportionally to the square of the distance[119], a significant contribution from the adjacent tissues seems unlikely. The distance from the surroundings to the microdialysis membrane is much longer compared to the distance from the bone to the membrane. Regarding the concern that the drill hole concentrations reflect that of a blood clot, a previous porcine study compared metabolite concentrations in an *in vitro* blood clot with measurements from a drill hole in the femoral head[120]. In this study, a wash-out pattern was seen within the *in vitro* blood clot; this was not the case for the drill hole in the femoral head. Furthermore, Tottrup et al. addressed the issue of potential influences from adjacent tissues by comparing the cefuroxime concentration in two symmetric cortical bone drill holes: one unsealed and one sealed with bone wax[58]. In this study, similar pharmacokinetic parameters were found for the sealed and unsealed drill holes[58]. As such, these studies indicate that the measured concentrations from bone drill holes do not reflect a blood clot or adjacent tissues. Whether the measurements obtained from bone drill holes reflect the true bone concentration remains unknown due to the lack of a validated reference method to quantify antimicrobial bone concentrations.

Nevertheless, measurements from drill holes seem to reflect the true orthopaedic peri- and post-operative conditions.

7.3 Evaluation of the porcine model

In the present PhD project, cefuroxime and ischemic metabolites concentrations in bone and soft tissues were evaluated in two experimental studies and one clinical study. In Study II, no differences were found in the T>MIC for the group receiving 1.5 g cefuroxime 15 min and 45 min prior to tourniquet inflation[2]. Consequently, 1.5 g of cefuroxime was administered 15 min prior to tourniquet inflation in the clinical study[3, 4]. Accordingly, the cefuroxime and ischemic metabolites concentrations from Group A in Study II are comparable to the clinical study in terms of study design[2-4]. An overview of the cefuroxime pharmacokinetic parameters and T>MIC from Study II and III can be found in Table 4. As the cefuroxime concentrations were obtained over 6 hours in the clinical study, the results of the porcine study have been modified to only include results from the first 6 out of 8 hours (Table 4). This makes the pharmacokinetic parameters and T>MIC results more comparable for the two studies.

Comparing the pharmacokinetic parameters between the porcine and clinical studies, substantial differences can be seen for AUC, T_{max} and $T_{1/2}$. However, the C_{max} and tissue penetration ratios are comparable for the two studies. The main reason for the pharmacokinetic differences between the two studies seems to be driven by a faster cefuroxime elimination (shorter $T_{1/2}$) in the porcine study. This seems to result in lower porcine AUC values and, correspondingly, lower T>MIC: ranging from 2.4 to 3.4 and 4.8 to 5.4 hours for the porcine and clinical studies, respectively. The weight difference between the two study populations did not differ significantly, which may be the reason for the comparable C_{max} values. The porcine study was conducted on young, healthy pigs (aged 5 months) with presumably good kidney function. Although all patients (mean age (range) = 58 (45–67) years) in the clinical study presented with normal creatinine levels (mean plasma creatinine (range) = 75 (60–90) µmol/L), it seems likely that young, healthy pigs display a faster elimination rate (either per se or age-dependent) compared to the included patients.

Comparable pharmacokinetic parameters were found between the two experimental studies (Studies I and II)[1, 2]. This indicates good reproducibility of the porcine model.

Table 4. Pharmacokinetic parameters and T>MIC for plasma, subcutaneous tissue, and calcaneal cancellous bone on both the tourniquet and non-tourniquet sides for Group A in Study II (for the first 6 out of 8 hours) and for Study III (first dosing interval).

Parameter	Porcine study (Study II)	Clinical Study (Study IV)
Plasma $AUC_{0\text{-}6h}$ (min µg/mL)	3863 (3227; 4624)	8198 (6611; 9785)
Non-tq subcutaneous tissue $AUC_{0\text{-}6h}$ (min µg/mL)	4274 (3570; 5116)	8538 (6952; 10125)
Tq subcutaneous tissue $AUC_{0\text{-}6h}$ (min µg/mL)	4104 (3428; 4912)	7548 (5962; 9135)
Non-tq calcaneal cancellous bone $AUC_{0\text{-}6h}$ (min µg/mL)	3807 (3180; 4557)	6648 (5061; 8235)
Tq calcaneal cancellous bone $AUC_{0\text{-}6h}$ (min µg/mL)	3767 (3147; 4510)	7107 (5561; 8694)
Plasma C_{max} (µg/mL)	131 (106; 161)	97 (84; 110)
Non-tq subcutaneous tissue C_{max} (µg/mL)	55 (45; 68)	58 (45; 70)
Tq subcutaneous tissue C_{max} (µg/mL)	53 (43; 65)	51 (38; 64)
Non-tq calcaneal cancellous bone C_{max} (µg/mL)	48 (39; 59)	59 (47; 72)
Tq calcaneal cancellous bone C_{max} (µg/mL)	32 (26; 39)	53 (40; 66)
Plasma T_{max} (min)	7.5 (7.5; 7.5)	7.5 (7.5; 7.5)
Non-tq subcutaneous tissue T_{max} (min)	25.3 (22.5; 45.0)	45.0 (22.5; 75.0)
Tq subcutaneous tissue T_{max} (min)	22.5 (22.5; 22.5)	48.8 (22.5; 105.0)
Non-tq calcaneal cancellous bone T_{max} (min)	22.5 (22.5; 22.5)	34.5 (22.5; 75.0)
Tq calcaneal cancellous bone T_{max} (min)	25.3 (22.5; 45.0)	84.0 (22.5; 135.0)
Plasma $T_{1/2}$ (min)	43 (36; 53)	74 (56; 93)
Non-tq subcutaneous tissue $T_{1/2}$ (min)	55 (45; 67)	94 (75; 113)
Tq subcutaneous tissue $T_{1/2}$ (min)	54 (45; 66)	99 (81; 118)
Non-tq calcaneal cancellous bone $T_{1/2}$ (min)	58 (48; 71)	86 (67; 105)
Tq calcaneal cancellous bone $T_{1/2}$ (min)	74 (61; 89)	95 (77; 114)
Non-tq subcutaneous tissue $AUC_{tissue}/AUC_{plasma}$	1.11 (0.90; 1.36)	1.09 (0.86; 1.32)
Tq subcutaneous tissue $AUC_{tissue}/AUC_{plasma}$	1.06 (0.87; 1.30)	0.96 (0.73; 1.19)
Non-tq calcaneal cancellous bone $AUC_{tissue}/AUC_{plasma}$	0.99 (0.80; 1.21)	0.84 (0.61; 1.07)
Tq calcaneal cancellous bone $AUC_{tissue}/AUC_{plasma}$	0.98 (0.80; 1.20)	0.88 (0.65; 1.11)
Plasma T>MIC (min)	145 (116; 174)	318 (297; 338)
Non-tq subcutaneous tissue T>MIC (min)	198 (169; 227)	312 (292; 333)
Tq subcutaneous tissue T>MIC (min)	198 (169; 226)	322 (302; 343)
Non-tq calcaneal cancellous bone T>MIC (min)	187 (158; 216)	306 (285; 326)
Tq calcaneal cancellous bone T>MIC (min)	208 (179; 237)	289 (269; 310)

Abbreviations: AUC, area under the concentration-time curve from 0 to 6 hours; C_{max}, peak drug concentration; T_{max}, time to C_{max}; $T_{1/2}$, half-life; $AUC_{tissue}/AUC_{plasma}$, area under the concentration-time curve ratio of tissue/plasma; tq, tourniquet.

AUC, C_{max}, and $T_{1/2}$ are given as medians (95% CI) in the porcine study and as means (95% CI) in the clinical study.

T_{max} given as means (ranges) in both studies.

T>MIC given as means (95% CI) in both studies.

When evaluating the ischemic metabolites for the porcine and clinical studies, comparable lactate/pyruvate ratios were found for subcutaneous tissue and cancellous bone[2, 4]. The recovery time of the lactate/pyruvate ratio was within 30 min (porcine study) and 55 min (clinical study) for subcutaneous tissue and within 165 min (porcine study) and 130 min (clinical study) for cancellous bone. Notwithstanding, the subcutaneous tissue glycerol ratio differed between the porcine and clinical studies, as the recovery time was within 60 min and 130 min, respectively. The recovery time for glycerol in cancellous bone was similar between the two studies. An explanation for the prolonged subcutaneous tissue glycerol recovery time in the clinical study could be attributed to the fact that young pigs have a thinner subcutaneous tissue layer than humans. The regulation of hypoglycaemia during tourniquet application initiates tissue catecholamine-induced lipolysis, which increases glycerol levels[121]. However, a direct comparison of the ischemic metabolites between the two studies should be taken with some precaution, as the tourniquet duration time was 25 min longer in the clinical study.

In regard to ischemic metabolites, the porcine model may represent a good model with limited interspecies differences. However, the differences in the pharmacokinetic parameters and T>MIC between the porcine and clinical studies indicate that the porcine cefuroxime pharmacokinetic data cannot readily be extrapolated to clinical settings.

7.4 Timing of tourniquet and cefuroxime administration

As the blood supply is occluded during surgery, and the tissues are exposed to ischemia, information regarding the optimal timing of antimicrobial prophylaxis and how this affects postoperative antimicrobial tissue concentrations may be very important[15, 64, 122-124]. The porcine study suggested that administering cefuroxime in a 15–45 min window prior to tourniquet inflation results in sufficient subcutaneous tissue and cancellous bone concentrations[2]. The clinical model confirmed that a time window of 15 min from cefuroxime administration to tourniquet inflation resulted in sufficient perioperative subcutaneous tissue, skeletal muscle and calcaneal cancellous bone concentrations in forefoot surgery[3]. These results are in line with the current, although sparse, literature[69, 70].

Previous studies have hypothesised that the tourniquet-induced ischemia reduces postoperative antimicrobial tissue concentrations[64, 124]. However, as the clinical study exhibited similar pharmacokinetic parameters and T>MIC results in terms of the first and second dosing intervals, this study does not indicate a decreased postoperative cefuroxime tissue penetration to the tourniquet-exposed leg following a tourniquet time of approximately 1 hour[3].

Interestingly, the porcine and clinical study indicate a potential benefit to T>MIC if the tourniquet application is used correctly[2, 3]. In both the porcine and clinical study, the cefuroxime half-life in the calcaneal cancellous bone tended to be increased in the tourniquet-exposed leg compared to the nonexposed leg. This could be explained by a limited elimination of cefuroxime from the tourniquet-exposed leg during tourniquet inflation[2, 3]. Furthermore, the clinical study demonstrated that the time to peak drug concentration (T_{max}) was prolonged in the tourniquet-exposed calcaneal cancellous bone compared to the non-tourniquet-exposed calcaneal cancellous bone[3]. These interesting findings are seemly induced by a limited cefuroxime elimination during tourniquet inflation combined with a second peak of the cefuroxime concentration at tourniquet release. In 5 out of 10 patients, the second peak was higher than the initial peak[3]. This favourable hyperaemic effect was also observed in the porcine study (Study II)[2].

In tourniquet-aided surgery, there may be a higher risk of haematoma formation at the end of surgery with the release of the tourniquet, which serves as a great growth medium for contaminant bacteria[71, 125]. Soriano et al., therefore, investigated whether administering antimicrobial prophylaxis at tourniquet release would be non-inferior to administering antimicrobial prophylaxis 10–30 min prior to tourniquet inflation, as they hypothesised that high plasma and tissue concentrations at wound closure are significantly important given the potential for haematoma formation[71]. Comparing these two groups, Soriano et al. found no differences in the surgical site infection rate[71]. Correspondingly, administering cefuroxime at tourniquet release was investigated as the third group in the porcine study (Study II) and compared to administering cefuroxime prior to tourniquet inflation[2]. For the tourniquet-exposed calcaneal cancellous bone, the T>MIC tended to be longer when administering cefuroxime at tourniquet

release compared to administering cefuroxime 15 min prior to tourniquet inflation[2]. However, no significant differences were found between the groups [2]. Nevertheless, there was a clear sign of a hyperaemic effect, demonstrated by an increased C_{max} and AUC values. Thus, administering antimicrobial prophylaxis both prior to tourniquet inflation and at tourniquet release may potentially decrease the risk of surgical site infections. However, the validity of this statement requires further investigation.

7.5 Cefuroxime dosing regimens

Cephalosporins are among the most common groups of antimicrobials used as antimicrobial perioperative prophylaxes[7]. The dosing regimen for the specific antimicrobial agent depends on the pharmacokinetic profile, and for time-dependent drugs, the drug half-life is crucial. For cefuroxime, it is generally recommended to administer 1.5 g 30–60 min prior to skin incision in order the achieve therapeutic plasma and tissue concentrations at the time of surgery[5-7, 9]. The dose should be intraoperatively repeated after 3–4 hours during prolonged surgeries or when the blood loss is greater than 2000 mL[5-7, 9].

Both the porcine and clinical studies indicate that cefuroxime penetrates very well into cancellous bone and soft tissues[2, 3]. For all the patients in the clinical study, a cefuroxime concentration of 4 µg/mL was reached within 22.5 min in all the investigated compartments and was maintained above this concentration for a minimum of 4.5 hours[3]. As such, the findings in this PhD project indicate that cefuroxime is a good choice for perioperative antimicrobial prophylaxis in terms of target tissue penetration and T>MIC. Moreover, a time frame of 30 min from cefuroxime administration to surgery and repeated dosing after 3–4 hours seems sufficient.

Cefuroxime is most commonly administered at a standard dose of 1.5 g when given as perioperative antimicrobial prophylaxis. This may seem irrational as it does not reflect the volume of distribution in obese patients. As such, it has been hypothesised that increased or weight-dependent dosing would increase the tissue concentrations in obese patients[49, 107, 126-128]. Tottrup et al. previously investigated both cancellous bone and subcutaneous tissue concentrations by means of microdialysis in a setup comparable to the clinical study[3, 28]. In their study, shorter T>MIC values

were obtained for both subcutaneous tissue and cancellous bone. The two study groups were comparable in most parameters, but Tottrup et al. had a case-mix of patients with substantially higher BMIs compared to the patients in the clinical study (31 vs 25)[3, 28]. These findings somewhat acknowledge the hypothesis that increased or weight-dependent dosing for obese patients may increase cefuroxime tissue concentrations. Further research investigating this matter is warranted.

7.6 Relevant targets

Whether cefuroxime appears to be a good choice as perioperative antimicrobial prophylaxis in terms of T>MIC is undeniably depended on the applied PK/PD index and MIC targets. In this context, it is important to remember that no definitive PK/PD index targets have been validated neither for perioperative prophylactic settings nor for therapeutic settings, as definitive *in vivo* targets are difficult to determine[5, 7, 31, 43-47]. Primarily, the perioperative antimicrobial prophylaxis target is based on expert opinions[5, 7].

An MIC target of 4 µg/mL of cefuroxime was chosen, as *S. aureus*, the most common aetiology of surgical wound contamination during orthopaedic surgeries, exhibits a clinical breakpoint MIC of 4 µg/mL for cefuroxime[16, 18]. However, *E. coli,* which exhibits a clinical breakpoint MIC of 8 µg/mL for cefuroxime, can also be found in orthopaedic departments[16-18]. Applying a MIC target of 8 µg/mL instead of 4 µg/mL will undoubtedly affect the interpretation of the pharmacokinetic results of the present PhD project. Moreover, the currently applied MIC values are based on the *in vitro* determination of a bacterium's sensitivity to a specific antimicrobial[18]. Whether these *in-vitro*-determined MIC values are representative of *in vivo* MIC values remains indefinite.

7.7 Selection of antimicrobial agents

During the 1990s and the early 2000s, cephalosporines were the first choice as perioperative antimicrobial prophylaxes in orthopaedic surgery and were used in up to 80–97% of elective surgeries[129-131]. The primary reasons for choosing cephalosporines were their presumed high tissue penetration, low costs and broad-spectrum antimicrobial activity[7, 132]. The cephalosporins most commonly used were cefuroxime and cefazolin[7, 129]. From 2005–2011, the use of cefuroxime decreased dramatically from 80% to 36%[129]. During the same time, the use of flucloxacillin (combined with gentamycin)

increased from 1% to 32%, and teicoplanin (combined with gentamycin) increased from 1% to 10%[129]. The primary reason for the decreasing use of cefuroxime as perioperative antimicrobial prophylaxis in elective surgeries was the assumed association to *Clostridium difficile* infections[129]. However, a study investigating the incidence of *C. difficile* infection in elective orthopaedic surgeries found an incidence of only 0.17%[133]. It was concluded that the use of cephalosporines was still safe and their association with *C. difficile* was insignificant[133]. Another reason for the decreased use of cephalosporines was, undoubtedly, the growing concerns about bacterial resistance[129].

A recent study investigated flucloxacillin's bone and soft tissue concentrations[134]. The use of flucloxacillin as perioperative antimicrobial prophylaxis has increased during recent decades given its narrow spectrum and presumably good effect against *S. aureus*. Surprisingly, this study demonstrated low bone and soft tissue concentrations[134]. A concentration of 2 µg/mL (flucloxacillin's clinical breakpoint MIC for *S. aureus*[18]) was not reached in tibial cancellous bone after administration of 1 g flucloxacillin intravenously[134]. This raises an interesting discussion regarding the drug of choice as perioperative antimicrobial prophylaxis. Based on these results, the authorities responsible for antimicrobial guidance may not only base their recommendation on a desire to reduce the use of a specific group of antimicrobials, or the spectrum of the antimicrobial agent, but also on the indispensable ability to provide adequate antimicrobial target-site concentrations.

7.8 Tourniquet-induced tissue ischemia

Local ischemic tissue metabolites have not previously been investigated in subcutaneous and bone tissues due to the lack of useful methods. However, two clinical studies have recently investigated ischemic metabolites in relation to tourniquet-exposed tissues[77, 78]. Only one of the two studies reported on the more precise ischemic marker, the lactate/pyruvate ratio, rather than individual metabolites[77]. In that study, the skeletal muscle recovered within 30 min of tourniquet release, which is in line with the Study IV[4].

As mentioned under section *7.3 Evaluation of the porcine model*, the lactate/pyruvate ratio between the tourniquet-exposed and non-tourniquet-exposed legs was

comparable between the porcine and clinical studies, indicating a tissue recovery time of approximately 30–60 min for subcutaneous tissue and 130–165 min for cancellous bone[2, 4]. However, when considering the lactate/pyruvate levels for the individual tourniquet-exposed tissues in relation to the lactate/pyruvate ischemic cutoff level of 25[76], only the tourniquet-exposed skeletal muscle significantly increased above the ischemic level in the clinical study (Study IV) and dropped below the ischemic cutoff level immediately after tourniquet release[4]. In the porcine study (Study II), both tourniquet-exposed subcutaneous tissue and cancellous bone increased significantly above the ischemic cutoff level during tourniquet application but dropped below 25 within 45 min for subcutaneous tissue and 75 min for cancellous bone[2]. The minor differences between the porcine (Study II) and clinical (Study IV) studies may be attributed to a longer tourniquet time in the porcine model (90 vs 65 min)[2, 4]. Still, both studies indicate that a tourniquet time of approximately 60–90 min results in limited tissue ischemia, when considering the lactate/pyruvate ratio difference between the tourniquet and non-tourniquet-exposed legs and the lactate/pyruvate ischemic cutoff level of 25. This is also consistent with the hyperaemic effect found in both the porcine (Study II) and clinical (Study III) studies as previously discussed in section *7.4 Timing of tourniquet and cefuroxime administration*[2, 3].

Several studies have previously associated tourniquet induced ischemia with multiple adverse events, such as pain, swelling, slow wound healing, compartment syndrome, and respiratory distress syndrome[64, 135, 136]. The above-mentioned adverse events cannot be directly correlated to ischemic metabolites. However, the ischemic metabolites evaluated in the present PhD project indicates only limited tissue ischemia following a tourniquet duration of 60–90 min[2, 4].

Despite the limited research on the ischemic metabolites' glucose, lactate, pyruvate and glycerol, in relation to tourniquet application, fairly extensive work on tourniquet ischemia in skeletal muscle was already performed during the 1980s, assessing the ultrastructural changes, tissue pH, creatine kinase leakage, and tissue desaturation[72-74]. These studies have led to the current recommendation of a maximum of 120 min continuous tourniquet inflation. This is in line with the present PhD project, suggesting limited tissue ischemia in

relation to a 60–90 min tourniquet duration time[2, 4].

The ischemic data in the present PhD project remains explorative. But it sheds light on both the peri- and post-operative tissue conditions in relation to tourniquet application. Furthermore, it presents microdialysis as an applicable method for investigating and monitoring the ischemic conditions of skeletal muscle, subcutaneous tissue and cancellous bone. Microdialysis offers the opportunity for bedside monitoring of ischemic tissue conditions in cases with vulnerable tissues, patient with decreased extremity blood flow, trauma patients with tissues exposed to an unknown duration of tissue ischemia, replantations, and monitoring of transplanted tissue vitality. Microdialysis is easily handled, relatively not time-consuming, and minimally invasive in soft tissues.

8 Conclusions

Meropenem was validated as a suitable internal standard for cefuroxime, and microdialysis was successfully applied for the evaluation of cefuroxime and ischemic metabolite concentrations before, during and after tourniquet application in both porcine and clinical studies.

Study II suggested that administering 1.5 g cefuroxime 15–45 min prior to tourniquet inflation seems to be a safe window for achieving bone and soft tissue concentrations above 4 µg/mL. The time window of 15 min was confirmed in the clinical study (Study III), acknowledging the conclusion from Study II. Furthermore, Study III demonstrated that a tourniquet application time of approximately 1 hour does not affect the cefuroxime tissue concentrations in the following dosing interval. Studies II and III demonstrated that cefuroxime penetrates very well into cancellous bone and soft tissues and suggests that cefuroxime is a good choice for perioperative antimicrobial prophylaxis in terms of tissue penetration and T>MIC. The current administration guidelines for cefuroxime as perioperative antimicrobial prophylaxis recommending a time frame of 30 min from cefuroxime administration to surgery and repeated intraoperative dosing after 3–4 hours are acknowledged by Study III.

When considering both the lactate/pyruvate ratio between the tourniquet and non-tourniquet-exposed legs and the lactate/pyruvate ischemic cutoff level of 25, Studies II and IV found that a tourniquet application time of approximately 60–90 min results in limited tissue ischemia and cell damage in subcutaneous tissue, skeletal muscle and calcaneal cancellous bone.

The porcine model may represent a good translational model with limited interspecies differences in terms of ischemic tissue metabolites. However, the present PhD project also indicates that porcine cefuroxime pharmacokinetic data cannot readily be extrapolated to clinical settings, as a substantially shorter cefuroxime half-life was found in the porcine study compared with the clinical study's patient group.

9 Perspectives and future research

The assessment of antimicrobial bone and soft tissue concentrations by means of tissue specimens is associated with considerable methodological challenges. As such, it has been argued that tissue pharmacokinetics obtained by means of tissue specimens may be misleading and, at worst, harmful to patients. The findings in the present PhD project suggest that microdialysis is a useful method for the evaluation of bone and soft tissue cefuroxime pharmacokinetics. An increased focus and knowledge regarding antimicrobial target-site concentrations may improve current antimicrobial dosing regimens of perioperative antimicrobial prophylaxes and in treatment settings, which, ultimately, could improve clinical outcomes. This is of great interest to patients and our healthcare system.

This PhD project describes a porcine model and a feasible clinical model for investigating cefuroxime and ischemic metabolite tissue concentrations before, during and after tourniquet application. Future studies investigating the optimal time interval between the administration of perioperative antimicrobial prophylaxis and tourniquet inflation for other antimicrobials seems relevant. Previous studies have demonstrated that different antimicrobials present tissue distribution heterogeneously. Moreover, future studies investigating how prolonged tourniquet times and tourniquet cuff pressures affect the peri- and post-operative antimicrobial and ischemic metabolite concentrations are warranted for different patient groups and surgical procedures.

Finally, the current setup and models allow for an evaluation of both systemic and local inflammatory responses and processes during and after a tourniquet application. The local inflammatory-marker composition in bone and subcutaneous tissue in relation to tourniquet application and its impact on, e.g. wound healing, is limited or even non-existent.

10 References

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11 Appendix

List of appendices:

11.1 Paper I

11.2 Paper II

11.3 Paper III

11.4 Paper IV

11.5 Co-authorship declarations

11.1 Paper I

Journal of Pharmaceutical Sciences xxx (2019) 1-7

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Pharmacokinetics, Pharmacodynamics and Drug Transport and Metabolism

Simultaneous Retrodialysis by Drug for Cefuroxime Using Meropenem as an Internal Standard—A Microdialysis Validation Study

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ABSTRACT

Microdialysis is a valuable pharmacokinetic tool for obtaining samples of drug concentrations from tissues of interest. When an absolute tissue concentration is needed, a calibration of the microdialysis catheter is required. The use of an internal standard offers a number of advantages compared to standard calibration methods. However, meticulous validation both *in vitro* and *in vivo* is needed, as this method requires an internal standard with physiochemical similarities to the analyte of interest with no interference. A series of *in vitro* and *in vivo* setups were conducted to determine the relative recovery by gain and by loss for cefuroxime, with and without a constant meropenem concentration. The cefuroxime and meropenem concentrations were determined using ultra-HPLC. The main finding was that cefuroxime and meropenem relative recovery behaved similarly both *in vitro* and *in vivo*, signifying that meropenem is a representative internal standard for cefuroxime. Furthermore, cefuroxime relative recovery *in vitro* was not affected by either the cefuroxime concentration or the presence of meropenem, and the *in vivo* meropenem relative recovery was constant over 6 h.

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Introduction

Traditionally, tissue specimens have been the predominant method for obtaining samples of drug concentrations in tissues. However, obtaining pharmacokinetic information by means of tissue specimens may be both misleading and ultimately harmful for patients.[1,2] When analyzing tissue specimens, no selective measures of free extracellular concentrations can be taken, and temporal resolution is poor to nonexistent.[1-3] As an alternative to tissue specimens, microdialysis has emerged in recent decades as a valuable tool for extracting samples of drugs from tissues of interest. Microdialysis is a minimally invasive technique which allows for the dynamic sampling of free and unbound fractions of drugs in the interstitial space. When investigating antimicrobial pharmacokinetics, this is favorable, as most pathogens reside in this compartment.[4-7] The microdialysis system is continuously perfused, and therefore, equilibrium across the semipermeable membrane cannot be achieved. Consequently, the concentration of the microdialysis samples, called dialysates, represents only a fraction of the total tissue concentration. This fraction is referred to as the relative recovery (RR). When absolute tissue concentrations are of interest—as they are in antimicrobial pharmacokinetic studies—it is imperative to determine the RR.

RR can be determined using various calibration methods.[5,6] In antimicrobial pharmacokinetic studies, retrodialysis by drug is the most commonly used calibration method.[8,9] This method is based on the fact that the transport of the analyte from the extracellular fluid to the microdialysis catheter, referred to as relative recovery by gain (RR_{gain}), is equal to the diffusion of the exogenous compound from the microdialysis catheter to the extracellular fluid, referred to as relative recovery by loss (RR_{loss}).[4,10,11] Most often, the

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exogenous compound used for calibration is the same as the analyte of interest. However, this method requires absence of the target compound in the tissues of interest, usually before administration of the first dosage. Calibration at the beginning of a study requires both absence and an additional washout period to prevent spillover of the analyte in the tissue of interest, whereas calibration at the end of a study requires only the absence of the analyte. Consequently, this method can be both time consuming and impractical in clinical settings where patients are being treated with the analyte of interest.

Alternatively, the RR can be determined by the use of retrodialysis by drug with an internal standard, also called the internal standard method.[11] However, the internal standard method requires an internal standard with physiochemical similarities to the analyte of interest with no interference. The internal standard method is based on the assumption that the RR_{loss} of the exogenous compound is equal to the RR_{gain} of the analyte of interest. The internal standard method is advantageous in that it allows for continuous calibration independent of the tissue concentration of the analyte and saves time. To facilitate and improve clinical cefuroxime pharmacokinetic studies, we aimed to evaluate meropenem as an internal standard for cefuroxime which is widely used for antimicrobial prophylaxis in surgery. Cefuroxime is a second-generation cephalosporin which efficacy is best related to the time for which the free drug concentration is maintained above the minimal inhibitory concentration (T > MIC).[12] Meropenem was chosen as an internal calibrator for cefuroxime, as they were both beta-lactam antibiotics with similar molecule size.

Materials and Methods

This study was conducted at the Institute of Clinical Medicine, Aarhus University Hospital, Denmark. The *in vivo* study was approved by the Danish Animal Experiments Inspectorate and was carried out according to existing laws (License No. 2017/15-0201-01184). All animals were cared for in accordance with the principles laid down by the European Union Directive 2010/63/EU for Protection of Animals used for Scientific Purposes. Chemical analyses were performed at the Department of Clinical Biochemistry, Aarhus University Hospital, Denmark.

Microdialysis

A brief description of the microdialysis method was given in the Section Introduction. An in-depth description of the microdialysis method can be found elsewhere.[4-7,10,13]

Microdialysis equipment from M Dialysis AB (Stockholm, Sweden) was used. Specifically, the catheter used was the CMA 63 (membrane length 30 mm with a 20-kilo Dalton molecule cutoff), and the CMA 107 precision pumps produced a flow rate of 2 μL/min.

The following equations were used to calculate the RR_{loss} and RR_{gain} for both calibration methods:

$$RR_{loss} = 1 - \frac{C_{dialysate}}{C_{perfusate}}$$

$$RR_{gain} = \frac{C_{dialysate}}{C_{medium}}$$

where $C_{dialysate}$ is the concentration of either the analyte or the internal standard in the dialysate, $C_{perfusate}$ is the concentration of either the analyte or the internal standard in the perfusate, and C_{medium} is the concentration of either the analyte or the internal standard in the surrounding medium.

For the *in vivo* study, absolute tissue concentrations of the analyte, C_{tissue}, were calculated by correcting for the RR obtained by both the retrodialysis by drug method and the internal standard method. The following equation was used:

$$C_{tissue} = \frac{C_{dialysate}}{RR}$$

where $C_{dialysate}$ is the concentration of the analyte.

In the *in vivo* pharmacokinetic data analysis, the measured concentrations of the analyte were attributed to the midpoint of the sampling intervals.

In Vitro *Study*

To evaluate whether meropenem could be used as an internal standard for cefuroxime, a series of *in vitro* experiments were conducted to determine RR_{gain} and RR_{loss} for cefuroxime with and without a constant meropenem concentration. Eight setups were conducted with 0.9% NaCl containing a cefuroxime concentration in the range of 1-30 μg/mL (mimicking the expected cefuroxime tissue concentrations) and a meropenem concentration of 5 μg/mL, as shown in Figure 1a. Each setup was performed with 4 microdialysis catheters, and 4 samples taken at 20-min intervals were collected from each setup and catheter. Before each setup, a 15-min equilibration period was allowed for. The same catheters were used for both RR_{gain} and RR_{loss} in all setups. A washout setup was conducted to evaluate the adherence of meropenem and cefuroxime to the microdialysis catheter; 0.9% NaCl holding 5 μg/mL meropenem was added to the perfusate; and 0.9% NaCl holding 30 μg/mL cefuroxime was added as the surrounding medium. After 1.5 h, both the perfusate and the surrounding medium were changed to blank 0.9% NaCl and five 20-min samples were obtained from each catheter.

In Vivo *Study*

Six female pigs were included in the study (Danish Landrace breed, weighing 78-82 kg). The pigs were kept under general anesthesia during the entire study with a combination of propofol (500 mg/h, continuous infusion) and fentanyl (0.5 mg/h, continuous infusion). Their pH and temperature were monitored and kept within the ranges of 7.31-7.53 and 37.4°C-38.8°C, respectively.

Surgery was initiated after the induction of anesthesia. With the pig in a supine position, the right calcaneus was exposed via a longitudinal plantar incision. Using fluoroscopic guidance, a diagonal drill hole with a diameter of 2 mm and a depth of 40 mm was made, starting at the inferior part of the calcaneal cuboid joint and ending at the proximal part of the calcaneus. The microdialysis catheter was placed in the drill hole and fixated with a single suture to the skin. Subsequently, a microdialysis catheter was placed in the subcutaneous adipose tissue of the thigh in accordance with the guidelines of the manufacturer. Correct locations of the bone catheters were evaluated using fluoroscopy.

After placement of the microdialysis catheters, all catheters were perfused with 0.9% NaCl containing 5 μg/mL meropenem, allowing for calibration with the internal standard method. Thirty minutes of tissue equilibration was allowed for. At time zero, 1500 mg cefuroxime was administered over 10 min. From time 0 to 180 min, dialysates were collected at 30-min intervals, and from time 180 to 360 min, dialysates were collected at 60-min intervals, producing a total of 9 dialysates over 6 h. Venous blood samples were drawn from a central venous catheter at the midpoint of the 9 sampling intervals. A dose of 1500 mg cefuroxime was chosen, as this is the most common dose for both intravenous perioperative

a

	Setup 1	Setup 2	Setup 3	Setup 4	Setup 5	Setup 6	Setup 7	Setup 8
RRgain	Cef 15 μg/mL	Cef 1 μg/mL	Cef 15 μg/mL	Cef 30 μg/mL	–	Mero 5 μg/mL	Mero 5 μg/mL	Mero 5 μg/mL
RRloss	–	Mero 5 μg/mL	Mero 5 μg/mL	Mero 5 μg/mL	Cef 15 μg/mL	Cef 1 μg/mL	Cef 15 μg/mL	Cef 30 μg/mL

b

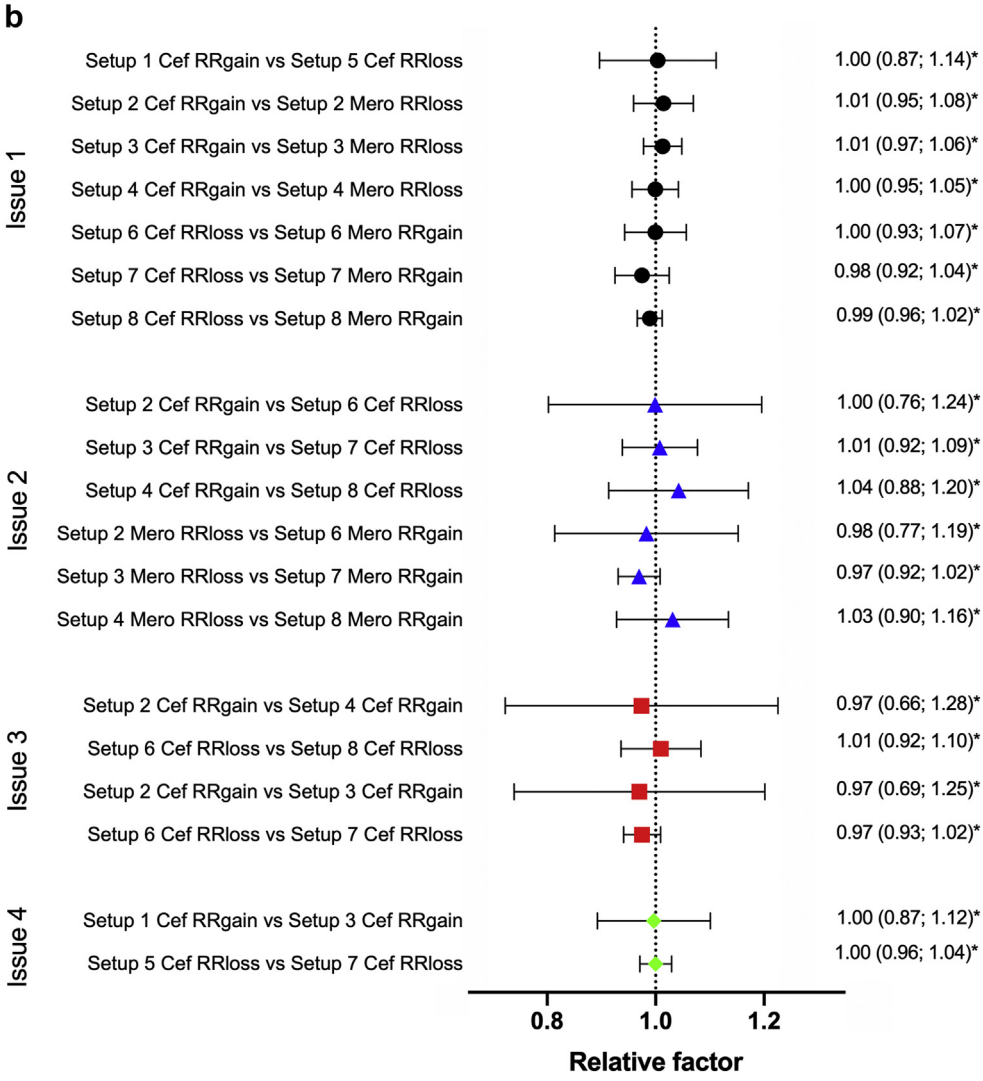

Figure 1. (a) Setup overview for the *in vitro* study. (b) Forest plot comparing RR values for the following 4 issues in the *in vitro* study: (1) Does cefuroxime RR resemble meropenem RR; (2) Does RR_{gain} resemble RR_{loss} for both cefuroxime and meropenem; (3) Does cefuroxime RR depend on the cefuroxime concentration; and (4) Does the presence of meropenem affect cefuroxime RR. Estimated mean relative factors are represented with 95% CI as bars. The mean values (95% CI) are given to the right. $^*p > 0.05$. Cef, cefuroxime; Mero, meropenem; RR, relative recovery; RRgain, relative recovery by gain; RRloss, relative recovery by loss.

prophylaxis and in the treatment of orthopedic infections. Subsequently, the perfusate was changed to 0.9% NaCl containing 100 μg/mL cefuroxime, and a 15-min microdialysis catheter equilibration was allowed for. All catheters were then calibrated with cefuroxime using the retrodialysis by drug method by collecting three 20-min samples. The high calibration concentration of cefuroxime was chosen to minimize the influence of possible residual tissue concentrations.

Handling of Samples

Venous blood samples were stored at 5°C for a maximum of 6 h before being centrifuged at 3000× *g* for 10 min. Plasma aliquots were then frozen and stored at −80°C until analysis. Dialysates were instantly frozen and stored at −80°C until analysis.

Ultrahigh Performance Liquid Chromatography Analysis of Cefuroxime and Meropenem

Cefuroxime and meropenem concentrations were determined using a validated ultrahigh performance liquid chromatography assay.[14] Inter-run imprecisions (percent coefficients of variation [%CV]) were 4.7% at 2.5 μg/mL for quantification of cefuroxime and 3.0% at 2.0 μg/mL for quantification of meropenem. The lower limits of quantification were 0.06 μg/mL and 0.5 μg/mL for cefuroxime and meropenem, respectively.

Table 1
Key Pharmacokinetic Parameters *In Vivo* for the Plasma, Subcutaneous Adipose Tissue, and Cancellous Bone Calculated Using Both the Retrodialysis by Drug Method and the Internal Standard Method

Pharmacokinetic Parameter	Plasma	Subcutaneous Adipose Tissue			Cancellous Bone			Overall Comparison[a]
		Internal Standard Method	Retrodialysis by Drug Method	*p* Value[b]	Internal Standard Method	Retrodialysis by Drug Method	*p* Value[b]	
AUC_{0-last} (min μg/mL)	4147 (3326; 4969)	4541 (3703; 5379)	4546 (3708; 5383)	$p = 0.98$	3654 (2832; 4475)	3730 (2909; 4552)	$p = 0.74$	$p = 0.41$
C_{max} (μg/mL)	115.5 (97.9; 133.1)	68.3 (50.7; 85.9)[d]	68.8 (51.2; 86.4)	$p = 0.91$	38.5 (21.0; 56.1)[e]	39.5 (21.9; 57.1)	$p = 0.70$	$p < 0.001$
T_{max} (min)[c]	15 (0)	15 (0)	15 (0)	–	45 (19.0)	45 (19.0)	–	–
$T_{½}$ (min)	48.5 (30.8; 66.2)	42.3 (24.6; 60.0)	42.3 (24.6; 60.0)	–	67.6 (49.9; 85.2)[f]	67.6 (49.9; 85.2)	–	$p = 0.04$
$AUC_{tissue}/AUC_{plasma}$	–	1.15 (0.66; 1.64)	1.16 (0.67; 1.66)	$p = 0.90$	0.92 (0.60; 1.25)	0.94 (0.66; 1.21)	$p = 0.87$	

AUC_{0-last}, area under the concentration-time curve from 0 to the last measured value; $AUC_{tissue}/AUC_{plasma}$, tissue penetration expressed as the ratio of $AUC_{tissue}/AUC_{plasma}$; C_{max}, peak drug concentration; T_{max}, time to C_{max}; $T_{1/2}$, half-life at β-phase.
Values are given as means (95% CI) unless stated otherwise.
[a] Overall comparison using an F test for the plasma, subcutaneous adipose tissue, and cancellous bone.
[b] *p*-values for comparison of the pharmacokinetic parameters using both retrodialysis by drug method and the internal standard method.
[c] Values are given as means (SD).
[d] $p < 0.01$ for comparisons with the plasma.
[e] $p < 0.05$ for comparisons with both the subcutaneous adipose tissue (calculated with the internal standard method) and plasma.
[f] $p = 0.03$ for comparisons with the subcutaneous adipose tissue (calculated with the internal standard method).

Pharmacokinetic Analysis and Statistics

Paired *t*-tests were used to compare the RR of the different *in vitro* setups and the RR of the different calibration methods in the *in vivo* study. For the *in vivo* study, the absolute tissue concentrations of cefuroxime in both the calcaneal cancellous bone and the subcutaneous adipose tissue were determined both by the retrodialysis by drug method (using cefuroxime) and by the internal standard method (using meropenem), providing 2 concentration-time profiles for each compartment. The standard pharmacokinetic parameters, area under the concentration-time curves (AUC_{0-last}), peak drug concentration (C_{max}), and times to C_{max} (T_{max}) and half-life ($T_{1/2}$) were determined separately for each compartment and calibration method and for each pig by non-compartmental analysis using the pharmacokinetic series of commands in Stata (v. 15.1, StataCorp LLC, College Station, TX). The AUC_{0-last} was calculated using the trapezoidal rule. C_{max} was calculated as the maximum of all the recorded concentrations, and T_{max} was calculated as the time to C_{max}. $T_{1/2}$ was calculated as $\ln(2)/\lambda_{eq}$, where λ_{eq} is the terminal elimination rate constant estimated by linear regression of the log concentration on time. Microsoft Excel was used to estimate the T > MIC for MIC 4 μg/mL separately using linear interpolation for each compartment and each animal. An overall comparison of the pharmacokinetic parameters and T > MIC values were conducted using a mixed model, considering the variance between pigs, followed by pairwise comparisons made by linear regression. The model assumptions were tested by visual diagnosis of residuals, fitted values, and estimates of random effects. A correction for degrees of freedom due to small sample size was performed using the Kenward-Roger approximation method. A *p*-value < 0.05 was considered significant. No correction for multiple comparisons was applied. The tissue AUC_{0-last} to plasma AUC_{0-last} ratio ($AUC_{tissue}/AUC_{plasma}$) was calculated as a measure of the tissue penetration. Statistical analyses were also performed using Stata. The mean values (95% CI or SD) of AUC_{0-last}, C_{max}, T_{max}, and $T_{1/2}$ are presented in Table 1. Values below the lower limits of quantification were set to zero.

Results

In Vitro *Study*

The mean RR for both cefuroxime and meropenem in each setup is presented in Figure 2. It should be noted that the spread of the RR in setup 2 is larger than the rest of the setups due to a RR ranging from 50% to 72%. However, we have no explanation for this phenomenon.

The following 5 issues were evaluated with the *in vitro* study. (1) Does cefuroxime RR resemble meropenem RR: The RR for cefuroxime and meropenem was similar for all tested concentrations ($p > 0.20$), indicating that meropenem RR_{loss} and RR_{gain} is representative of cefuroxime RR_{gain} and RR_{loss}, respectively. (2) Does RR_{gain} resemble RR_{loss} for both cefuroxime and meropenem: no differences were found between RR_{gain} and RR_{loss} for either cefuroxime or meropenem ($p > 0.09$). (3) Does cefuroxime RR depend on the cefuroxime concentration: The concentration of cefuroxime, ranging from 1 to 30 μg/mL, had no impact on the cefuroxime RR ($p > 0.10$). (4) Does the presence of meropenem affect cefuroxime RR: Cefuroxime RR_{gain} and RR_{loss} were not affected by the presence of meropenem ($p > 0.90$). (5) Do meropenem and cefuroxime adhere to the microdialysis catheter: For the drug adherence test, all concentrations were 0 μg/mL in all samples for all catheters; as such, no drug adherence problems were found.

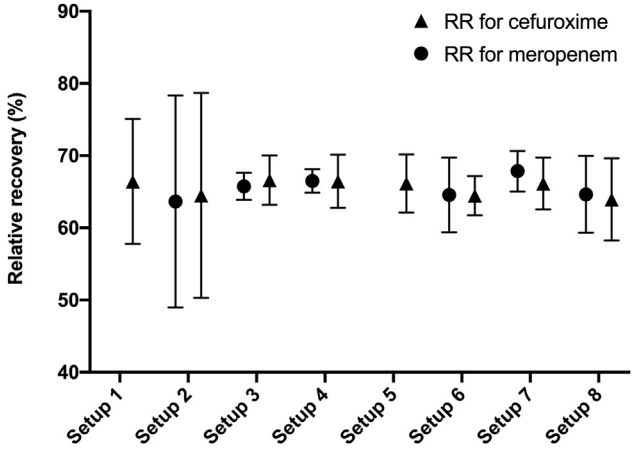

Figure 2. Mean RR for cefuroxime and meropenem for the different setups *in vitro*. Bars represent 95% CI. No significant differences were found between setups. RR, relative recovery.

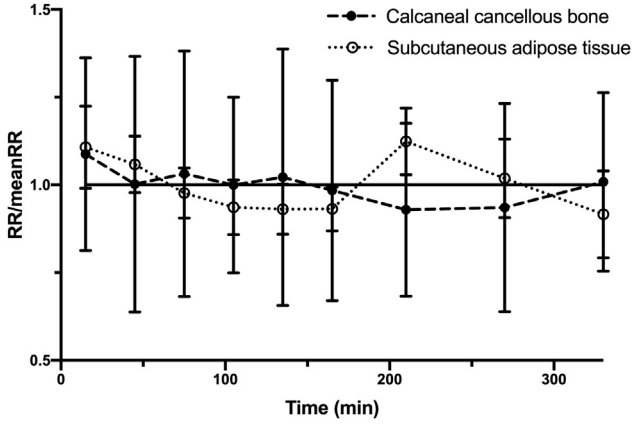

Figure 3. The relationship of the RR/mean RR for meropenem over 6 h *in vivo* for the subcutaneous adipose tissue and calcaneal cancellous bone. Bars represent 95% CI. No distinct patterns were found. RR, relative recovery.

A forest plot comparing RR values for the first 4 issues with estimated mean relative factors with a 95% CIs is presented in Figure 1b.

In Vivo *Study*

All 6 pigs completed the study and all catheters were functioning and located correctly. For subcutaneous adipose tissues, the mean RR (95% CI) was 0.28 (0.23; 0.35) for the retrodialysis by drug method using cefuroxime and 0.29 (0.22; 0.37) for the internal standard method using meropenem ($p = 0.84$). For the calcaneal cancellous bone, the mean RR (95% CI) was 0.30 (0.25; 0.36) for the retrodialysis by drug method using cefuroxime and 0.32 (0.23; 0.40) for the internal standard method using meropenem ($p = 0.38$). When calculating the pharmacokinetic parameters for the subcutaneous adipose tissue and calcaneal cancellous bone using both calibration methods, no differences were found ($p > 0.70$) (Table 1).

Furthermore, over 6 h, the relationship of the RR/mean RR for meropenem ranged from 0.92 to 1.12 for the subcutaneous adipose tissue and 0.93-1.09 for the calcaneal cancellous bone (Fig. 3). No distinct patterns were found.

The standard concentration-time profiles for the plasma, subcutaneous adipose tissue, and calcaneal cancellous bone calculated using the internal standard method are presented in Figure 4. Regarding the pharmacokinetic parameters, no differences were found among AUC_{0-last} for the plasma, subcutaneous adipose tissue, and calcaneal cancellous bone, as illustrated in Table 1. However, there was a difference in C_{max} ranging from 38.5 to 115.5 μg/mL with the highest value in the plasma and the lowest value in the calcaneal cancellous bone ($p < 0.05$). Furthermore, prolonged half-life was found in the calcaneal cancellous bone compared with the subcutaneous adipose tissue ($p = 0.03$). A delayed penetration of cefuroxime from plasma to the calcaneal cancellous bone was found (Table 1). The T > MIC 4 μg/mL (95% CI) was 149 min (138; 160) for the plasma, 183 min (153; 214) for the subcutaneous adipose tissue, and 213 min (195; 230) for the calcaneal cancellous bone. The T > MIC 4 μg/mL was significantly higher in the calcaneal cancellous bone compared with both the subcutaneous adipose tissue and plasma ($p < 0.05$), whereas the T > MIC in the subcutaneous adipose tissue was higher compared with the plasma ($p = 0.02$).

Discussion

This is the first study to evaluate meropenem as an internal standard for cefuroxime. The main finding was that cefuroxime and meropenem RR behaved similarly *in vitro* and *in vivo*, signifying that meropenem is a representative internal standard for cefuroxime. Furthermore, cefuroxime RR *in vitro* was not affected by either the cefuroxime concentration or the presence of meropenem, and the *in vivo* meropenem RR was constant over 6 h.

When investigating antimicrobial pharmacokinetics, most studies have used the retrodialysis by drug method for calibration. However, the applicability of the retrodialysis by drug method is limited by the fundamental need for the target compound to be absent from the tissues. Consequently, clinical studies investigating exogenous steady-state compounds as well as endogenous compound concentrations using the retrodialysis by drug method are challenging, if not impossible.

Calibration with the internal standard method allows for calibration independent of the study drug concentration. Furthermore, it allows for detection of RR changes during the experiment, thus serving as a quality control tool for changes in the RR.[6] However, using an internal standard requires thorough validation due to the fact that the effective membrane diffusion of the internal standard is considered equal to the analyte of interest for *in vivo* conditions. When it comes to antimicrobials, to the best of our knowledge, internal standards have only been validated by *in vitro* studies.[15-17] Although *in vitro* validation of an internal standard provides information on important issues, the *in vivo* physiochemical effects on the RR can only be addressed in *in vivo* studies.[6] As such, *in vitro* RR does not necessarily reflect the *in vivo* RR, as exemplified by Stahle,[18] suggesting that the suitability of an internal standard should be explored and validated not only in *in vitro* studies but also in *in vivo* studies to provide the most viable microdialysis setup. This calls for a standard validation model when validating internal standards for exogenous compounds. For this matter, we believe aspects of the present methodological setup can be used as inspiration.

RR is determined by the effective diffusion of the environment.[11] As such, tissue characteristics play a compelling role on the RR, which have been demonstrated in previous antimicrobial microdialysis studies.[8,9,19] Although the physiology of the subcutaneous adipose tissue and cancellous bone differs in many ways, the present study found comparable mean RR across tissues. The same tendency was found in a previous microdialysis validation study, investigating RRs of cefuroxime both *in vitro* and *in vivo*, with a

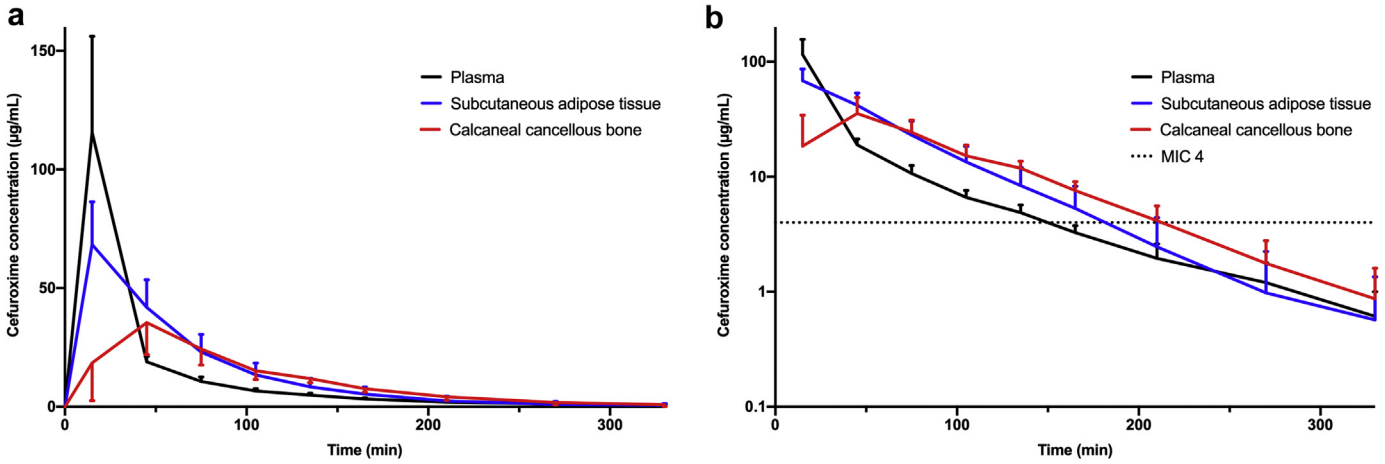

Figure 4. Mean concentration-time profiles *in vivo* for the plasma, subcutaneous adipose tissue, and calcaneal cancellous bone. Bars represent 95% CI. Cefuroxime concentrations were calculated using the internal standard method. (a) The y-axis is in normal scale. (b) The y-axis is in log scale, and the dotted line represents an MIC value of 4 µg/mL. MIC, minimal inhibitory concentration.

mean RR of 0.29 in both the subcutaneous adipose tissue and calcaneal cancellous bone.[20] These ambiguous results emphasize the need for individual microdialysis catheter calibration in all tissues.

The present study does not clarify all the methodological aspects of microdialysis calibration techniques, such as the impact of flow rate, air in the microdialysis system, temperature, and so on. Some of these matters have been investigated in previous studies.[15,17,20] Although increasing flow rates and the presence of air in the microdialysis system were found to decrease RR,[15,17] cefuroxime RR was not affected by physiological temperature changes.[20] Furthermore, it is important to recognize the specific limitations of the microdialysis method, the most profound of which are associated with the calibration of the microdialysis catheters. *In vivo* RR has been found to range from 20% to 60%, meaning that accurate pharmacokinetic data can only be obtained through calibration.[4,5,11,17] In addition, the compulsory correction for RR is associated with a magnification of the variations associated with preanalytical sample handling and the chemical assay. Furthermore, small dialysate volumes and low dialysate concentrations call for accurate, sensitive, and precise assays. To minimize the variation increasing with decreasing RR, it is commonly recommended that the RR exceeds 20%.[5] A thorough assessment and validation of the chosen calibration method must therefore be performed before conducting microdialysis antimicrobial pharmacokinetic studies.

Microdialysis has previously been used to determine the tissue pharmacokinetics of cefuroxime, given its important role as perioperative antimicrobial prophylaxis in different surgical settings.[9,20-23] Studies have reported both complete and incomplete penetrations of cefuroxime to the cancellous bone and subcutaneous adipose tissue.[9,20] In the present study, all compartments displayed comparable AUC_{0-last} values with complete cefuroxime tissue penetration to both the calcaneal cancellous bone and subcutaneous adipose tissue. Calcaneal cancellous bone exhibited the lowest C_{max} value. However, on account of a prolonged elimination rate, T > MIC 4 µg/mL was highest for the calcaneal cancellous bone compared with both the subcutaneous adipose tissue and plasma. It is generally recommended that both plasma and tissue concentrations of cefuroxime exceed the MIC values of relevant pathogens throughout the surgical procedure.[24] For cefuroxime, the clinical breakpoint MIC for *Staphylococcus aureus* is 4 µg/mL.[25] For an MIC of 4 µg/mL, 1500 mg of cefuroxime may provide sufficient concentrations after 15 min and last for 2.5 to 3.5 h, which may suffice for most orthopedic procedures. However, for long-lasting procedures, additional doses should be given after 2.5 h to ensure sufficient concentrations throughout the procedures. Further investigation of cefuroxime tissue pharmacokinetics both perioperatively, under different surgical settings, as well as in steady-state conditions with and without tissue infections, and so on, are warranted. For this purpose, meropenem can be used as an internal standard for cefuroxime.

Conclusion

In conclusion, we found meropenem suitable as an internal standard for cefuroxime in the subcutaneous adipose tissue and calcaneal cancellous bone under the investigated experimental conditions. This was observed in both the *in vitro* and *in vivo* studies. Furthermore, the cefuroxime RR *in vitro* was not affected by either the cefuroxime concentration or the presence of meropenem. *In vivo*, the meropenem RR remained constant for a minimum of 6 h.

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11.2 Paper II

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Timing of Antimicrobial Prophylaxis and Tourniquet Inflation

A Randomized Controlled Microdialysis Study

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Background: Tourniquets are widely used during extremity surgery. In order to prevent surgical site infection, correct timing of antimicrobial prophylaxis and tourniquet inflation is important. We aimed to evaluate the time for which the free drug concentration of cefuroxime is maintained above the minimum inhibitory concentration (t > MIC) in porcine subcutaneous adipose tissue and calcaneal cancellous bone during 3 clinically relevant tourniquet application scenarios.

Methods: Twenty-four female Danish Landrace pigs were included. Microdialysis catheters were placed bilaterally for sampling of cefuroxime concentrations in calcaneal cancellous bone and subcutaneous adipose tissue, and a tourniquet was applied to a randomly picked leg of each pig. Subsequently, the pigs were randomized into 3 groups to receive 1.5 g of cefuroxime by intravenous injection 15 minutes prior to tourniquet inflation (Group A), 45 minutes prior to tourniquet inflation (Group B), and at the time of tourniquet release (Group C). The tourniquet duration was 90 minutes in all groups. Dialysates and venous blood samples were collected for 8 hours after cefuroxime administration. Cefuroxime and various ischemic marker concentrations were quantified.

Results: Cefuroxime concentrations were maintained above the clinical breakpoint MIC for *Staphylococcus aureus* (4 µg/mL) in calcaneal cancellous bone and subcutaneous adipose tissue throughout the 90-minute tourniquet duration in Groups A and B. Cefuroxime administration at the time of tourniquet release (Group C) resulted in concentrations of >4 µg/mL for approximately of 3.5 hours in the tissues on the tourniquet side. Furthermore, tourniquet application induced ischemia (increased lactate:pyruvate ratio) and cell damage (increased glycerol) in subcutaneous adipose tissue and calcaneal cancellous bone. Tissue ischemia was sustained for 2.5 hours after tourniquet release in calcaneal cancellous bone.

Conclusions: Administration of cefuroxime (1.5 g) in the 15 to 45-minute window prior to tourniquet inflation resulted in sufficient concentrations in calcaneal cancellous bone and subcutaneous adipose tissue throughout the 90-minute tourniquet application. Furthermore, tourniquet-induced tissue ischemia fully resolved 2.5 hours after tourniquet release.

Clinical Relevance: Cefuroxime administration 15 to 45 minutes prior to tourniquet inflation seems to be a safe window. If the goal is to maintain postoperative cefuroxime concentrations above relevant MIC values, our results suggest that a second dose of cefuroxime should be administered at the time of tourniquet release.

Tourniquets are widely used in extremity surgery to reduce perioperative bleeding and improve visualization[1,2]. However, in order to prevent surgical site infection, correct timing of administration of antimicrobial prophylaxis and tourniquet inflation is imperative as antimicrobial plasma and tissue concentrations are recommended to exceed minimum inhibitory concentration (MIC) values of relevant bacteria throughout surgery[3]. Current guidelines are ambiguous[4,5]. Whereas Johnson stated that administration of antimicrobial prophylaxis (cefuroxime) 10 minutes prior to tourniquet inflation is sufficient[6], Deacon et al. advocated a 30 to 60-minute interval between antimicrobial prophylaxis (cefazolin) and tourniquet

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inflation[7]. In contrast, Soriano et al. suggested that administration of antimicrobial prophylaxis at the time of tourniquet release had a beneficial effect[8].

Tourniquet use has been linked to adverse effects resulting from perioperative and postoperative ischemia, including nerve paralysis, soft-tissue damage, thromboembolism, slow wound-healing, increased postoperative pain, longer recovery, and reduced muscle strength[2]. For skeletal muscle, ischemic and metabolite changes during tourniquet application have been well described[9,10]. However, to our knowledge, these changes have not been investigated in subcutaneous adipose tissue and cancellous bone.

In the present randomized study, we evaluated the time for which the free drug concentration of cefuroxime is maintained above the MIC (t > MIC) in porcine subcutaneous adipose tissue and calcaneal cancellous bone. Cefuroxime (1.5 g) was administered 15 minutes prior to tourniquet inflation (Group A), 45 minutes prior to tourniquet inflation (Group B), and at the time of tourniquet release (Group C). Furthermore, we aimed to describe ischemic markers in subcutaneous adipose tissue and calcaneal cancellous bone before, during, and after tourniquet application.

Materials and Methods

The present experimental study was conducted at the Institute of Clinical Medicine, Aarhus University Hospital, Denmark. The study was carried out according to existing laws and was approved by the Danish Animal Experiments Inspectorate (license no.: 2017/15-0201-01184). Chemical analyses were performed at the Department of Clinical Biochemistry, Aarhus University Hospital, Denmark.

Study Procedure

Microdialysis

Microdialysis is a catheter-based technique that allows water-soluble molecules, such as antimicrobial agents, to diffuse across a semipermeable membrane at the tip of the catheter[11-13]. Because of the continuous perfusion of the semipermeable membrane, concentrations in the dialysates only represent a fraction of the actual tissue concentration. This fraction is referred to as the relative recovery (RR) and can be determined by means of various calibration methods[11]. In the present study, meropenem was used as an internal calibrator for cefuroxime[14]. RR was not determined for the ischemic markers. When changes in the concentration ratios between interventions or compartments are of interest, RR determination is unnecessary[15].

Microdialysis equipment from M Dialysis was used. The microdialysis catheters consisted of CMA 63 membranes (membrane length: 30 mm, 20-kD cutoff), and CMA 107 precision pumps produced a flow rate of 2 μL/min.

Animals, Anesthetic, and Surgical Procedure

Twenty-four female Danish Landrace pigs were included in the present study (weight, 73 to 77 kg; age, 5 months). Prior to anesthesia, the pigs were sedated with an intramuscular injection of a porcine zoletil mix (1 mL/15 kg). The pigs were then kept under general anesthesia throughout the entire study with a combination of propofol (400 to 600 mg/hr, continuous infusion) and fentanyl (0.45 to 0.75 mg/hr, continuous infusion). Their pH levels and temperatures were monitored and kept within the ranges of 7.37 to 7.53 and 35.1°C to 39.6°C, respectively. A bolus infusion of 1,000 mL of 0.9% NaCl followed by continuous infusion (150 mL/hr) was administered to maintain normohydration, and glucose was substituted when needed.

After the induction of anesthesia, the surgical procedure was initiated. With the pig in a supine position, both calcanei were exposed via a longitudinal plantar incision. With use of fluoroscopic guidance, a drill-hole (diameter, 2 mm; length, 40 mm) was made from the inferior part of the calcaneocuboid joint to the proximal part of the calcaneus. Catheters were placed in the drill-holes and were fixed with a single skin suture. Subsequently, catheters were placed in the subcutaneous adipose tissue of the plantar sides of both hind feet. Correct

TABLE I Time with Cefuroxime Concentration Above the MIC (4 μg/mL) for Plasma, Subcutaneous Adipose Tissue, and Calcaneal Cancellous Bone on Tourniquet and Non-Tourniquet Sides

Parameter	Time with Concentration Above 4 μg/mL* *(min)*		
	Group A	Group B	Group C
Plasma	145 (116 to 174)†	147 (118 to 175)†	142 (123 to 171)†
Subcutaneous adipose tissue			
Non-tourniquet side	198 (169 to 227)	207 (178 to 236)	204 (175 to 233)
Tourniquet side	198 (169 to 226)	204 (175 to 233)	226 (197 to 255)
Calcaneal cancellous bone			
Non-tourniquet side	187 (158 to 216)	213 (184 to 242)	206 (177 to 235)
Tourniquet side	208 (179 to 237)	245 (216 to 273)	240 (211 to 269)

*The values are given as the mean and the 95% confidence interval. †Comparisons within the group: $p < 0.05$ for comparison with all compartments.

bone catheter placement was documented with use of fluoroscopy. All catheters were perfused with 0.9% NaCl containing 5 μg/mL meropenem, allowing for calibration with the internal standard method. A 30-minute period of tissue equilibration was allowed for[16]. Meanwhile, a tourniquet-cuff (VBM Medizintechnik) (width, 6 cm) connected to a manual tourniquet hand inflator (VBM Medizintechnik) was placed on the lower leg of a randomly picked side by drawing a note from an opaque envelope containing a total of 24 notes (12 marked *right leg* and 12 marked *left leg*). Each pig was then randomized to either Group A, B, or C by drawing a note from an opaque envelope containing a total of 24 notes (8 marked *Group A,* 8 marked *Group B,* and 8 marked *Group C*). Both randomization procedures were performed after the surgical procedures.

Sampling Procedures

Irrespective of the assigned group, 1.5 g of cefuroxime was administered intravenously over a period of 10 minutes, with the initiation of cefuroxime infusion marking time 0. Cefuroxime was used as the drug of choice because first or second-generation cephalosporins are recommended for antimicrobial prophylaxis during orthopaedic procedures[3]. The weight of the pigs was chosen to resemble that of an average human, for whom 1.5 g cefuroxime is the standard dose. Cefuroxime was administered 15 minutes prior to tourniquet inflation (Group A), 45 minutes prior to tourniquet inflation (Group B), and at the time of tourniquet release (Group C). The duration of the tourniquet application was 90 minutes in all 3 groups. Because of the anatomy of the pigs' hind legs, a tourniquet pressure of 400 mm Hg was chosen to ensure occlusion. Dialysates were collected at 15-minute intervals from 0 to 30 minutes, at 30-minute intervals from 30 to 180 minutes, and at 60-minute intervals from 180 to 480 minutes, producing a total of 12 dialysates from each catheter over an 8-hour period. At the midpoints of the sampling intervals, venous blood samples were drawn from a central venous catheter that was placed in the external jugular vein.

Handling of Samples

The venous blood samples were stored at 5°C for a maximum of 6 hours before being centrifuged at 3,000 rpm for 10 minutes. Plasma aliquots were then stored at −80°C until analysis. Dialysate samples were instantly stored at −80°C until analysis.

Quantification Techniques

Concentrations of Cefuroxime and Meropenem

The concentrations of cefuroxime and meropenem were quantified with use of a validated ultra-high-performance liquid chromatography assay[17]. Inter-run imprecisions (percent coefficients of variation) were 4.7% at 2.5 μg/mL for the quantification of cefuroxime and 3.0% at 2.0 μg/mL for the quantification of meropenem. The lower limits of quantification were 0.06 μg/mL for cefuroxime and 0.5 μg/mL for meropenem.

Assessment of Ischemic Markers

The concentrations of glucose, lactate, pyruvate, and glycerol were determined with use of the CMA 600 Microdialysis Analyzer with Reagent Set A (M Dialysis). As the duration of tourniquet application was equal in all groups, these markers were assessed in dialysates from Group A only.

Ischemia occurs when the supply of oxygen to tissues is reduced, forcing the cells to change from oxidative phosphorylation to anaerobic glycolysis in order to generate energy. Consequently, glucose and pyruvate concentrations decrease while lactate concentrations increase[18]. Ultimately, this process will lead to an increased lactate:pyruvate ratio[18]. Furthermore,

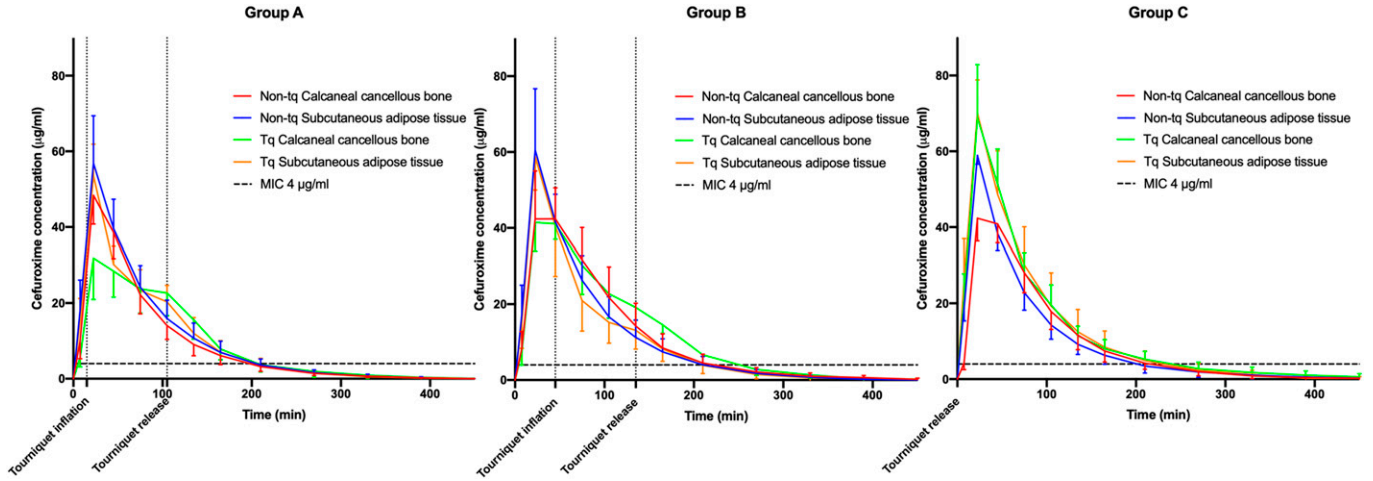

Fig. 1

Line graphs showing mean concentration-time profiles for Groups A, B, and C for cefuroxime in subcutaneous adipose tissue and calcaneal cancellous bone on both the tourniquet and non-tourniquet sides. The dotted line represents the clinical breakpoint minimal inhibitory concentration (MIC) of cefuroxime for *S. aureus* (4 μg/mL). The I-bars represent the 95% confidence intervals. Tq = tourniquet.

TABLE II Pharmacokinetic Parameters for Plasma, Subcutaneous Adipose Tissue, and Calcaneal Cancellous Bone on Tourniquet and Non-Tourniquet Sides*

Parameter	Group A	Group B	Group C
AUC *(min × [μg/mL])*			
Plasma	3,887 (3,239 to 4,664)	3,845 (3,204 to 4,614)	3,559 (2,965 to 4,270)
Subcutaneous adipose tissue			
Non-tourniquet side	4,303 (3,586 to 5,164)	4,521 (3,768 to 5,426)	4,262 (3,551 to 5,115)
Tourniquet side	4,123 (3,436 to 4,948)†	4,240 (3,533 to 5,088)	5,353 (4,461 to 6,424)†§
Calcaneal cancellous bone			
Non-tourniquet side	4,303 (3,586 to 5,164)	4,631 (3,859 to 5,558)	4,246 (3,538 to 5,095)
Tourniquet side	3,829 (3,191 to 4,596)#	5,190 (4,325 to 6,229)†	5,539 (4,616 to 6,648)†**
C_{max} *(μg/mL)*			
Plasma	131 (106 to 161)††	115 (93 to 141)††	121 (98 to 149)††
Subcutaneous adipose tissue			
Non-tourniquet side	55 (45 to 68)	58 (47 to 71)	59 (48 to 72)
Tourniquet side	53 (43 to 65)†	58 (47 to 72)	72 (59 to 89)
Calcaneal cancellous bone			
Non-tourniquet side	48 (39 to 59)	45 (37 to 55)	44 (36 to 55)
Tourniquet side	32 (26 to 39)#**	45 (36 to 55)††	67 (55 to 83)**
T_{max} *(min)*			
Plasma	7.5 (7.5 to 7.5)	7.5 (7.5 to 7.5)	7.5 (7.5 to 7.5)
Subcutaneous adipose tissue			
Non-tourniquet side	25.3 (22.5 to 45.0)	25.3 (22.5 to 45)	22.5 (22.5 to 22.5)
Tourniquet side	22.5 (22.5 to 22.5)	25.3 (22.5 to 45.0)	25.3 (22.5 to 45.0)
Calcaneal cancellous bone			
Non-tourniquet side	22.5 (22.5 to 22.5)	30.9 (22.5 to 45.0)	30.9 (22.5 to 45.0)
Tourniquet side	25.3 (22.5 to 45.0)	34.7 (22.5 to 75.0)	25.3 (22.5 to 45.0)
$T_{1/2}$ *(min)*			
Plasma	45 (37 to 54)	43 (35 to 52)††	46 (38 to 56)††
Subcutaneous adipose tissue			
Non-tourniquet side	55 (45 to 66)†	61 (51 to 74)	59 (49 to 71)
Tourniquet side	53 (44 to 64)	58 (48 to 70)	60 (50 to 72)
Calcaneal cancellous bone			
Non-tourniquet side	59 (49 to 71)†	64 (53 to 77)	57 (47 to 68)
Tourniquet side	70 (59 to 86)†	78 (65 to 94)**	66 (55 to 80)
$AUC_{tissue}/AUC_{plasma}$			
Subcutaneous adipose tissue			
Non-tourniquet side	1.11 (0.90 to 1.35)	1.17 (0.96 to 1.44)	1.20 (0.98 to 1.47)
Tourniquet side	1.06 (0.87 to 1.30)†	1.10 (0.90 to 1.35)†	1.50 (1.22 to 1.84)§
Calcaneal cancellous bone			
Non-tourniquet side	0.99 (0.81 to 1.21)	1.20 (0.98 to 1.48)	1.19 (0.97 to 1.46)
Tourniquet side	0.98 (0.80 to 1.21)#	1.35 (1.10 to 1.65)	1.56 (1.27 to 1.91)**

*AUC = area under the concentration-time curve from 0 to the last measured value, C_{max} = peak drug concentration, T_{max} = time to C_{max}, $T_{1/2}$ = half-life, and $AUC_{tissue}/AUC_{plasma}$ = area under the concentration-time curve ratio of tissue/plasma. AUC, C_{max}, and $T_{1/2}$ are given as the median, with the 95% confidence interval in parentheses. T_{max} is given as the mean, with the range in parentheses. †P < 0.05 for comparison with Group C. ‡P < 0.05 for comparison with plasma. §P < 0.05 for comparison with subcutaneous adipose tissue on the non-tourniquet side. #P < 0.05 for comparison with both Groups B and C. **P < 0.01 for comparison with calcaneal cancellous bone on the non-tourniquet side. ††P < 0.001 for comparison with all tissues within the group. ‡‡P < 0.01 for comparison with Group C.

the concentration of glycerol, which is a marker of cell damage, increases as a consequence of increased cell damage during ischemia[18].

Pharmacokinetic Analysis and Statistics

Pharmacokinetic parameters were determined for each compartment in all animals with use of noncompartmental analysis

TABLE III Concentration Difference of Ischemic Markers in Tourniquet Side Relative to Non-Tourniquet Side for Both Subcutaneous Adipose Tissue and Calcaneal Cancellous Bone*†

Time (min)	Concentration Difference in Tourniquet Side Relative to Non-Tourniquet Side (%)									
	Glucose		Lactate		Glycerol		Pyruvate		Lactate/Pyruvate	
	SCT	CCB	SCT	CCB	SCT	CCB	SCT	CCB	SCT	CCB
7.5	104 (71 to 137)	96 (82 to 109)	112 (94 to 130)	103 (87 to 119)	114 (99 to 129)	91 (76 to 106)	108 (89 to 127)	112 (76 to 149)	105 (90 to 120)	113 (95 to 131)
22.5	83 (65 to 101)	57 (35 to 78)	137 (95 to 179)	103 (85 to 120)	109 (94 to 123)	101 (77 to 125)	101 (80 to 122)	86 (72 to 101)	110 (84 to 135)	121 (106 to 136)
45	51 (22 to 79)	17 (12 to 23)	185 (134 to 236)	129 (90 to 169)	112 (80 to 145)	110 (84 to 136)	92 (74 to 110)	94 (73 to 115)	190 (142 to 238)	143 (124 to 162)
75	30 (17 to 42)	8 (5 to 12)	222 (174 to 270)	131 (99 to 164)	132 (78 to 186)	158 (119 to 196)	69 (49 to 90)	61 (48 to 74)	323 (213 to 432)	217 (184 to 249)
105	35 (23 to 46)	12 (9 to 15)	219 (199 to 240)	157 (120 to 194)	134 (89 to 178)	179 (129 to 229)	77 (56 to 98)	53 (40 to 66)	321 (217 to 425)	324 (259 to 389)
135	111 (77 to 145)	75 (48 to 101)	201 (129 to 273)	151 (118 to 185)	137 (114 to 161)	151 (117 to 185)	129 (84 to 174)	78 (52 to 103)	140 (87 to 194)	217 (174 to 259)
165	102 (70 to 135)	105 (75 to 135)	144 (91 to 198)	142 (105 to 179)	117 (98 to 135)	126 (96 to 156)	110 (96 to 125)	113 (78 to 148)	116 (69 to 163)	143 (101 to 186)
210	103 (69 to 136)	81 (62 to 101)	107 (77 to 137)	110 (86 to 134)	108 (91 to 125)	101 (83 to 118)	101 (90 to 112)	93 (74 to 113)	102 (72 to 132)	123 (101 to 145)
270	97 (71 to 124)	81 (67 to 95)	93 (72 to 115)	115 (86 to 134)	98 (80 to 117)	95 (81 to 108)	111 (96 to 125)	100 (80 to 120)	82 (56 to 108)	122 (94 to 149)
330	111 (77 to 145)	85 (69 to 101)	108 (78 to 138)	113 (91 to 135)	110 (92 to 127)	92 (79 to 106)	113 (95 to 131)	104 (83 to 124)	95 (61 to 129)	112 (96 to 128)
390	113 (80 to 145)	86 (70 to 102)	107 (82 to 133)	103 (73 to 133)	103 (82 to 124)	92 (76 to 107)	107 (93 to 122)	102 (77 to 127)	90 (66 to 114)	105 (84 to 126)
450	103 (83 to 124)	88 (62 to 114)	86 (68 to 104)	112 (80 to 145)	105 (81 to 129)	96 (83 to 109)	114 (92 to 137)	106 (86 to 125)	83 (67 to 99)	104 (84 to 124)

*SCT = subcutaneous adipose tissue, and CCB = calcaneal cancellous bone. Values are given as the mean, with the 95% confidence interval in parentheses. †The markers were assessed for Group A only (tourniquet inflation time, 15 minutes; tourniquet release time, 105 minutes).

in Stata (version 15.1; StataCorp). The areas under the concentration-time curves ($AUC_{0\text{-last}}$) were calculated with use of the trapezoidal rule. The maximum of all of the recorded concentrations was defined as the peak drug concentration (C_{max}), enabling calculation of the time to C_{max} (T_{max}). Half-life ($T_{1/2}$) was calculated as $\ln(2)/\lambda_{eq}$, where λ_{eq} is the terminal elimination rate constant estimated by means of linear regression of the log of the concentration on time. The AUC_{tissue}:AUC_{plasma} ratio was calculated as a measure of tissue penetration. Microsoft Excel was used to estimate t > MIC with use of linear interpolation. A general comparison of the pharmacokinetic parameters and t > MIC was conducted with use of a repeated-measurements analysis of variance followed by pairwise comparisons made by means of linear regression. The Kenward-Roger approximation method was used for degrees-of-freedom correction because of the small sample size. The model assumptions were tested with use of visual assessment of residuals, fitted values, and estimates of random effects. The pharmacokinetic parameters were log-transformed to improve normality. A significance level of 5% was used. Microsoft Excel was used to calculate the mean concentration difference in percent between the tourniquet and non-tourniquet sides for the ischemic markers. The measured cefuroxime and ischemic marker concentrations of the dialysate were attributed to the midpoint of the sampling intervals.

Results

All pigs completed the study. The mean RR for the investigated compartments ranged from 23% to 29%.

t > MIC

The t > MIC results are shown in Table I. The cefuroxime clinical breakpoint MIC for *Staphylococcus aureus* (4 μg/mL) was used to evaluate t > MIC[19]. In Groups A and B, cefuroxime concentrations in both subcutaneous adipose tissue and calcaneal cancellous bone on the tourniquet side were >4 μg/mL throughout the entire 90-minute duration of tourniquet application and for approximately 1 hour after tourniquet release (Fig. 1). In Group C, cefuroxime concentrations were >4 μg/mL for approximately 3.5 hours after tourniquet release in both subcutaneous adipose tissue and calcaneal cancellous bone on the tourniquet side. There were no significant differences in t > MIC (4 μg/mL) in subcutaneous adipose tissue or calcaneal cancellous bone between the 3 groups. However, Group A tended toward shorter t > MIC in calcaneal cancellous bone on the tourniquet side compared with Group C ($p = 0.08$). Furthermore, intragroup

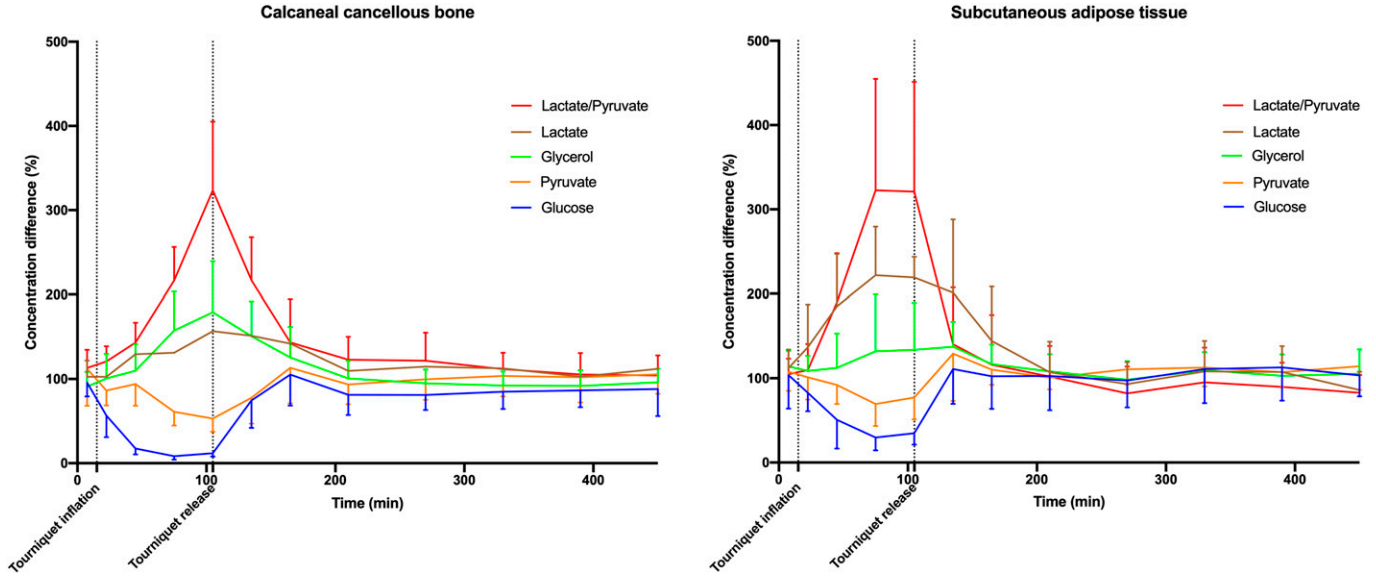

Fig. 2

Line graphs showing the mean ischemic marker concentration differences between the tourniquet and non-tourniquet sides for both calcaneal cancellous bone and subcutaneous adipose tissue. The I-bars represent the 95% confidence intervals.

tendencies toward higher t > MIC in calcaneal cancellous bone on the tourniquet side than on the non-tourniquet side were found in Group B ($p = 0.08$) and Group C ($p = 0.06$). In all groups, the t > MIC was lower in plasma than in the investigated tissues.

Pharmacokinetic Parameters

The pharmacokinetic parameters are shown in Table II, and the concentration-time profiles are depicted in Figure 1. The administration of cefuroxime 15 minutes (Group A) and 45 minutes (Group B) prior to tourniquet release resulted in comparable pharmacokinetic parameters, except for a lower AUC and C_{max} value in calcaneal cancellous bone on the tourniquet side for Group A as compared with Group B. Furthermore, in an intragroup comparison in Group A, calcaneal cancellous bone exhibited lower C_{max} values on the tourniquet side than on the non-tourniquet side. Calcaneal cancellous bone on the tourniquet side showed a tendency toward a prolonged half-life compared with the non-tourniquet side, but the differences were not significant ($p = 0.08$ and 0.06 for Groups A and B, respectively).

Administering cefuroxime at the time of tourniquet release (Group C) resulted in higher AUC and C_{max} values in both calcaneal cancellous bone and subcutaneous adipose tissue on the tourniquet side in comparison with the values in Group A and also resulted in a higher C_{max} value in calcaneal cancellous bone on the tourniquet side in comparison with the value in Group B. An intragroup comparison showed higher AUC values in both cancellous bone and subcutaneous adipose tissue on the tourniquet side than on the non-tourniquet side. Furthermore, the C_{max} value for calcaneal cancellous bone was higher on the tourniquet side than on the non-tourniquet side.

Ischemic Markers

The mean differences in the concentration percentages of the ischemic markers between the tourniquet side and the non-tourniquet side are depicted for subcutaneous adipose tissue and calcaneal cancellous bone in Table III and Figure 2. Shortly after tourniquet inflation, a threefold increase in the lactate:pyruvate ratio was found in both subcutaneous adipose tissue and calcaneal cancellous bone. While the lactate:pyruvate ratio for subcutaneous adipose tissue decreased to baseline immediately after tourniquet release, the lactate:pyruvate ratio was normalized in calcaneal cancellous bone after 2.5 hours. Furthermore, calcaneal cancellous bone was exposed to a decreased glucose ratio and an increased glycerol ratio during tourniquet application.

Discussion

To our knowledge, the present study is the first to simultaneously investigate cefuroxime concentrations and ischemic markers in subcutaneous adipose tissue and calcaneal cancellous bone before, during, and after tourniquet application in legs with and without a tourniquet. Three clinically relevant scenarios were tested: administration of 1.5 g of cefuroxime 15 minutes prior to tourniquet inflation (Group A), 45 minutes prior to tourniquet inflation (Group B), and at the time of tourniquet release (Group C). The duration of the tourniquet application was 90 minutes in all 3 groups. The main finding was that cefuroxime concentrations were maintained above the clinical breakpoint MIC for *S. aureus* (4 μg/mL) throughout the 90-minute tourniquet duration in Groups A and B. Administering cefuroxime at the time of tourniquet release (Group C) provided concentrations of >4 μg/mL for approximately 3.5 hours in the tissues on the tourniquet side. Furthermore, tourniquet use was found to induce ischemia and cell damage (increased glycerol) in both subcutaneous adipose tissue and calcaneal cancellous bone. While ischemia was maintained in calcaneal cancellous bone for 2.5 hours after tourniquet release, subcutaneous adipose tissue recovered instantly after tourniquet release.

The timing of antimicrobial prophylaxis may be very important in tourniquet-aided surgical procedures as the blood supply to the tissue in the operative field is occluded during surgery[20-23]. The findings of the present study suggest that administration of cefuroxime (1.5 g) 15 and 45 minutes prior to tourniquet inflation results in sufficient antimicrobial concentrations in subcutaneous adipose tissue and calcaneal cancellous bone throughout a 90-minute surgical procedure. These findings are in line with those of previous studies[6,7].

Across all 3 groups, calcaneal cancellous bone on the tourniquet side demonstrated a tendency toward higher t > MIC values than calcaneal cancellous bone on the non-tourniquet side. This finding could be explained by the prolonged cefuroxime-elimination rate in calcaneal cancellous bone during tourniquet application in Groups A and B and by an increased peak drug concentration in Group C due to hyperemia following ischemia. Interestingly, this finding implies that correct tourniquet usage could potentiate the effect of the antimicrobial prophylaxis.

Recently, Soriano et al. demonstrated that the administration of antimicrobial prophylaxis (cefuroxime) just prior to tourniquet release was non-inferior to the administration of standard antimicrobial prophylaxis 10 to 30 minutes prior to tourniquet inflation in terms of the development of surgical site infection[8]. Soriano et al. argued that high plasma and tissue concentrations at the time of wound closure are important as hematomas are formed at the end of surgical procedures, especially in tourniquet-aided surgical procedures, providing excellent growth conditions for contaminant bacteria[8]. This statement was supported by Zelenitsky et al.[24]. Also, as demonstrated in the present study, the administration of antimicrobial prophylaxis at the time of tourniquet release may provide a beneficial hyperemia effect, leading to higher peak drug concentrations and AUC values, better tissue penetration, and prolonged t > MIC postoperatively. As such, the present study suggests that there is an advantage to administering antimicrobial prophylaxis both prior to tourniquet inflation and at the time of tourniquet release. However, this possibility needs further investigation.

Interestingly, the present study indicates that bone is more vulnerable than subcutaneous adipose tissue to ischemia. Previous studies have demonstrated full recovery of tourniquet-induced ischemia in skeletal muscle within 2 to 3 hours after tourniquet release[9,10]. This finding is comparable with the findings in calcaneal cancellous bone in the present study. Moreover, we found increased glycerol levels during ischemia, indicating increased cell turnover. Wound-healing disorders are a known complication of tourniquet application that may be related to increased cell turnover/damage[2]. In the present study, all ischemic markers were normalized after 2.5 hours, suggesting that there is no association between tourniquet application and wound and bone-healing complications. However, factors such as prolonged tourniquet duration and disorders involving decreased tissue blood flow might increase the risk of tissue-healing complications.

Until now, the few studies investigating the timing of antimicrobial prophylaxis prior to tourniquet inflation have involved the use of tissue specimens[6,7]. However, that approach has important methodological limitations: the free extracellular concentration cannot be selectively measured, antimicrobial concentration is given in terms of mass rather than volume, and temporal resolution is poor or nonexistent and is limited to the time of surgery[25]. In contrast, microdialysis allows for simultaneous, serial sampling of the free and active fraction of drugs in the interstitial space from multiple compartments. Such features are desirable as the majority of infections occur in the interstitial space. However, microdialysis remains a sampling technique and is prone to limitations associated with calibration procedures and chemical assays[15,26-29].

Although pigs resemble humans in terms of physiology and anatomy, important interspecies differences must be taken into account[30]. The present study used juvenile pigs (age, 5 months), and one could speculate that the young pigs exhibited greater cefuroxime tissue penetration and faster ischemic marker recovery after tourniquet release than would be seen in middle-aged and old humans. Furthermore, the stress and weight-bearing impact of the calcaneal bone differs between humans and pigs. Finally, the applied tourniquet pressure, which is higher than in normal clinical use, may impact the amount of ischemia and cell damage during and after tourniquet application.

In summary, cefuroxime concentrations were maintained above the clinical breakpoint MIC for *S. aureus* (4 μg/mL) throughout the 90-minute tourniquet duration in both subcutaneous adipose tissue and calcaneal cancellous bone regardless of whether cefuroxime was administered 15 minutes (Group A) or 45 minutes (Group B) prior to tourniquet inflation. Administration of cefuroxime at the time of tourniquet release resulted in a hyperemic effect demonstrated by higher AUC, higher peak drug concentrations, and increased tissue penetration in comparison with those seen with cefuroxime administration prior to tourniquet inflation. Furthermore, tourniquet application induced ischemia and cell damage in both subcutaneous adipose tissue and calcaneal cancellous bone; tissue ischemia resolved 2.5 hours after tourniquet release. Future studies investigating the benefits of antimicrobial prophylaxis administered both prior to tourniquet inflation and at the time of tourniquet release are warranted. ■

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11.3 Paper III

Effects of Tourniquet Inflation on Peri- and Postoperative Cefuroxime Concentrations in Bone and Tissue

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[Word count: 3152**]**

Abstract

Background and purpose: Tourniquet is widely used in orthopedic surgery to reduce intraoperative bleeding and improve visualization. We evaluated the effect of tourniquet application on peri- and postoperative cefuroxime concentrations in subcutaneous tissue, skeletal muscle, calcaneal cancellous bone, and plasma. The primary endpoint was the time for which the free cefuroxime concentration was maintained above the clinical breakpoint minimal inhibitory concentration (T>MIC) for *Staphylococcus aureus* (4 µg/mL).

Patients and methods: Ten patients scheduled for hallux valgus or hallux rigidus surgery were included. Microdialysis catheters were placed for sampling of cefuroxime concentrations bilaterally in subcutaneous tissue, skeletal muscle, and calcaneal cancellous bone. A tourniquet was applied on the thigh of the leg scheduled for surgery (tourniquet duration time (range): 65 (58; 77) minutes). Cefuroxime (1.5 g) was administered intravenously 15 minutes prior to tourniquet inflation, followed by a second dose 6 hours later. Dialysates and venous blood samples were collected for 12 hours.

Results: A cefuroxime concentration of 4 µg/mL was reached within 22.5 minutes in all compartments and patients. For cefuroxime the T>MIC (4 µg/mL) ranged between 4.8–5.4 hours across compartments, with similar results for the tourniquet and non-tourniquet leg. Comparable T>MIC and penetration ratios were found for the first and second dosing intervals.

Interpretation: Administration of cefuroxime (1.5 g) 15 minutes prior to tourniquet inflation is safe in order to achieve tissue concentrations above 4 µg/mL throughout surgery. A tourniquet application time of approximately 1 hour did not affect the cefuroxime tissue penetration in the following dosing interval.

Introduction

Tourniquet (tq) is widely used in orthopedic surgery due to its ability to reduce intraoperative bleeding and improve visualization(Rama et al. 2007). However, as the blood supply to the operating field is occluded during surgery, correct timing of the antimicrobial prophylaxis administration and tq inflation is essential in order to ensure therapeutic tissue concentrations at the site of surgery. Only few studies have investigated the ideal time interval from perioperative antimicrobial prophylaxis administration to tq inflation, resulting in ambiguous guidelines(Johnson 1987, Deacon et al. 1996, Prokuski 2008, Ochsner et al. 2016). With regard to cefuroxime in particular, a recent randomized controlled microdialysis study in a porcine model suggested that a window of 15–45 min between cefuroxime administration and tq inflation results in sufficient perioperative tissue concentrations throughout a 90-minute tq application(Hanberg et al. 2020b).

Tq induce peri- and postoperative ischemia(Ejaz et al. 2015), which may result in decreased postoperative tissue perfusion and antimicrobial tissue exposure(Smith and Hing 2010). A recent experimental study on a rat model demonstrated a reduced distribution of antimicrobials to tq-affected tissues for up to 72 hours after tq release(Mangum et al. 2019). Decreased postoperative antimicrobial tissue exposure may ultimately increase the risk of surgical site infection.

The aim of this study was therefore to dynamically evaluate the effects of tq application on both peri- and postoperative *in situ* cefuroxime concentrations in subcutaneous tissue, skeletal muscle, calcaneal cancellous bone, and plasma. Cefuroxime (1.5 g) was administered intravenously as a bolus 15 minutes prior to tq inflation and followed by a subsequent dose 6 hours later. The primary endpoint was the time for which the free drug concentration of cefuroxime was maintained above the clinical breakpoint minimal inhibitory concentration (T>MIC) for *Staphylococcus aureus* (4

µg/mL)(EUCAST 2021), which we hypothesized was maintain throughout surgery in the tourniquet-exposed tissues when administering cefuroxime 15 min prior to tourniquet inflation.

Materials and Methods

This study was conducted at the Department of Orthopedic Surgery, Horsens Regional Hospital, Denmark. Chemical analyses were performed at the Department of Clinical Biochemistry, Aarhus University Hospital, Denmark. This study was performed in the same setting as another study, which investigated tissue ischemic metabolites(Hanberg et al. 2021).

Study procedure

Microdialysis

The microdialysis catheter consists of a semipermeable membrane at the tip of the catheter, which allows for sampling of water-soluble molecules such as antimicrobials(Hanberg et al. 2016, Kho et al. 2017, Bue et al. 2018, Hanberg et al. 2019b). However, as the semipermeable membrane is continuously perfused, equilibrium across the semipermeable membrane cannot be attained. Consequently, the dialysates represent only a fraction of the actual tissue concentration. This fraction is referred to as the relative recovery that can be determined by different calibration methods(Kho et al. 2017). For this study, meropenem was used as an internal calibrator for cefuroxime(Hanberg et al. 2019a). An in-depth description of the microdialysis technique can be found elsewhere(Kho et al. 2017).

Microdialysis equipment from M Dialysis AB (Stockholm, Sweden) was used. The microdialysis catheters consisted of CMA 63 membranes and CMA 107 precision pumps (flow rate: 2 µL/min).

Study design and patients

10 patients were included in a prospective observational cohort study. The effects of tq application on both peri- and postoperative cefuroxime concentrations were evaluated in subcutaneous tissue, skeletal muscle, and calcaneal cancellous bone in a simultaneous paired comparison of the tq and non-tq leg during 12 hours of continuous microdialysis sampling (Figure 1).

Patients scheduled for hallux valgus or hallux rigidus surgery were offered enrolment in the study. A single surgeon recruited 10 patients who attended the outpatient clinic. Written informed consent was obtained from all patients prior to study enrolment. Inclusion criteria were as follows: age ≥ 18 years, normal distal blood pressure bilaterally, normal creatinine levels, and use of contraception for fertile women. Exclusion criteria were as follows: previous arterial surgery in either of the legs, previous surgery on either of the calcaneal bones, previous fracture or bone infection in either of the calcaneal bones, diabetes, unsuccessful spinal anesthesia, and allergy to cefuroxime. All patients asked for enrolment were included in the study and all completed the study.

After placement of the 6 microdialysis catheters, 1.5 g of cefuroxime (Fresenius Kabi AB, Sweden) was administered intravenously over 10 minutes, marking time zero. Fifteen minutes after initiation of the cefuroxime administration, the tq cuff was inflated (Pressure: 260 mmHg) on the thigh of the leg scheduled for surgery. Prior to tq inflation, the leg was elevated for 1 minute. The planned surgical procedure was performed after tq inflation. When the surgical procedure was completed, the tq cuff was released (mean tq inflation time [range]: 65 [58; 77] min). A second dose of 1.5 g cefuroxime was administered at 6 hours. As first- and second-generation cephalosporins are recommended for antimicrobial prophylaxis in orthopedic surgeries, cefuroxime was the drug of choice(Mangram et al. 1999). For adults, 1.5 g cefuroxime is the standard dose.

Surgery

Before the surgical procedure, microdialysis catheters were placed similarly in both legs: in the subcutaneous tissue (membrane length: 30 mm), at the posterior site of the mid-lower leg, in the gastrocnemius muscle of the medial head (membrane length: 30 mm), and in the calcaneal cancellous bone (membrane length: 10 mm) via drill holes (ø: 2 mm; depth 30 mm) made on the posterolateral side aiming at the anteromedial side of the calcaneal bone (Figure 1). After placement of the microdialysis catheters, all catheters were perfused with 0.9% NaCl containing 5 µg/mL meropenem, allowing for continuous calibration with meropenem as an internal calibrator.

Sampling procedures

Dialysates were collected from all 6 microdialysis catheters at 15-minute intervals from time 0–30 minutes, at 30-minute intervals from time 30–180 minutes, and at 60-minute intervals from both time 180–240 minutes and time 300–360 minutes. Following administration of the second dose of 1.5 g cefuroxime at time 360 minutes, dialysates were collected at 30-minute intervals from time 360–540 minutes, and at 60-minute intervals from both time 540–600 minutes and time 660–720 minutes. A total of 17 samples from each microdialysis catheter were collected over the 12-hour period. Venous blood samples were collected at the midpoint of the sampling intervals drawn from a peripheral catheter in the cubital vein. After the last sample was collected, all microdialysis catheters were removed.

Handling of samples

The venous blood samples were stored at 5°C for a maximum of 10 hours before being centrifuged at 3,000*g* for 10 minutes. The plasma aliquots were then stored at -80°C until analysis. The dialysate samples were immediately stored at -80°C until analysis.

Quantification of cefuroxime and meropenem concentrations

The concentrations of cefuroxime and meropenem were quantified using a validated ultra-high-performance liquid chromatography assay(Hanberg et al. 2018). Inter-run imprecisions (percent coefficients of variation) were 4.7% at 2.5 µg/mL for quantification of cefuroxime and 3.0% at 2.0 µg/mL for quantification of meropenem. The lower limits of quantification were 0.06 µg/mL for cefuroxime and 0.5 µg/mL for meropenem.

Pharmacokinetic analysis and statistics

Pharmacokinetic parameters were determined for each compartment in all patients using noncompartmental analysis in Stata (v. 15.1, StataCorp, College Station, TX, United States). The areas under the concentration-time curves (AUC) were calculated using the trapezoidal rule. The maximum of all the recorded concentrations was defined as peak drug concentration (C_{max}), enabling calculation of the time to C_{max} (T_{max}). The half-life ($T_{1/2}$) was calculated as $\ln(2)/\lambda_{eq}$, where λ_{eq} is the terminal elimination rate constant estimated by linear regression of the log concentration on time. The $AUC_{tissue}/AUC_{plasma}$ ratio was calculated as a measure of tissue penetration. Microsoft Excel (v. 16.16.11, Microsoft Corporation, Redmond, Washington) was used to estimate the T>MIC (4 µg/mL) using linear interpolation. The pharmacokinetic parameters and T>MIC were calculated separately for both the first (time 0–6 hours) and second (time 6–12 hours) dosing intervals. A general comparison of the pharmacokinetic parameters and T>MIC was conducted using a repeated measurements analysis of variance followed by pairwise comparisons made by linear regression. The

Kenward-Roger approximation method was used for degrees of freedom correction due to the small sample size. The model assumptions were tested using visual diagnosis of residuals, fitted values, and estimates of random effects. A significance level of 5% was used. The cefuroxime concentrations of the dialysate were attributed to the midpoint of the sampling intervals.

Ethics, registration, data sharing plan, funding, and potential conflicts of interest

The study was approved by the Danish Medicines Agency (EudraCT number 2018-000217-21), the Central Denmark Region Committees on Health Research Ethics (Registration number 1-10-72-47-18), and the Danish Data Protection Agency (Registration number 1-16-02-88-18). The study was registered at www.clinicaltrialsregister.eu (number 2018-000217-21) and conducted in accordance with the Declaration of Helsinki and the ICH Harmonized Tripartite Guideline for Good Clinical Practice. The Good Clinical Practice Unit at Aalborg and Aarhus University Hospitals conducted the mandatory monitoring procedures.

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The authors have no conflicts of interest.

Results

No adverse events related to the microdialysis technique or cefuroxime infusion occurred. The patients' characteristics are presented in Table 1.

The mean relative recovery (SD) values were 23% (9%) for tq subcutaneous tissue, 20% (7%) for non-tq subcutaneous tissue, 39% (4%) for tq skeletal muscle, 33% (12%) for non-tq skeletal muscle, 21% (8%) for tq calcaneal cancellous bone, and 19% (7%) for non-tq calcaneal cancellous bone.

T>MIC

Similar results were observed for T>MIC (4 µg/mL) between the first and second dosing intervals. Therefore, the T>MIC results are only presented for the first dosing interval in Table 2. A cefuroxime concentration of 4 µg/mL was reached within 22.5 minutes in all compartments and patients. The T>MIC (4 µg/mL) ranged between 4.8–5.4 hours across compartments, with similar results for the tq and non-tq leg (Figure 2 and Table 2). When comparing tq and non-tq legs separately, lower T>MIC values were found for calcaneal cancellous bone compared to the remaining compartments in the tq leg, including plasma ($P < 0.05$). No differences were found between the compartments in the non-tq leg.

Pharmacokinetic parameters

Similar pharmacokinetic results were seen between the first and second dosing intervals in all investigated compartments. Only the tq calcaneal cancellous bone T_{max} was longer in the first dosing interval (mean [range], 84.0 [22.5; 135.0]) compared to the second dosing interval (mean [range], 51.0 [15.0; 75.0]) ($P < 0.01$). The pharmacokinetic parameters are presented only for the first dosing interval in Table 3. The concentration time profiles are depicted for both the first and second dosing interval in Figure 2.

Similar AUC, C_{max}, $T_{1/2}$, and tissue penetrations were observed when comparing the tq and non-tq leg. Only the calcaneal cancellous bone T_{max} was longer in the tq leg (mean [range], 84.0 [22.5; 135.0]) compared to the non-tq leg (mean [range], 34.5 [22.5; 75.0]) ($P < 0.01$) in the first dosing interval. No differences were found for the remaining compartments.

When comparing the tq and non-tq leg separately, a lower AUC was found for the non-tq calcaneal cancellous bone compared to non-tq subcutaneous tissue. Plasma C_{max} was higher compared to all investigated compartments. Moreover, the tq skeletal muscle C_{max} was higher compared to both tq calcaneal cancellous bone and tq subcutaneous tissue. Finally, plasma T_{max} was shorter compared to all tissues in both the tq and non-tq leg, and the tq calcaneal cancellous bone was longer than both tq subcutaneous tissue and tq skeletal muscle.

Discussion

This is the first clinical study to investigate the effects of tq application on both peri- and postoperative cefuroxime concentrations in subcutaneous tissue, skeletal muscle, and calcaneal cancellous bone in a simultaneous paired comparison of the tq and non-tq leg. The main finding of this study was a cefuroxime T>MIC (4 μg/mL) range between 4.8–5.4 hours across compartments, with similar results for the tq and non-tq leg. Furthermore, similar T>MIC and penetration ratios were found for the first and second dosing intervals.

Tq is widely used in orthopedic surgery, but only a few studies have investigated antimicrobial tissue concentrations during the tq application, and no clinical studies have investigated antimicrobial tissue concentrations after tq release. Using bone and fat tissue specimens, Johnson investigated different

time intervals from administration of cefuroxime (1.5 g) to tq inflation, and concluded that a time interval of 10 minutes was sufficient to achieve tissue concentrations above 4 µg/mL(Johnson 1987). Recently, a randomized controlled microdialysis study in a porcine model suggested that a window of 15–45 minutes between cefuroxime (1.5 g) administration and tq inflation was sufficient to achieve calcaneal cancellous bone and subcutaneous tissue concentrations above 4 µg/mL(Hanberg et al. 2020b). The present clinical study confirms these findings, suggesting that cefuroxime has fully penetrated the investigated tissues after 15 minutes.

It has previously been hypothesized that perioperative ischemia reduces the postoperative antimicrobial tissue penetration(Smith and Hing 2010, Mangum et al. 2019). However, studies investigating tissue ischemia during and after tq application found that ischemia-exposed tissue fully recovers 2.5 hours after tq release(Ejaz et al. 2015, Hanberg et al. 2020b). The findings from the present study do not indicate any decreased postoperative cefuroxime penetration in the tq exposed tissues for a tq application of approximately 1 hour.

Interestingly, the present study showed that tq calcaneal cancellous bone T_{max} is longer than in non-tq calcaneal cancellous bone. Furthermore, a wider range of the T_{max} values was found for both subcutaneous tissue and skeletal muscle in the tq leg compared to the non-tq leg. These T_{max} results may be attributed to a combination of the limited elimination rate of cefuroxime during tq time and a second peak in the cefuroxime concentration after tq release. For 5 patients, this peak was higher than the initial peak prior to tq inflation in tq calcaneal cancellous bone. This may indicate a favorable hyperemic effect when the tq is released, which was also observed in a porcine model(Hanberg et al. 2020b).

For antimicrobial prophylaxis it is generally recommended that the antimicrobial plasma and tissue concentrations exceed the MIC values of relevant bacteria throughout surgery(Mangram et al. 1999). In the present study a tq cuff was inflated 15 minutes after initiation of the cefuroxime administration and a cefuroxime concentration of 4 µg/mL was reached within 22.5 minutes in all tissues and patients, which was maintained above this target for a minimum of 4.5 hours in all the investigated compartments. As such, these findings indicate that cefuroxime appears as a good choice for antimicrobial prophylaxis in terms of tissue penetration and T>MIC. Only one clinical study has previously investigated cefuroxime bone tissue concentrations by means of microdialysis(Tottrup et al. 2019). Tottrup et al. found a shorter T>MIC in plasma, subcutaneous tissue, and tibial cancellous bone after a postoperative intravenous bolus administration of 1.5 g cefuroxime compared to the present study compartments(Tottrup et al. 2019). While the plasma creatinine was comparable between the patient groups in the two studies, Tottrup et al. recorded a substantially higher mean BMI compared to the present study (30.6 vs 25.0)(Tottrup et al. 2019). Weight-based dosing of cefuroxime, in addition to consideration of renal function, may therefore be considered in order to achieve therapeutic tissue concentrations in heavy weighing patients.

The few clinical studies that have investigated antimicrobial concentrations during tq application have been based on tissue specimens(Johnson 1987, Deacon et al. 1996). However, this approach suffers from important methodological limitations because sampling in clinical studies is limited to the time of surgery, biopsy studies are likely confined to the surgical side, free extracellular concentrations cannot be measured selectively, and drug concentrations are given by mass rather than volume(Landersdorfer et al. 2009). Microdialysis, on the other hand, allows for simultaneous and serial sampling of the free and active fraction of drugs in the interstitial space from multiple compartments, both peri- and postoperative(Tottrup et al. 2016, Hanberg et al. 2020a). These features

278 are desirable, as the majority of infections occur in the interstitial space. However, microdialysis
279 remains a sampling technique that has limitations associated with calibration procedures and chemical
280 assays(Landersdorfer et al. 2009, Kho et al. 2017).
281
282 In summary, cefuroxime T>MIC (4 μg/mL) ranged between 4.8–5.4 hours in subcutaneous tissue,
283 skeletal muscle, and calcaneal cancellous bone, with similar results for the tq and non-tq leg.
284 Furthermore, similar T>MIC and penetration ratios were found for the first and second dosing
285 intervals. This study therefore suggests that, for this patient population, administering cefuroxime
286 (1.5 g) 15 minutes prior to tq inflation is safe in order to achieve tissue concentrations above 4 μg/mL
287 throughout surgery and that a tq application time of approximately 1 hour does not affect the
288 cefuroxime tissue penetration in the following dosing interval.
289

290 ## Contribution of authors

291 PH, MB, JK, KS, and MS initiated and designed the study. PH, JK, and CJ conducted the surgery and
292 placed all the probes. PH, MB and ARJ collected the data. Statistical analysis and interpretation of
293 data was done by PH, MB, JK, KS, and MS. All authors drafted and revised the manuscript.
294

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Figures and tables

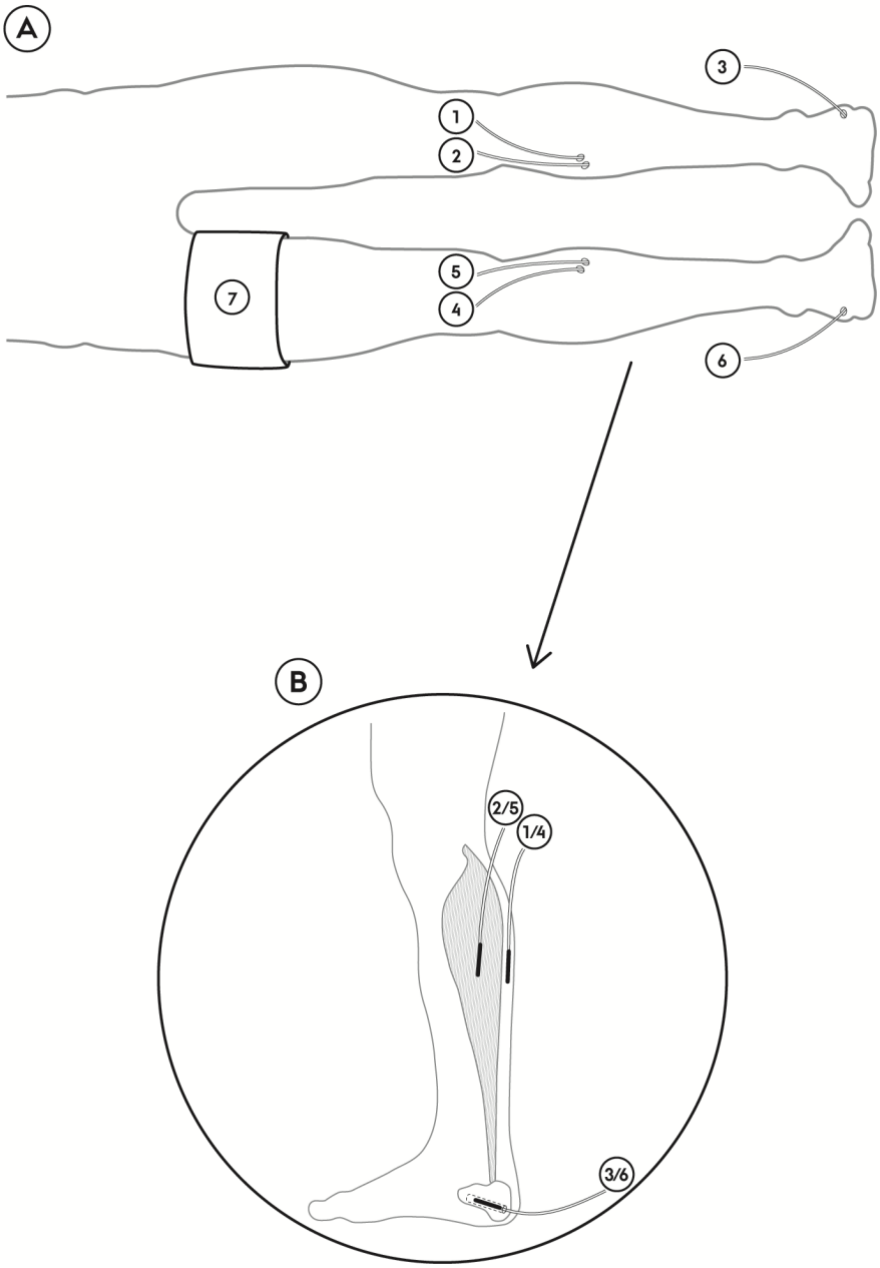
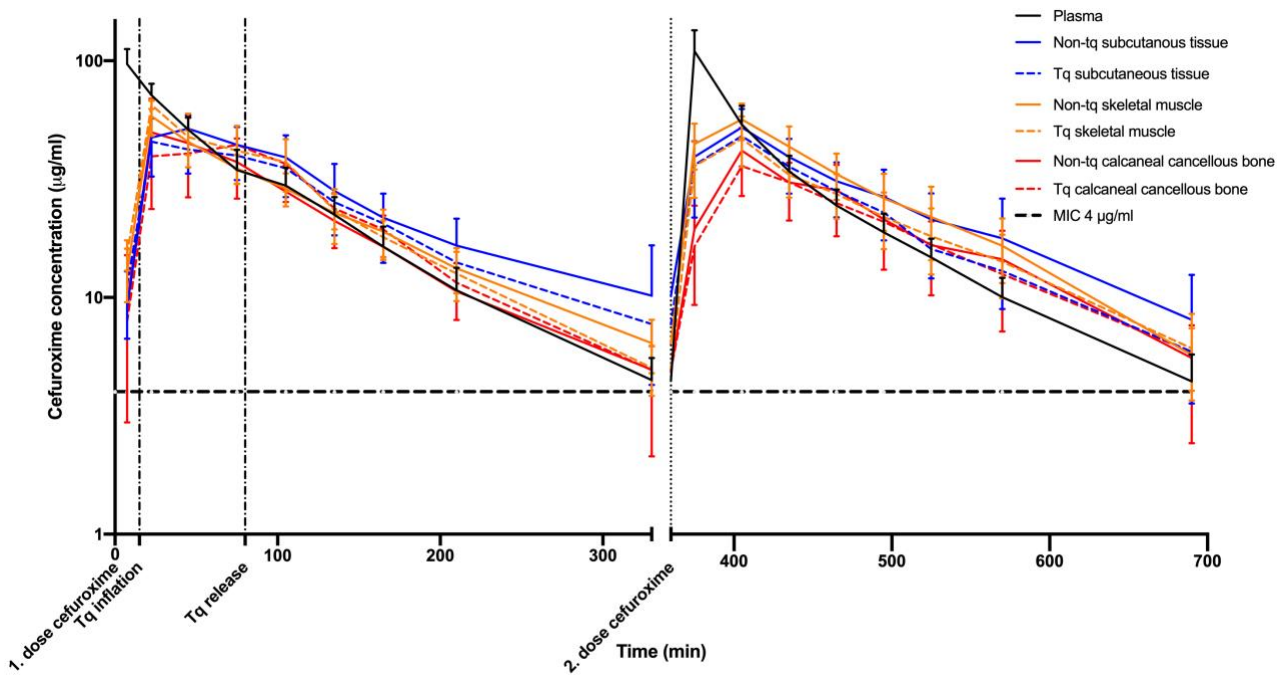


Figure 1

Illustration of the inserted microdialysis catheters. Cefuroxime concentrations were obtained by means of microdialysis catheters placed in non-tourniquet subcutaneous tissue (1), non-tourniquet skeletal muscle (2), non-tourniquet calcaneal cancellous bone (3), tourniquet subcutaneous tissue (4), tourniquet skeletal muscle (5), and tourniquet calcaneal cancellous bone (6). A tourniquet cuff (7) was placed on the leg scheduled for surgery.



306
307 **Figure 2**

308 Mean concentration–time profiles of cefuroxime for plasma, subcutaneous tissue, skeletal muscle, and calcaneal cancellous bone on
309 both the tourniquet and non-tourniquet leg. Bars represent 95% CI. The y-axis is in log scale. The first and second dose of 1.5 g
310 cefuroxime was administered at time 0 and 6 h, respectively. Tourniquet inflation and mean release times were 15 and 80 min,
311 respectively.

312 Abbreviations: Tq, Tourniquet; MIC, minimal inhibitory concentration.

313 **Table 1**

314 Patients' characteristics

Parameter	
Sex (female/male)	7/3
Age (y), mean (range)	58 (45-67)
Height (cm), mean (range)	169 (156-185)
Weight (kg), mean (range)	72 (56-89)
Body mass index (kg/m^2), mean (range)	25 (20-33)
Plasma creatinine (µmol/L), mean (range)	75 (60-90)
Tourniquet duration (min), mean (range)	65 (58-77)
Ankle-brachial index tourniquet leg, mean (range)	1.11 (0.90-1.28)
Ankle-brachial index non-tourniquet leg, mean (range)	1.08 (0.91-1.28)

315 Normal range: Plasma creatinine (males), 60–106 µmol/L; Plasma creatinine (females), 45–90 µmol/L; Ankle-brachial index, ≥ 0.9

316

317 **Table 2**

318 The time with concentrations above the minimal inhibitory concentration (T>MIC) (4 µg/mL) in min for plasma, subcutaneous tissue,
319 skeletal muscle, and calcaneal cancellous bone on both the tourniquet and non-tourniquet leg from the first dosing interval.

Compartment	Time (min) Non-tourniquet leg	Time (min) Tourniquet leg	*P* values
Plasma	318 (297; 338)	-	-
Subcutaneous tissue	312 (292; 333)	322 (302; 343)	0.4
Skeletal muscle	320 (300; 341)	316 (295; 336)	0.7
Calcaneal cancellous bone	306 (285; 326)	289 (269; 310)[a]	0.2

320 Time given as mean (95% CI)

321 [a] $P < 0.05$ for comparison with all compartments in the tourniquet side and with plasma.

322

Table 3

Pharmacokinetic parameters for plasma, subcutaneous tissue, skeletal muscle, and calcaneal cancellous bone on both the tourniquet and non-tourniquet leg.

Compartment	Non-tourniquet	Tourniquet	*P* values
Plasma $AUC_{0\text{-}6h}$ (min µg/mL)	8198 (6611; 9785)	-	-
Subcutaneous tissue $AUC_{0\text{-}6h}$ (min µg/mL)	8538 (6952; 10125)	7548 (5962; 9135)	0.3
Skeletal muscle $AUC_{0\text{-}6h}$ (min µg/mL)	7280 (5693; 8866)	7785 (6198; 9372)	0.6
Calcaneal cancellous bone $AUC_{0\text{-}6h}$ (min µg/mL)	6648 (5061; 8235)[a]	7107 (5561; 8694)	0.6
Plasma C_{max} (µg/mL)	97 (84; 110)[b]	-	-
Subcutaneous tissue C_{max} (µg/mL)	58 (45; 70)	51 (38; 64)	0.4
Skeletal muscle C_{max} (µg/mL)	61 (48; 73)	70 (60; 83)[c]	0.3
Calcaneal cancellous bone C_{max} (µg/mL)	59 (47; 72)	53 (40; 66)	0.4
Plasma T_{max} (min)	7.5 (7.5; 7.5)[b]	-	-
Subcutaneous tissue T_{max} (min)	45.0 (22.5; 75.0)	48.8 (22.5; 105.0)	0.7
Skeletal muscle T_{max} (min)	27.0 (22.5; 45.0)	33.0 (22.5; 105.0)	0.5
Calcaneal cancellous bone T_{max} (min)	34.5 (22.5; 75.0)	84.0 (22.5; 135.0)[d]	< 0.01
Plasma $T_{1/2}$ (min)	74 (56; 93)	-	-
Subcutaneous tissue $T_{1/2}$ (min)	94 (75; 113)	99 (81; 118)	0.7
Skeletal muscle $T_{1/2}$ (min)	97 (78; 116)	87 (68; 105)	0.4
Calcaneal cancellous bone $T_{1/2}$ (min)	86 (67; 105)	95 (77; 114)	0.5
Subcutaneous tissue $AUC_{tissue}/AUC_{plasma}$	1.09 (0.86; 1.32)	0.96 (0.73; 1.19)	0.3
Skeletal muscle $AUC_{tissue}/AUC_{plasma}$	0.92 (0.69; 1.15)	0.98 (0.75; 1.21)	0.6
Calcaneal cancellous bone $AUC_{tissue}/AUC_{plasma}$	0.84 (0.61; 1.07)	0.88 (0.65; 1.11)	0.8

AUC, area under the concentration–time curve from 0 to 6 h; C_{max}, peak drug concentration; T_{max}, time to C_{max}; $T_{1/2}$, half-life; $AUC_{tissue}/AUC_{plasma}$, area under the concentration–time curve ratio of tissue/plasma.

AUC, C_{max}, and $T_{1/2}$ are given as mean (95% CI).

T_{max} given as mean (ranges).

[a] $P = 0.04$ for comparison with non-tourniquet subcutaneous tissue.

[b] $P < 0.05$ for comparison with all tissues.

[c] $P < 0.05$ for comparison with tourniquet subcutaneous tissue and calcaneal cancellous bone.

[d] $P < 0.01$ for comparison with tourniquet subcutaneous tissue and skeletal muscle.

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11.4 Paper IV

Tourniquet Induced Ischemia and Reperfusion in Subcutaneous Tissue, Skeletal Muscle, and Calcaneal Cancellous Bone

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Summary

Authors

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Title

Tourniquet Induced Ischemia and Reperfusion in Subcutaneous Tissue, Skeletal Muscle, and Calcaneal Cancellous Bone

Abstract

This study aimed to evaluated ischemic metabolites in subcutaneous tissue, skeletal muscle, and calcaneal cancellous bone before, during, and after tourniquet application in a simultaneous paired comparison of tourniquet-exposed and non-tourniquet-exposed legs. Ten patients scheduled for hallux valgus or hallux rigidus surgery were included. Microdialysis catheters were placed to simultaneously and continuously sample the metabolites glucose, lactate, pyruvate, and glycerol bilaterally for 12 hours in subcutaneous tissue, skeletal muscle, and calcaneal cancellous bone. A tourniquet was applied on the leg planned for surgery (inflation time: 15 minutes, mean tourniquet duration time (range): 65 (58;77) minutes). During tourniquet inflation, a 2- to 3-fold increase of the mean lactate/pyruvate ratio was found for all investigated tissues in the tourniquet-exposed leg compared to the non-tourniquet-exposed leg. The lactate/pyruvate ratio recovery time after tourniquet release was within 30 minutes for skeletal muscle, 60 minutes for subcutaneous tissue, and 130 minutes for calcaneal cancellous bone. Only the tourniquet-exposed skeletal muscles were found to be ischemic during tourniquet inflation, defined by a significant increase of the lactate/pyruvate ratio exceeding the ischemic cutoff level of 25; however, this level decreased below 25 immediately after tourniquet release. The glycerol ratio increased instantly after inflation in the tourniquet-exposed leg in skeletal muscle and subcutaneous tissue, and recovered within 60 (skeletal muscle) and 130 minutes (subcutaneous tissue) after tourniquet release. These findings suggest that applying tourniquet for approximately 1-hour results in limited tissue ischemia and cell damage in subcutaneous tissue, skeletal muscle, and calcaneal cancellous bone.

Keywords. Bone, ischemic metabolites, microdialysis, soft tissues, tourniquet.

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1 Introduction

Tourniquet is widely used in orthopedic surgery in order to improve visualization and reduce perioperative bleeding(1). However, tourniquet has been associated with multiple adverse effects, including thromboembolism, nerve paralysis, postoperative pain, longer recovery time, reduced muscle strength, soft tissue damage, and slow wound healing(2). Although many of these adverse effects may be related to tourniquet-induced ischemia, only few studies have investigated local tissue metabolite changes during and after tourniquet application(3-5). While only clinical studies have investigated ischemic changes in tourniquet-exposed skeletal muscle(3, 4), one porcine study has evaluated ischemic changes in subcutaneous tissue and cancellous bone(5). For skeletal muscle (the clinical studies) and cancellous bone (the porcine study) ischemic changes were reported last for approximately 2.5 hours after tourniquet release, whereas the porcine study found that subcutaneous tissue recovered immediately after tourniquet release(3-5).

Microdialysis is a membrane-bearing method allowing for sampling of metabolites (e.g., glucose, lactate, pyruvate, and glycerol) from the interstitial space of various tissues. These metabolites can be easily and promptly analyzed when linked to an appropriate analytical assay, which in some settings have been used to monitor the vitality of transplanted tissue(6). Ischemia occurs when the supply of oxygen and substrates to the cells is reduced(7). This drives the ischemic cells to change from oxidative phosphorylation to anaerobic glycolysis in order to maintain energy production. Anaerobic glycolysis causes glucose and pyruvate concentrations to decrease and lactate concentrations to increase, ultimately leading to an increased lactate/pyruvate ratio(7). A lactate/pyruvate ratio above 25 is considered to signify ischemia(8). Glycerol, a basic component of cell membranes, is released when the cell membrane is damaged and is therefore used as a marker of cell damage(7).

In this clinical study, we evaluated glucose, lactate, pyruvate, glycerol, and the lactate/pyruvate ratio *in situ* in subcutaneous tissue, skeletal muscle, and calcaneal cancellous bone before, during, and after tourniquet application in a simultaneous paired comparison of tourniquet-exposed and non-tourniquet-exposed legs.

2 Materials and Methods

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This study was conducted at the Department of Orthopaedic Surgery, Horsens Regional Hospital, Denmark. The analyses of the ischemic metabolites were performed at the Institute of Clinical Medicine, Aarhus University Hospital, Denmark. The study was approved by the Danish Medicines Agency (EudraCT number 2018-000217-21), the Central Denmark Region Committees on Health Research Ethics (Registration number 1-10-72-47-18), and the Danish Data Protection Agency (Registration number 1-16-02-88-18). The study was registered at www.clinicaltrialsregister.eu (number: 2018-000217-21, Date: 2018-02-01) and conducted in accordance with the Declaration of Helsinki and the ICH Harmonized Tripartite Guideline for Good Clinical Practice. The Good Clinical Practice Unit at Aalborg and Aarhus University Hospital conducted the mandatory monitoring procedures. This study was performed in the same setting as another study, which investigated tissue concentrations of cefuroxime(9).

2.1 Study procedure

2.1.1 Microdialysis

Microdialysis is a catheter-based technique wherein a semipermeable membrane is held at the tip of the catheter, allowing for sampling of water-soluble molecules from the interstitial space of various tissues(Figure 1)(10-15). The semipermeable membrane is continuously perfused which prevent equilibrium across the membrane(10-14). The catheters are normally calibrated to compensate for this effect(15, 16). However, when changes in the concentration ratios and variation between interventions or compartments are of interest, as they are for comparing ischemic marker concentrations between tourniquet-exposed and non-tourniquet-exposed leg and for ratios between markers (i.e., lactate/pyruvate), this is not essential.

Microdialysis equipment from M Dialysis AB (Stockholm, Sweden) was used. The microdialysis catheters consisted of CMA 63 membranes (molecule cutoff: 20 kDa) and CMA 107 precision pumps (flow rate: 2 µL/min).

2.1.2 Study design, patients, and surgical procedure

The study design, patient characteristics, and surgical procedure have been described in detail in a previous study(9). Ten patients were included in a prospective observational cohort study evaluating the effect of tourniquet application on local ischemic metabolites in subcutaneous tissue, skeletal muscle, and calcaneal cancellous bone before, during, and after tourniquet

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application. In a simultaneous paired design, a tourniquet-exposed and non-tourniquet-exposed leg were compared during 12 hours of continuous microdialysis sampling.

Patients scheduled for hallux valgus or hallux rigidus surgery were offered enrolment in the study. A single surgeon recruited all 10 patients, who attended the outpatient clinic. Written informed consent was obtained from all patients prior to enrollment in the study. Inclusion criteria were as follows: age ≥ 18 years, normal distal blood pressure bilaterally, normal kidney function (normal creatinine levels), and use of contraception for fertile women. Exclusion criteria were: previous arterial surgery in either leg, previous surgery on either calcaneal bone, previous fracture or bone infection in either calcaneal bone, diabetes, unsuccessful spinal anesthesia, and allergy to cefuroxime. All patients that were offered study enrolment accepted participation, and they all completed the study.

The microdialysis catheters were placed similarly in both legs: in the subcutaneous tissue (membrane length: 30 mm) at the posterior site of the mid-lower leg, in the medial head of the gastrocnemius muscle (membrane length: 30 mm), and in the calcaneal cancellous bone (membrane length: 10 mm) via lateral drill holes (ø 2 mm; depth 30 mm). A detailed description of the catheter placement can be found in a previous study(9). After placement, all catheters were perfused with 0.9% NaCl containing 5 µg/mL meropenem, which was used as an internal calibrator for the pharmacokinetic study(9). Preoperatively, all patients received 1.5 g cefuroxime intravenously as antimicrobial prophylaxis. Subsequently, dialysates were collected from all 6 microdialysis catheters at 15-minute intervals from time 0–30 minutes, at 30-minute intervals from time 30–180 minutes, and at 60-minute intervals from both time 180–240 minutes and time 300–360 minutes. Following the administration of a second dose of 1.5 g cefuroxime at time 360 minutes, dialysates were collected at 30-minute intervals from time 360–540 minutes and at 60-minute intervals from both time 540–600 minutes and time 660–720 minutes. A total of 17 samples from each microdialysis catheter were collected over the 12-hour period. After collection, all dialysate samples were immediately stored at -80°C until analysis.

The tourniquet cuff was inflated (pressure: 260 mmHg) at time 15 minutes on the thigh of the leg planned for surgery. Prior to tourniquet inflation the leg was elevated for 1 minute. The planned

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surgical procedure was performed after tourniquet inflation. The tourniquet cuff was released (mean tourniquet time [range]: 65 [58; 77] minutes) at the end of the surgical procedure.

2.2 Assessment of ischemic metabolites

Concentrations of glucose, lactate, pyruvate, and glycerol were determined using a CMA 600 Microdialysis Analyzer using Reagent Set A (M Dialysis AB, Sweden).

2.3 Analysis and statistics

Microsoft Excel (v. 16.16.11, Microsoft Corporation, Redmond, Washington) was used to calculate both the mean concentration difference in percentage of ischemic metabolites between the tourniquet-exposed and non-tourniquet-exposed leg and the mean lactate/pyruvate ratio for each compartment separately. The measured metabolite concentrations were attributed to the midpoint of the sampling intervals. A conclusion of no difference between the tourniquet-exposed and non-tourniquet-exposed leg (tourniquet/non-tourniquet) was defined as a ratio including 100% in the CI 95%.

3 Results

All 10 patients completed the study with no adverse events related to the microdialysis catheters, and all microdialysis catheters functioned properly. The patients' characteristics are presented in Table 1.

The mean concentration differences in percentage between the tourniquet-exposed and non-tourniquet-exposed leg for glucose, lactate, pyruvate, glycerol, and the lactate/pyruvate ratio for subcutaneous tissue, skeletal muscle, and calcaneal cancellous bone are presented in Table 2 and Figure 2. During the tourniquet inflation time, a 2- to 3-fold increase of the mean lactate/pyruvate ratio between the tourniquet-exposed and non-tourniquet-exposed leg was found for all investigated tissues. The highest and most prompt lactate/pyruvate ratio increase was found in skeletal muscle and the lowest and most delayed increase was found in calcaneal cancellous bone (Table 2 and Figure 2). As a measure of the tissue recovery time after tourniquet release, the lactate/pyruvate ratio between the tourniquet-exposed and non-tourniquet-exposed leg was analogous within 30 minutes for skeletal muscle, 60 minutes for subcutaneous tissue, and 130 minutes for calcaneal cancellous bone. Considering the lactate/pyruvate ischemic cutoff level of

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25 for each compartment separately, only the tourniquet-exposed skeletal muscle increased significantly above the ischemic cutoff level during tourniquet inflation; however, this level decreased below 25 immediately after tourniquet release (Figure 3). Within statistical significance, tourniquet-exposed subcutaneous tissue and calcaneal cancellous bone failed to reach the ischemic cutoff level during tourniquet inflation. The increased lactate/pyruvate ratios were primarily caused by increases in lactate concentrations (Table 2). For all investigated tissues, decreased glucose ratios were found shortly after tourniquet inflation and were normalized immediately after tourniquet release.

The glycerol ratios in skeletal muscle and subcutaneous tissue increased immediately after tourniquet inflation and recovered within 60 (skeletal muscle) and 130 minutes (subcutaneous tissue) after tourniquet release (Table 2). For calcaneal cancellous bone, glycerol ratios tended to increase during tourniquet inflation, but only significantly at time 105 minutes (Table 2).

4 Discussion

This is the first clinical study to investigate the effect of tourniquet application on *in situ* ischemic metabolites in subcutaneous tissue, skeletal muscle, and calcaneal cancellous bone in a simultaneous paired comparison of tourniquet-exposed and non-tourniquet-exposed legs. The main finding was that the use of a tourniquet increased the lactate/pyruvate ratio in all tissues. However, the lactate/pyruvate ratio fully recovered within 30 minutes for skeletal muscle, 60 minutes for subcutaneous tissue, and 130 minutes for calcaneal cancellous bone after tourniquet release. Only tourniquet-exposed skeletal muscle increased significantly above the lactate/pyruvate ischemic cutoff level of 25 during inflation of the tourniquet. The findings of the present study remain explorative, but brings important knowledge to understand postoperative tissue condition. Furthermore, it presents a new method to investigate the ischemic conditions in bone and soft tissues, which can be used in future studies investigating other orthopedically relevant settings, patient groups, and for different tourniquet duration time/cuff pressure.

Two clinical studies have previously investigated the ischemic metabolites glucose, lactate, pyruvate, and glycerol in skeletal muscle during and after tourniquet application, both of which reported a recovery time for all metabolites of approximately 150 minutes after tourniquet release(3, 4). However, these recovery times were based on the individual ischemic metabolites

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rather than the more precise ischemic marker, the lactate/pyruvate ratio. Only two studies have compared the lactate/pyruvate ratio between a tourniquet-exposed and a non-tourniquet-exposed extremity – one clinical study investigating skeletal muscle and one porcine study investigating subcutaneous tissue and cancellous bone(3, 5). The recovery time from tourniquet release was within 30 minutes for skeletal muscle and subcutaneous tissue and within 150 minutes for cancellous bone. These results are consistent with the findings of the present study.

A tourniquet duration time of approximately 1 hour resulted in limited tissue ischemia when considering the lactate/pyruvate ischemic cutoff level of 25. Only tourniquet-exposed skeletal muscle increased significantly above the ischemic cutoff level during tourniquet inflation; however, it dropped below 25 immediately after tourniquet release, indicating an instant reperfusion of the tourniquet-exposed tissues. This is consistent with previous studies that documented a hyperemic effect and increase in antimicrobial tissue concentrations after tourniquet release(5, 9). Furthermore, these results acknowledge the current literature investigating the tourniquet induced ischemia by looking at the ultrastructural changes, tissue pH, creatine kinase leakage, and tissue desaturation in skeletal muscle(17-19), which recommends a maximum of 2 hour continuous tourniquet inflation.

The glycerol ratios in all the investigated tissues were affected, which may be due to cell damage. However, it could also be explained by the hormonal regulation of hypoglycemia during tourniquet application, which initiates catecholamine-induced lipolysis in tissues(20). Subcutaneous tissue is the major site of glycerol production, which could explain the prolonged glycerol recovery found in subcutaneous tissue compared to skeletal muscle and calcaneal cancellous bone(20). The prolonged glycerol ratio recovery time in subcutaneous tissue differs from recent porcine findings, in which the glycerol ratio was fully recovered within 60 minutes after tourniquet release(5). This could partly be explained by the fact that pigs have a thinner subcutaneous tissue layer than humans. With regard to calcaneal cancellous bone, the clinical glycerol recovery time was comparable to that of pigs(5). In a clinical study Ejaz et al. found that glycerol ratios were increased for 150 minutes after tourniquet release, which is a substantially longer time than was observed in the present study(3). The study by Ejaz et al. is comparable with the present study in many ways, e.g., the skeletal muscle measurements were performed in the same locations and in patients without considerable comorbidities and with comparable BMIs.

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However, the mean age of the patient population in the preceding clinical study was higher (68 years) compared to the present study (58 years) and the patients' distal blood pressure was not measured prior to inclusion(3). Combined with the higher degree of data variation found in the present study, these differences could explain the different recovery times for glycerol in skeletal muscle.

Tourniquet-induced ischemia has been associated with pain, swelling, slow wound healing, compartment syndrome, and respiratory distress syndrome(2, 21, 22). As all of the investigated ischemic metabolites in all tissues were normalized within 130 minutes, the findings of the present study suggest no association between approximately 1 hour of tourniquet application and the above-mentioned complications when glucose, lactate, pyruvate, glycerol, and the lactate/pyruvate ratio are used as indicators of tissue ischemia. The present study was performed on patients with a mean age of 58 years and a mean BMI of 25, with normal kidney function and normal distal blood pressure. These results may therefore only be extrapolated to patients without significant comorbidities.

The present study demonstrates that microdialysis can be used to monitor ischemic metabolites in the interstitial space of subcutaneous tissue, skeletal muscle, and calcaneal cancellous bone. Handling and implanting a microdialysis catheter are simple, little time consuming, and minimally invasive, at least in subcutaneous tissue and skeletal muscle. As such, microdialysis allows for the bedside monitoring of ischemic metabolites in the interstitial space of relevant tissues and may be considered in situations where prolonged tourniquet application is necessary, for patients with decreased extremity blood flow, for trauma patients where the duration of tissue ischemia is unknown, for amputations and replantations, and for monitoring the vitality of transplanted tissues.

Some limitations should be mentioned. The present study was part of a larger pharmacokinetic study investigating the influence of tourniquet application on cefuroxime tissue penetration as primary effect parameter, and so a flow rate of 2 μL/min was used(9). Sufficient quantification of absolute concentrations requires calibration or complete (or at least a high degree of) equilibrium across the microdialysis membrane, which in turn depends on several factors, e.g., flow rate, membrane size and length, and the diffusivity of the substances in the tissue(15, 23). The

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combination of long/large membranes and a low flow rate provides a high degree of equilibrium and is preferable for bedside monitoring. In the present study it was not possible to acquire absolute concentrations, but the comparison of ischemic marker concentrations between the tourniquet-exposed and non-tourniquet-exposed leg and assessment of ratios between markers (lactate/pyruvate) are considered valid. Moreover, surgery was performed only on the forefoot of the tourniquet-exposed leg, distally to the investigated tissues. Even though neither of the investigated tissues on the tourniquet-exposed leg were subjects to the primary surgery, a potential influence of the surgical response on the ischemic metabolites in the investigated tourniquet-exposed tissues cannot be omitted.

In conclusion, we found that a tourniquet application time of approximately 1 hour resulted in limited tissue ischemia and cell damage in subcutaneous tissue, skeletal muscle, and calcaneal cancellous bone. All investigated ischemic metabolites were fully recovered within 130 minutes after tourniquet release in all tissues.

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Table 1. Patient characteristics

Parameter	
Gender (women/men)	7/3
Age (years), mean (range)	58 (45–67)
Height (cm), mean (range)	169 (156–185)
Weight (kg), mean (range)	72 (56–89)
Body mass index (kg/m^2), mean (range)	25 (20–33)
Plasma creatinine (μmol/L), mean (range)	75 (60–90)
Tourniquet duration (min), mean (range)	65 (58–77)
Ankle index tourniquet leg, mean (range)	1.11 (0.90–1.28)
Ankle index non-tourniquet leg, mean (range)	1.08 (0.91–1.28)

Normal range: Plasma creatinine men, 60–106 μmol/L; Plasma creatinine women, 45–90 μmol/L; Ankle-brachial index, ≥ 0.9

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Table 2. The mean concentration difference (%) of ischemic metabolites between the tourniquet-exposed and non-tourniquet-exposed leg (tourniquet/non-tourniquet).

	Glucose			Lactate			Glycerol			Pyruvate			Lactate/pyruvate		
Time (min)	SCT (%)	Muscle (%)	Bone (%)	SCT (%)	Muscle (%)	Bone (%)	SCT (%)	Muscle (%)	Bone (%)	SCT (%)	Muscle (%)	Bone (%)	SCT (%)	Muscle (%)	Bone (%)
7,5	108 (74; 141)	138 (110; 167)	96 (46; 146)	128 (45; 211)	92 (61; 122)	98 (71; 125)	105 (90; 120)	107 (84; 131)	123 (95; 151)	110 (49; 70)	95 (63; 127)	109 (44; 173)	117 (90; 143)	103 (70; 137)	116 (71; 160)
22,5	113 (80; 147)	131 (83; 173)	50 (7; 94)	134 (58; 210)	159 (81; 237)	150 (108; 192)	123 (108; 139)	141 (110; 173)	132 (89; 175)	105 (46; 163)	107 (65; 148)	147 (88; 206)	138 (114; 161)	173 (61; 284)	127 (66; 187)
45	72 (53; 91)	74 (61; 87)	64 (32; 95)	125 (58; 192)	255 (170; 340)	120 (89; 152)	132 (113; 150)	214 (157; 270)	151 (90; 212)	97 (36; 158)	84 (58; 110)	100 (80; 121)	203 (100; 305)	318 (221; 416)	122 (100; 144)
75	65 (43; 86)	70 (58; 83)	58 (30; 87)	199 (108; 290)	379 (277; 482)	173 (117; 229)	135 (115; 155)	241 (147; 335)	218 (85; 351)	97 (44; 149)	119 (95; 142)	99 (79; 120)	310 (156; 464)	336 (131; 540)	192 (118; 265)
105	97 (64; 130)	145 (121; 170)	75 (26; 124)	141 (85; 197)	162 (102; 222)	142 (89; 196)	126 (106; 145)	147 (102; 193)	242 (117; 366)	128 (65; 191)	187 (96; 278)	93 (61; 125)	142 (101; 182)	108 (64; 153)	169 (101; 237)
135	96 (57; 135)	135 (94; 175)	75 (12; 138)	119 (59; 180)	111 (69; 152)	139 (87; 191)	125 (105; 146)	126 (81; 171)	147 (97; 197)	141 (78; 203)	121 (86; 157)	104 (54; 153)	97 (69; 126)	90 (71; 109)	153 (102; 204)
175	100 (54; 145)	115 (98; 132)	137 (34; 241)	121 (64; 178)	107 (83; 131)	103 (78; 128)	140 (103; 177)	113 (80; 145)	117 (45; 189)	123 (63; 183)	104 (80; 129)	82 (55; 108)	116 (87; 146)	106 (84; 129)	147 (105; 189)
210	108 (59; 156)	115 (88; 141)	113 (45; 180)	128 (48; 208)	97 (65; 130)	121 (66; 177)	125 (89; 161)	114 (90; 138)	103 (43; 163)	111 (66; 155)	103 (65; 141)	102 (40; 163)	107 (82; 131)	101 (78; 125)	140 (98; 183)
330	107 (62; 152)	112 (81; 143)	100 (72; 129)	110 (61; 160)	120 (101; 139)	103 (71; 135)	115 (85; 145)	99 (69; 128)	106 (77; 134)	121 (80; 161)	93 (72; 114)	83 (51; 115)	84 (70; 99)	146 (91; 202)	142 (92; 192)
375	134 (79; 189)	109 (78; 140)	84 (57; 112)	111 (62; 161)	104 (71; 136)	78 (50; 107)	109 (75; 142)	98 (71; 125)	80 (50; 110)	121 (92; 149)	96 (63; 129)	69 (38; 100)	90 (59; 122)	115 (81; 149)	125 (66; 184)
405	128 (63; 194)	104 (75; 134)	73 (44; 102)	116 (54; 178)	103 (76; 129)	93 (50; 136)	105 (80; 130)	80 (56; 105)	97 (58; 136)	114 (78; 150)	94 (64; 125)	88 (56; 119)	91 (68; 115)	116 (86; 146)	104 (80; 128)
435	96 (66; 125)	98 (63; 132)	78 (42; 114)	114 (62; 166)	88 (63; 113)	99 (63; 135)	114 (82; 145)	105 (56; 155)	89 (64; 115)	122 (85; 159)	87 (71; 104)	93 (62; 124)	87 (68; 107)	108 (67; 148)	110 (78; 142)
465	102 (57; 147)	100 (68; 132)	92 (50; 135)	110 (63; 157)	94 (70; 118)	102 (58; 147)	110 (77; 143)	77 (44; 111)	88 (62; 114)	105 (80; 130)	87 (66; 108)	103 (57; 148)	98 (76; 121)	113 (84; 141)	104 (79; 118)
495	105 (64; 146)	97 (65; 130)	103 (42; 164)	102 (60; 144)	93 (66; 120)	110 (46; 174)	108 (74; 143)	79 (56; 103)	97 (60; 133)	104 (83; 125)	88 (65; 111)	112 (58; 167)	93 (70; 117)	112 (79; 146)	99 (79; 118)
525	94 (66; 122)	92 (61; 123)	86 (38; 133)	109 (66; 152)	92 (68; 116)	114 (58; 170)	106 (77; 134)	81 (57; 105)	105 (63; 148)	112 (85; 138)	88 (74; 102)	95 (60; 130)	93 (73; 113)	114 (79; 149)	119 (98; 141)
570	88 (64; 111)	108 (76; 140)	81 (44; 119)	107 (75; 139)	103 (74; 132)	117 (72; 162)	105 (71; 139)	80 (56; 105)	100 (68; 132)	106 (86; 126)	87 (70; 105)	97 (69; 125)	99 (77; 120)	117 (90; 145)	123 (94; 153)
690	131 (56; 206)	111 (79; 143)	104 (45; 163)	101 (67; 135)	98 (72; 124)	103 (40; 165)	108 (77; 140)	90 (48; 132)	191 (68; 113)	110 (80; 139)	93 (68; 117)	111 (85; 137)	94 (68; 120)	110 (79; 140)	93 (51; 136)

Abbreviations: Bone, calcaneal cancellous bone; Muscle, skeletal muscle; SCT, subcutaneous tissue.

The mean tourniquet duration is marked in gray. Tourniquet inflation time: 15 minutes, mean (range) tourniquet release time: 85 (73; 92) minutes.

Values are given as mean (95% CI)

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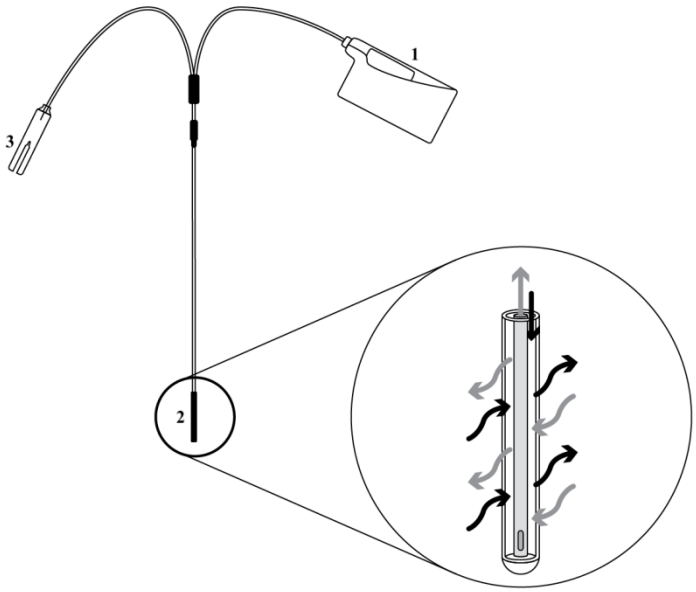

Figure 1. Illustrative drawing of the microdialysis system with an enlargement of the membrane. The microdialysis system consists of a (1) precision pump, a (2) semipermeable membrane, and a (3) sample container system (microvials).

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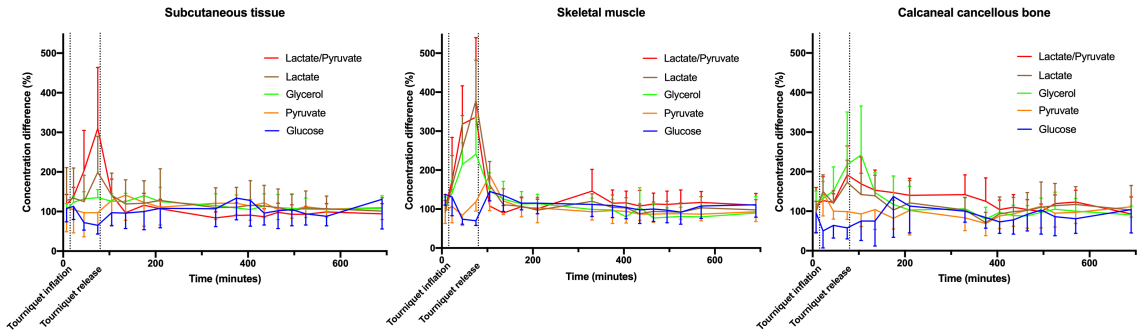

Figure 2. The mean concentration difference (%) of ischemic metabolites between the tourniquet-exposed and non-tourniquet-exposed leg (tourniquet/non-tourniquet). Tourniquet inflation time: 15 minutes, mean (range) tourniquet release time: 85 (73; 92) minutes (both are marked with vertical dotted lines). Bars represent the 95% CI.

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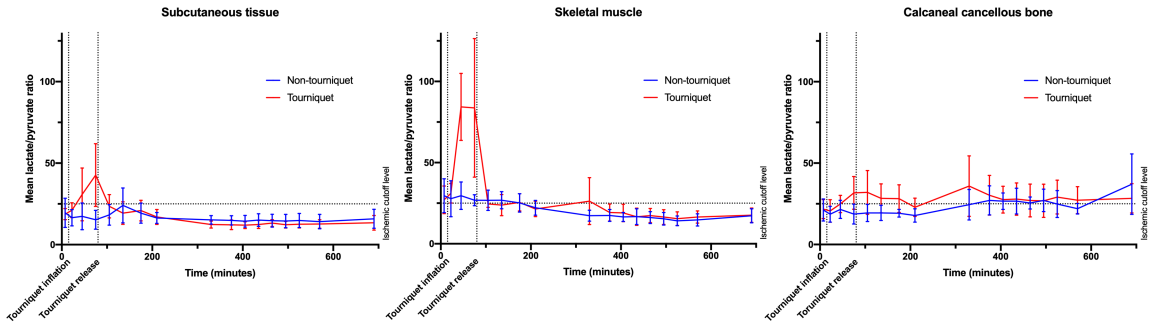

Figure 3. The mean lactate/pyruvate ratios for both the tourniquet-exposed and non-tourniquet-exposed legs. Tourniquet inflation time: 15 minutes, mean (range) tourniquet release time: 85 (73; 92) minutes (both are marked with vertical dotted lines). The ischemic cutoff level of 25 is marked with horizontal dotted lines. Bars represent the 95% CI.

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11.5 Co-authorship declarations

AARHUS UNIVERSITY

Declaration of co-authorship concerning article for PhD dissertations

Full name of the PhD student: Pelle Emil Hanberg

This declaration concerns the following article/manuscript:

Title:	Simultaneous Retrodialysis by Drug for Cefuroxime Using Meropenem as an Internal Standard-A Microdialysis Validation Study
Authors:	Hanberg P, Bue M, Öbrink-Hansen K, Kabel J, Thomassen M, Tøttrup M, Søballe K, Stilling M

The article/manuscript is: Published ☒ Accepted ☐ Submitted ☐ In preperation ☐

If published, state full reference: J Pharm Sci. 2020 Mar;109(3):1373–1379. doi: 10.1016/j.xphs.2019.11.014. Epub 2019 Nov 20

If accepted or submitted, state journal:

Has the article/manuscript previously been used in other PhD or doctoral dissertations?

No ☒ Yes ☐ If yes, give details:

Your contribution

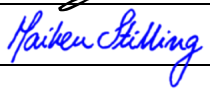
Please rate (A-F) your contribution to the elements of this article/manuscript, **and** elaborate on your rating in the free text section below.

A. Has essentially done all the work (>90%)
B. Has done most of the work (67-90 %)
C. Has contributed considerably (34-66 %)
D. Has contributed (10-33 %)
E. No or little contribution (<10%)
F. N/A

Category of contribution	Extent (A-F)
The conception or design of the work:	B
Free text description of PhD student's contribution ***(mandatory)*** This study concept was designed in coorporation with supervisors.	
The acquisition, analysis, or interpretation of data:	A
Free text description of PhD student's contribution ***(mandatory)*** The PhD student collected, analysed and intepreted the data with little help.	
Drafting the manuscript:	A
Free text description of PhD student's contribution ***(mandatory)*** The first draft of the manuscripet was written by the PhD student without any help.	
Submission process including revisions:	B

Free text description of PhD student's contribution ***(mandatory)***
The manuscript was revised by all co-authors prior to submission. The PhD student was responsible for the submission process.

Signatures of first- and last author, and main supervisor

Date	Name	Signature
25/1-21	Pelle Hanberg (first author)	
25/1-21	Maiken Stilling (last author and main supervisor)	

Date: 25/1-21

Signature of the PhD student

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Full name of the PhD student: Pelle Emil Hanberg

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Title:	Timing of Antimicrobial Prophylaxis and Tourniquet inflation - A Randomized Controlled Microdialysis Study
Authors:	Hanberg P, Bue M, Öbrink-Hansen K, Thomassen M, Søballe K, Stilling M

The article/manuscript is: Published ☒ Accepted ☐ Submitted ☐ In preperation ☐

If published, state full reference: J Bone Joint Surg Am. 2020 Aug 4. doi: 10.2106/JBJS.20.00076. Online ahead of print

If accepted or submitted, state journal:

Has the article/manuscript previously been used in other PhD or doctoral dissertations?

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The acquisition, analysis, or interpretation of data:	B
Free text description of PhD student's contribution ***(mandatory)*** The PhD student collected, analysed and intepreted the data with some help to handle the animals and with the analysis of the antibiotic concentrations.	
Drafting the manuscript:	A
Free text description of PhD student's contribution ***(mandatory)*** The first draft of the manuscript was written by the PhD student without any help.	
Submission process including revisions:	B

Free text description of PhD student's contribution ***(mandatory)***
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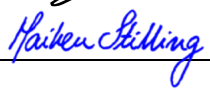
25/1-21	Maiken Stilling (last author and main supervisor)	

Date: 25/1-21

Signature of the PhD student

AARHUS UNIVERSITY

Declaration of co-authorship concerning article for PhD dissertations

Full name of the PhD student: Pelle Emil Hanberg

This declaration concerns the following article/manuscript:

Title:	Effects of tourniquet inflation on peri- and post operative cefuroxime concentrations in bone and tissue
Authors:	Hanberg P, Bue M, Kabel J, Jørgensen AR, Jessen C, Søballe K, Stilling M

The article/manuscript is: Published ☐ Accepted ☐ Submitted ☒ In preperation ☐

If published, state full reference:

If accepted or submitted, state journal: Acta Orthopaedica

Has the article/manuscript previously been used in other PhD or doctoral dissertations?

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Signature of the PhD student

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Declaration of co-authorship concerning article for PhD dissertations

Full name of the PhD student: Pelle Emil Hanberg

This declaration concerns the following article/manuscript:

Title:	Tourniquet Induced Ischemia and Reperfusion in Subcutaneous Tissue, Skeletal Muscle, and Calcaneal Cancellous Bone
Authors:	Hanberg P, Bue M, Kabel J, Jørgensen AR, Søballe K, Stilling M

The article/manuscript is: Published ☐ Accepted ☒ Submitted ☐ In preperation ☐

If published, state full reference:

If accepted or submitted, state journal: Acta Pathologica et Microbiologica Scandinavica

Has the article/manuscript previously been used in other PhD or doctoral dissertations?

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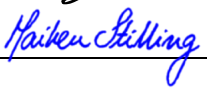
25/1-21	Maiken Stilling (last author and main supervisor)	

Date: 25/1-21

Signature of the PhD student