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Intracellular uropathogenic *Escherichia coli* are undetectable in urinary bladders after oral mecillinam treatment: An experimental study in a pig model of cystitis

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1. Introduction

Urinary tract infection (UTI) in humans is often followed by relapse within 2–6 months [1]. Historically, the assumption has been that recurrent UTIs (rUTIs) are caused by *de novo* inoculations with bacteria from an intestinal reservoir. In later years this view has been challenged...
by research documenting epithelial invasion and intracellular colonization by uropathogenic *Escherichia coli* (UPEC) during cystitis in mice and in human cell-culture based assays [2]. In these studies, intracellular UPEC survive antibiotic treatment, and it is hypothesized that UPEC in this niche serve as reservoirs for rUTIs as the intracellular bacteria can break out and re-infect the bladder [3]. Although microscopy studies have shown intracellularly colonized exfoliated cells in urine specimens from patients with UTI, conclusive evidence that supports a similar pathogenesis in humans has been difficult to obtain [4,5].

To come closer to answer this important question, we investigated UPEC persistence in a porcine model of cystitis after oral treatment with the extracellular antibiotic mecillinam: a drug that has previously been used to document the intracellular pathogenesis in mice [3]. Compared to mice, pigs are naturally susceptible to *Escherichia coli* UTI and likely more accurately reflect the course of infection in humans [6–10]. Moreover, pigs are generally considered a reliable model for human oral drug bioavailability and renal clearance due to the high similarity to humans in terms of gastrointestinal- and renal anatomy and function [11]. The aim of this study was first, to investigate if standard mecillinam treatment effectively clears urine- and bladder tissue-associated *Escherichia coli* persistence in a porcine model of cystitis after oral treatment with mecillinam. Moreover, pigs are generally considered a reliable model for human oral drug bioavailability and renal clearance due to the high similarity to humans in terms of gastrointestinal- and renal anatomy and function [11]. The aim of this study was first, to investigate if standard mecillinam treatment effectively clears urine- and bladder tissue-associated *Escherichia coli* persistence in a porcine model of cystitis after oral treatment with mecillinam. Moreover, pigs are generally considered a reliable model for human oral drug bioavailability and renal clearance due to the high similarity to humans in terms of gastrointestinal- and renal anatomy and function [11]. The aim of this study was first, to investigate if standard mecillinam treatment effectively clears urine- and bladder tissue-associated *Escherichia coli* persistence in a porcine model of cystitis after oral treatment with mecillinam. Moreover, pigs are generally considered a reliable model for human oral drug bioavailability and renal clearance due to the high similarity to humans in terms of gastrointestinal- and renal anatomy and function [11]. The aim of this study was first, to investigate if standard mecillinam treatment effectively clears urine- and bladder tissue-associated *Escherichia coli* persistence in a porcine model of cystitis after oral treatment with mecillinam. Moreover, pigs are generally considered a reliable model for human oral drug bioavailability and renal clearance due to the high similarity to humans in terms of gastrointestinal- and renal anatomy and function [11].

## 2. Materials and methods

### 2.1. Animals

Nine female pigs (Landrace x Yorkshire, mix) were purchased from a vendor with red SPF-X status, the highest health status according to the Danish SPF system (Kokkenborg, ApS) and acclimatized for 7 days. Female pigs were chosen, as male pigs are troublesome to catheterize through the penile opening, due to the shape of the penis and a preputial diverticulum. The average weight when experiments began was 45 kg (range 43–48 kg). The animals were grouped housed in 3 m² enclosures with sawdust bedding and enrichment in the form of various toys, music and daily human interaction. The pigs were given a standard feed and free access to water and attended at least twice daily. Experiments were approved by the Danish Animal Experiments Inspectorate, license number: 2017-15-0201-01271.

### 2.2. Bacteria and inoculum preparation

To infect the pigs, we used the *Escherichia coli* cystitis isolate UTI89, a prototypical strain used previously in cell culture and murine UTI model studies by us and others, and lately also in a pig model of cystitis [7,9,12,13]. To prepare the inoculum, 25 mL of Lysogeny broth (LB) was inoculated with a single colony from an overnight (ON) agar plate-culture and incubated for 24 h. From the broth culture, 25 μL were used to inoculate a new LB of 25 mL and incubated 24 h. On the day of the infection, the LB was centrifuged at 2500 g for 20 min and the bacterial pellet resuspended in saline and adjusted to an optical density of 1.0 at 600 nm. This suspension was diluted 1:1000 to reach a final inoculum of 10<sup>6</sup> CFU·mL<sup>-1</sup> which was verified by plating (mean 2.75 · 10<sup>6</sup> CFU·mL<sup>-1</sup>, range: 1.5 to 4.5 · 10<sup>6</sup> CFU·mL<sup>-1</sup>). For the experiment using the green fluorescence protein (GFP) expressing *E. coli* we used the UTI89pMAN01 previously constructed by transforming UTI89 with the pMAN01 plasmid which contains a constitutively active promoter driving gfp expression [14]. UTI89pMAN01 was cultured as above but in LB containing 30 μg·mL·<sup>-1</sup> chloramphenicol to maintain the pMAN01 plasmid.

### 2.3. Anesthesia

Pigs were pre-medicated with medetomidine 0.12 mg kg<sup>-1</sup> (Cepetor), butorphanol (Butomidor 0.2 mg·kg<sup>-1</sup>) and Midazolam (Midazolam 0.1 mg·kg<sup>-1</sup>). Anesthesia was induced and maintained on propofol 10 mg·mL<sup>-1</sup>. After the procedure, the pigs were immediately given intramuscular antisedan (0.2 mg·kg<sup>-1</sup>) to reverse the α2-adrenergic effects of medetomidine.

### 2.4. Porcine UTI model and mecillinam treatment

Seven pigs were catheterized with a charrière 10 Foley catheter (Rüsch) and the bladder emptied and inoculated with 100 mL bacterial suspension. Hereafter, the catheter was pinched for 1 h to avoid immediate voiding of the inoculum and ensure consistent and equal inoculation between pigs. Then the bladders were emptied, catheters were removed, and the pigs returned to their stables. In three of the animals, 6 biopsies were collected with a Storz C-VIEW video cystoscope immediately before inoculation and again 24 h post infection to induce damage to the urothelium. One pig (from the control group) developed sepsis and was euthanized on the day of the infection.

At 5 days post infection (dpi), an oral treatment was initiated consisting of 400 mg pivmecillinam (Selexid, Leo Pharma), three times a day for three consecutive days, corresponding to the recommended oral treatment for humans with acute uncomplicated UTI according to Danish clinical guidelines. The tablets were concealed inside banana pieces and the pigs were trained beforehand to eat the banana pieces readily. After the treatment, i.e. at 8 dpi, pigs were euthanized with pentobarbital and the urinary bladders were aseptically removed within 5 min. The bladders were immediately transferred to a laminar airflow cabinet, opened by cutting between the ureters and gently washed in sterile PBS. The bladders were then divided into smaller pieces by punching out a total of 12 round-shaped specimens using a drive-punch (Ø = 10 mm), corresponding to approximately half of the bladder surface. From each bladder, six tissue specimens were left untreated, whereas 3 were incubated for 1 h in DMEM supplemented with 10% fetal bovine serum and 2 μg·mL·<sup>-1</sup> ciprofloxacin, an intracellularly active antibiotic, to kill potential intracellular bacteria or with the extracellular drug gentamicin (400 μg·mL·<sup>-1</sup>) as control. Following incubation all tissue specimens were washed thoroughly in sterile PBS and homogenized in 5 mL PBS using a rod disperser at 22,000 rpm (IKA Ultra-Turrax T25) and plated on LB agar (SSI Diagnostica) with a limit of detection of 50 CFU·mL·<sup>-1</sup>. After plating, remaining bladder-tissue homogenates and urine specimens were sterile filtered and inoculated with UTI89, incubated ON and plated to assess if any traces of mecillinam would inhibit UTI89 growth.

### 2.5. Urine collection and bacterial quantification

Urine specimens were collected at baseline, 1, 5, 6 and 8 dpi to monitor the infection and treatment outcome. The urine sample at 8 dpi was taken >12 h after the last antibiotic dosage given the night before to assure that no antibiotic was left in the urine. Urine samples were collected by bladder catheter and bacterial quantification was performed by plating urine aliquots on LB agar. Specimens below the limit of detection (10 CFU·mL<sup>-1</sup>) is referred to as sterile. Urine specific gravity (urine density) was measured by a digital refractometer (UG-a, Atago).

### 2.6. Determination of urine mecillinam concentration

Urine samples were collected after the first antibiotic dose on the 2nd day of treatment (i.e., 6 dpi) to determine the biologically active concentrations of mecillinam in the urine. To do so, 200 μL of the urine sample was added to the first well of 96 well plate and from this a series of 2-fold dilutions were made in 100 μL of Müller Hinton II (MH) bouillon (SSI Diagnostica). A bacterial suspension was prepared by suspending an ON UTI89 agar plate-colony in sterile NaCl and adjusting to McFarland 0.5. From this suspension, 100 μL was transferred to 9.9 mL of MH bouillon to make the inoculum. Each well was inoculated with
100 μL of bacterial inoculum and incubated ON at 35 °C. The first well without bacterial growth represents the minimum inhibitory concentration (MIC). Using the same assay with known concentrations of mecillinam in MH bouillon, the MIC of UTI89 was determined to be 0.03 μg·mL⁻¹. The urine mecillinam concentration was calculated by multiplying the MIC with the number of serial dilutions before reaching the MIC in urine samples.

2.7. Blood collection and analysis

Blood samples were collected from the jugular vein under sedation at baseline, 1, 5, 6 and 8 dpi. Whole blood samples were analyzed in a SCIL Vet animal blood counter (ABX diagnostics, France). Subsequently, plasma was isolated and stored at −80 °C for further analysis (see below).

2.8. Whole-cell ELISA

To assess if bladder colonization had been recognized by the immune system, a whole cell ELISA was performed to measure the potential presence of antibodies specific for UTI89. Microtiter plates were coated with 100 μL per well of 1 μg·mL⁻¹ poly-γ-L-lysine (Sigma) for 2 h. Plates were washed and 100 μL bacterial suspension of UTI89 adjusted to OD600 = 0.2 was added to each well. Plates where then centrifuged at 900 g for 20 min. The bacteria were fixed by adding 0.2% formaldehyde for 20 min. The plates where then washed and blocked with 10% foetal bovine serum-PBS for 1 h. After removing the blocking buffer wells were loaded with 100 μL of 2-fold serial dilutions of standards (made by pooled porcine plasma from 9 infected pigs and assigned a value of 1000 μL of bacterial inoculum and incubated ON at 35 °C. The first well without bacterial growth represents the minimum inhibitory concentration (MIC). Using the same assay with known concentrations of mecillinam in MH bouillon, the MIC of UTI89 was determined to be 0.03 μg·mL⁻¹). The urine mecillinam concentration was calculated by multiplying the MIC with the number of serial dilutions before reaching the MIC in urine samples.

2.9. GFP-expressing UTI89 and confocal laser scanning microscopy

To investigate the tissue-associated localization of UPEC during the acute stages of UTI, two additional pigs were inoculated as described above with 100 mL of UTI89pMAN01 in saline (1·10⁸ CFU·mL⁻¹). The pigs were euthanized after 12 h and the bladders prepared for microscopy as described previously for mouse bladders with some exceptions [14]. In short, whole bladders were removed and splayed onto a silicone support followed by immersion in neutral buffered formalin solution where they were left ON at 4 °C. Prior to microscopy, 20 × 40 mm pieces were cut-out and mounted on microscope slides. The samples were either inspected directly with CLSM for visualization of UTI89pMAN01 or additionally stained: Samples were washed in PBS and bladder cell membranes permeabilized for 5 min with 0.5% Triton X-100 (Sigma-Aldrich). The tissue specimens were then stained for 30 min at room temperature with 100 nM Acti-stain 555 phalloidin (Cytoskeleton, Inc.). CLSM was conducted using an Olympus FX1000MPE CLSM equipped with and an Olympus UPlanSapo 20 × /0.85 objective. Images were captured using the Olympus FX10–ASW software.

2.10. Statistical analysis

Statistical analysis was performed with GraphPad Prism 8 software version 8.9.1. Comparisons between two groups were performed with paired Wilcoxon signed rank test and comparisons between three or more groups were performed with One-Way ANOVA and Tukey’s multiple comparisons test.

3. Results

3.1. In vivo beta-lactam treatment study

3.1.1. Oral mecillinam eliminates UPEC from the urine of infected pigs

The experimental porcine infection protocol is summarized in Fig. 1. Seven pigs were inoculated through a transurethral bladder catheter with UTI89 to induce cystitis. Since urothelial damage and complement opsonization have been suggested to influence the invasive capacity of UPEC, the animals were divided into a control group (n = 4) and a biopsy group (n = 3): the latter in which the bladders were biopsied immediately prior to inoculation and 24 h after to induce damage to the urothelium with the purpose of promoting intracellular colonization [15,16]. At 5 dpi, oral treatment was initiated consisting of 400 mg mecillinam three times per day for three consecutive days. Urine samples were collected sparingly to minimize the risk of influencing the course of infection by frequent catheterization.

At baseline, i.e., before inoculation, all pigs had sterile urine. At 1 dpi, all pigs had developed bacteriuria with titers above 10⁹ CFU·mL⁻¹ and this level of bacteriuria persisted until 5 dpi when mecillinam treatment was initiated (Fig. 2A). Treatment with mecillinam resulted in sterile urine in all pigs after 24 h, and mecillinam concentrations in urine samples at this timepoint was determined to be averagely 12.3 mg·L⁻¹ (Range: 1–64), corresponding to a minimum of 32x MIC. At 8 dpi, 12 h after the last dose of mecillinam, urine remained sterile, indicating that mecillinam had effectively cleared the infection. The mean urine specific gravity was 1.0096 (SD: 0.0067).

During the course of infection, no significant change was observed in white blood cells or differential count (Fig. 2C and D), suggesting localized disease. All pigs showed increased levels of serum anti-UTI89 IgG at 8 dpi compared to 0 dpi (p = 0.03) measured by whole-cell ELISA, suggesting that the pigs have responded to the infection by raising pathogen-specific antibodies (Fig. 2B). This indicates that the observed bacteriuria represents symptomatic UTI, as previous studies in this animal have shown that asymptomatic colonization was not correlated with increased IgG [9].

3.1.2. Viable UPEC are undetectable in the bladders of mecillinam-treated pigs

To investigate the presence of intracellular UPEC in the tissue we used a well-established protocol of homogenizing whole-bladders which is the methodological approach used in murine studies as well [3,14].

Our results showed that no viable bacteria could be detected from any of the tissue-samples. To confirm that the absence of viable bacteria was not a result of remaining traces of mecillinam that may kill bacteria released from intracellular niches upon homogenization, all urine samples and aliquots of bladder homogenates were inoculated with UTI89. After incubation ON, bacterial growth was observed in all samples.

3.2. Microscopy analysis of pig bladders infected with GFP-expressing UTI89

3.2.1. Intracellular bacterial communities and bacterial filaments were not detected in the pig bladders

During murine UTI, UPEC can proliferate inside the superficial epithelial cells forming distinct bacterial aggregates called intracellular bacterial communities (IBC). When the IBCs eventually burst, the bacteria are released as long filamentous phenotypes which are suggested to promote adhesion by increasing the surface area and thus adhesion-receptor interactions [17]. To investigate the localization of UPEC during acute infection in the pigs and, specifically, to reveal whether the hallmarks of the intracellular uropathogenesis cascade characterized in mice, i.e., intracellular bacterial communities (IBC) and filamentous bacteria, could be identified in the pigs, a separate experiment was conducted in which two pigs were infected with
GFP-tagged UTI89 (UTI89pMAN01) and bladders investigated by microscopy. To reflect earlier experiments in mice, a higher inoculum of $10^8$ CFU mL$^{-1}$ in 100 mL was used and the pigs were terminated after 12 h: at this timepoint IBC's are at the most pronounced stage in mice and can be easily identified by fluorescence microscopy [14, 17]. The pig bladders were splayed and their whole area meticulously screened for IBC's using epifluorescence microscopy and by personnel experienced in identifying IBC's in mice. In areas of high bacterial densities, CLMS z-stacks were recorded and analyzed to reveal the potential presence of deeply located colonies. While bacteria were present in high numbers on the bladder surface, they were exclusively rod shaped and no IBCs were detected in the pig bladders (Figs. 3 and 4). While these hallmarks of infection in mice were not identified at the 12-h time point in the pig, the CLSM analysis did reveal individual bacteria that might reside intra-cellularly (Fig. 4D).

**Fig. 1.** Flowchart of the pig experiments. One animal in the control group was euthanized due to symptoms of septicemia.

**Fig. 2.** A. Pigs were divided into a control group (n = 4) and a biopsy group (n = 3), the latter in which 6 bladder biopsies were collected immediately before inoculation and at 1 dpi to induce damage to the urothelium. All animals developed bacteriuria above $10^3$ CFU mL$^{-1}$ in response to inoculation with UTI89. Bacteriuria was still present at 5 dpi, but could not be detected after the onset of mecillinam-treatment. B. Anti-UTI89 IgG in the pig’s plasma was increased at 8 dpi in all pigs compared to baseline, p = 0.03, paired Wilcoxon signed rank test. C. No significant difference in total WBC (blood) or (D) differential count was detected in response to inoculation, One-way ANOVA. CFU, colony forming units; WBC, white blood cells; Gra, granulocytes; Lym, lymphocytes; Mon, monocytes.
In this study, we treated infected pigs with the extracellularly active β-lactam antibiotic, mecillinam, a first-line treatment of UTI in Scandinavian countries, to evaluate whether such a treatment completely eradicates UPEC from the bladder, or if the bacteria are able to survive such a treatment in bladder tissue-embedded niches, similar to what has been documented in mice [3]. Orally administered mecillinam was detected in urine at levels above MIC and effectively cleared the infection in all treated pigs. The urine mecillinam concentrations in the pigs were similar to studies in human volunteers after consumption of the same drug, demonstrating the pig as an appropriate model for oral antibiotic bioavailability [18]. Previous studies using identical inoculation procedures have shown persistent bladder infection in pigs untreated with antibiotics for at least 14 days, supporting that the result in this study is not a result of spontaneous clearing of the infection [7,9]. We found that none of the pigs, including animals biopsied to expose the underlying epithelium, contained persistent tissue-associated reservoirs of UTI89 at detectable levels after treatment with mecillinam.

In a separate experiment in two pigs we analyzed the potential presence of intracellular bacterial communities (IBCs) and bacterial filaments, which are associated with the intracellular infection cascade observed in mice [2,17]. Neither of these hallmarks of acute murine UTI were found in the pig bladders. Although only two pigs were analyzed and only at the 12-h time point, the absence of these characteristics indicate differences in the UTI pathogenesis pathway in these two animal species. Although bacterial filaments are absent in most urine samples from human UTI patients, they have been occasionally observed [19]. The exact causality of UPEC filaments in humans, however, is not clear but may be induced in urine of high osmolality as demonstrated previously [20]. Consequently, the relatively low osmolality of pig urine, which is similar to humans, but below the range of mice, may explain why UPEC filaments were not observed in this study [9]. In the murine bladder urine where osmolality is much higher, considerable filamentation is typically present owing to altered or arrested cell-division [14,17,21]. Since β-lactams such as mecillinam mainly target dividing bacteria, UPEC growing in the murine bladder may be less susceptible to this drug, which could be another explanation for UPEC surviving in mice but not in the current pig model [22].

In human studies, intracellular bacteria have been observed in exfoliated bladder epithelial cells in urine from women and children suffering from acute UTI [19,23,24]. Although exfoliated cells are not representative of the viable urothelium, these observations indicate that epithelial invasion may take place at some time point during the initial stages of UTI in humans but yields no information on the persistence of such intracellular bacteria and their potential role in antibiotic survival.

![Fig. 3. Microscopy analysis of two pig bladders infected with UTI89pMAN01. At 12 h post infection, bladders were removed, splayed, fixed, and cut into approximately 20 × 40mm pieces. These pieces were systematically scanned for bacterial location using an epifluorescence microscope equipped with a motorized stage (A) and subsequently by confocal laser scanning microscopy (B–D). UTI89pMAN01 were mainly found in inflamed regions, where the bacteria were located near cell junctions (B) or together with clusters of exfoliated cells (C–D). Green colors are UTI89pMAN01; red color is background laser signal indicating bladder tissue (B–D). Scalebar: 10 μm.](image-a)

![Fig. 4. Microscopy analysis of the deeper location of bacteria in pigs infected with UTI89pMAN01. Confocal laser scanning microscopy of bladders after staining with Acti-stain 555 phalloidin (Cytoskeleton, Inc.) visualizes the large superficial umbrella cells (A, white arrow) and deeper cells of the transitional epithelium (A, yellow arrow). Bacteria were mainly associated to the bladder epithelial cell-surface (B) or junctional borders between cells (C, D, white arrows). Occasionally, single bacteria were observed surrounded by actin seemingly within the cytoplasm of epithelial cells, indicating endocytosed bacteria (D, yellow arrows). Most bacteria were located in protected niches such as in epithelial pockets or folds (E). Scalebar: 100 μm.](image-b)
and rUTI. It has been suggested that damaged or sensitized mucosa from rUTI episodes may predispose to more severe UTI and perhaps more intracellular colonization, and thus, experimental animal models, like the one used in this study in which naïve pigs are used, may not accurately reflect the mucosal susceptibility of certain patients [16,25]. However, in murine models, where IBCs are extensively observed, naïve animals are also used.

Based on this study, we cannot rule out that IBC-formation may have happened transitory, at some point during the infection. The 12-h timepoint where IBCs are extensively observed in mice, may be different in pigs or humans where intracellular colonization may take longer as a result of thicker urothelial- and mucus barrier and thus may have been missed in the current study. However, the eradication of planktonic and tissue associated UPEC to below detectable levels after three days of oral mecillinam treatment, as opposed to findings in similarly performed experiments in mice, indicates that potential IBCs formed up until the initiation of mecillinam treatment, did not lead to bacterial persistence that could survive the CLSM treatment.

Although IBCs were undetected in the CLSM analysis of infected pig bladders, we could not rule out the occasional presence of individual bacteria inside the cytoplasm of the epithelial cells. The individual cases of possibly endocytosed bacteria, which were very few in numbers, could represent part of a natural defensive mechanism against invading microorganisms more so than early-stage IBCs. The finding, however, is in accordance with our previous studies using the porcine UTI-model, where low numbers of tissue-associated UPEC remained viable in bladders after 90 min ex vivo gentamicin treatment [8,9]. Alternatively, as discussed in these earlier studies, identified bacteria may have been biofilm-associated or mucus embedded-rather-than intracellular, that might be able to survive short term ex vivo treatment.

This study has the following weaknesses that limits the extent to which solid conclusions can be drawn from the study. Since we only report negative findings, our study lacks a proper positive control to verify the sensitivity of our methods to detect potential intracellular bacteria. Although, a control experiment in mice would more clearly demonstrate major differences in the pathogenesis of these two species, a direct back-to-back comparison would inevitably be associated with a high degree of uncertainties because critical methodological steps used in the pig model, such as inoculation, urine collection and oral treatment is not easily performed in mice. We used growth conditions and inoculum identical to our previous study in mice to provide optimal conditions for detecting IBCs and filamentous bacteria, as these are easily identified in murine bladders under these conditions [14]. Finally, we report only on a single strain, UTI89, which has been the major model organism in the investigation of UPEC intracellular persistence in mice and other model systems. However, the ability to make general conclusions regarding UPEC pathogenesis is limited by using only one strain.

In conclusion, this pilot study demonstrates the efficacy of mecillinam for treating UTI caused by UPEC but raises questions about the extent to which the intracellular uropathogenic cascade as observed in mice can be transferred to larger mammals and possibly humans with respect to its timing and significance. Our results suggest that more studies are needed in large animal models, preferably in direct comparison to the mouse, to obtain deeper knowledge about the E. coli UTI pathogenesis in humans and in particular the mechanisms that underlie the frequent UTI recurrences with this pathogen.

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**CRediT authorship contribution statement**

**Kristian Stærk:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Rasmus Birkholm Grønnesmo:** Writing – review & editing, Resources, Methodology, Investigation. **Yaseelan Palarasah:** Writing – review & editing, Methodology, Investigation. **Lars Lund:** Writing – review & editing, Resources. **Thomas Emil Andersen:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

**Declaration of competing interest**

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Thomas Emil Andersen reports financial support was provided by Coloplast

**Data availability**

Data will be made available on request.

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**References**


