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Original article

# Biofilm formation on endovascular aneurysm repair (EVAR) grafts—a proof of concept *in vitro* model

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ABSTRACT

*Objectives:* An endovascular aneurysm repair (EVAR) graft is a catheter-implanted vascular prosthesis and is the preferred treatment for patients with aortic aneurysm. If an EVAR graft becomes the focus of infection, the treatment possibilities are limited because it is technically difficult to remove the graft to obtain source control. This study examines whether *Pseudomonas aeruginosa* and *Staphylococcus aureus* form biofilm on EVAR prostheses.

*Methods:* EVAR graft sections were exposed to bacteria at $10^2$ or $10^8$ colony forming units (CFU)/mL in lysogeny broth and Krebs-Ringer at 37°C, bacterial biofilm formation was evaluated by scanning electron microscopy and counting CFU on the graft sections after antibiotic exposure at × 10 minimal inhibitory concentration. Bacteria were tested for tolerance to benzylpenicillin, tobramycin, and ciprofloxacin.

*Results:* Bacterial exposure for 15 minutes established biofilms on all prosthesis fragments (6/6 replicates). After 4 hours, bacteria were firmly attached to the EVAR prostheses and resisted washing. After 18–24 hours, the median CFU/g of EVAR graft reached $5.2 \times 10^8$ ($1.15 \times 10^8$–$1.1 \times 10^9$) for *S. aureus* and $9.1 \times 10^7$ ($3.5 \times 10^7$–$6.25 \times 10^8$) for *P. aeruginosa*. Scanning electron microscopy showed bacterial attachment to the graft pieces. There was a time-dependent development of tolerance with approximately 20 (tobramycin), 560 (benzylpenicillin), and 600 (ciprofloxacin) times more *S. aureus* surviving antibiotic exposure in 24- compared with 0-hour-old biofilm. Five (tobramycin) and 170 times (ciprofloxacin) more *P. aeruginosa* survived antibiotic exposure in 24- compared with 0-hour-old biofilms.

*Discussion:* Our results show that bacteria can rapidly adhere to and subsequently form antibiotic-tolerant biofilms on EVAR graft material in concentrations equivalent to levels seen in transient bacteraemia *in vivo*. Potentially, the system can be used for identifying optimal treatment combinations for infected EVAR prosthesis. **Torgny Sunnerhagen, Clin Microbiol Infect 2023;▪:1**



## Introduction

Endovascular aneurysm repair (EVAR) is recommended for many cases of abdominal aortic aneurysms and dissections. EVAR graft implant increases with the incidence of abdominal aortic dissections and aneurysm ruptures being up to 70 per 100 000 person-years and EVAR being used more frequently over time [1–3]. As the number of patients with EVAR graft implants increases and with EVAR also being used in the management of infected aortic aneurysms, infections of these grafts are becoming a recurring and increasing problem. This important clinical problem is insufficiently studied, with only sparsely documented effective solutions investigated.

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Studies indicate that infections after EVAR implantation often happen during the first 2 years post-implantation and are often preceded by bacteraemia [4–9]. Although bacteraemia often precedes an EVAR-related infection, it is not always clear whether the present bacteraemia causes the EVAR graft infection or originates from an already infected prosthesis. The treatment of endovascular graft infections (EVAR and non-EVAR) is difficult, because surgical removal of the grafts has a high morbidity and mortality, and antibiotic treatment alone rarely leads to bacterial eradication, often necessitating life-long suppressive antibiotics. The reason for this difficulty of treating the infections with antibiotics is believed to be because of biofilm formation on the EVAR grafts, leading to antibiotic tolerance and challenges for the immune system to clear the infection. Treatments used are often based on beta-lactams, with additions of quinolones, aminoglycosides, and lincosamides being common [5,10–15].

The microbiology of EVAR graft infections has not been extensively studied. The data that exist support Gram-positive bacteria being the most common, with *Staphylococcus aureus* as well as coagulase-negative *Staphylococcus* being the most common of those, and with *Pseudomonas aeruginosa* being found among the Gram-negative bacteria isolated. This is similar to what is seen in native and mycotic aortic aneurysms for the Gram-positive bacteria, whereas Gram-negative rods such as *P. aeruginosa* are more often observed with endovascular prostheses [16,17].

The purpose of this study is to investigate if biofilms actually form on endovascular prosthesis using two of the most frequent pathogens reported from these infections—*S. aureus* as the most frequent Gram-positive pathogen identified and *P. aeruginosa* as the Gram-negative pathogen with extra challenges of high intrinsic antibiotic resistance and ability to further develop antibiotic resistance, not the least in a biofilm setting. Although this is a proof-of-concept study, we aim to establish a model for identifying the most effective treatment strategies against these difficult-to-treat infections.

## Materials and methods

### *Preparing assay medium*

Bacteria were grown in a medium consisting of 50% Krebs-Ringer solution (SSC Panum, Copenhagen, Denmark; containing 0.2% [5.2 mM] D-Glucose [Merck, Darmstadt, GER]), and 50% Lysogeny broth (SSC Panum, Copenhagen, Denmark).

### *Bacterial preparation*

The strains *Staphylococcus aureus* NCTC 8325-4 and *Pseudomonas aeruginosa* PAO1 were used, both without acquired antibiotic resistance. The minimal inhibitory concentrations for ciprofloxacin, tobramycin and (for *S. aureus*) penicillin were confirmed with Etests (Biomérieux, Ballerup, Denmark). The bacteria were incubated overnight, shaking 85 rounds per minute (similar to previous studies [18,19]), at 37°C, and subsequently diluted in the assay medium. These settings were used for all incubations.

### *Preparation of antibiotics*

Ciprofloxacin (Fresenius Kabi, Uppsala, Sweden), tobramycin (Eurocept international, Ankeveen, Netherlands), and penicillin (Panpharma, Luitré, France) were diluted in assay medium to a concentration of ten times the minimal inhibitory concentration (MIC) of the respective bacterial strain, the concentration chosen to ensure killing of planktonic growing bacteria but also to obtain anti-biofilm effect. This concentration remains within peak concentrations obtained in humans.

### *EVAR graft biofilm model*

To produce graft pieces of suitable size, EVAR prostheses (Zenith Flex, Cook Medical, Bloomington, IN, USA) were cut into pieces of approximately 0.1 g (approximately 1 × 2 cm) using surgical wire cutters (DP512R B. Braun Medical AB, Danderyd, Sweden), in a biological safety cabinet to minimize contamination. The EVAR grafts are made of steel wire, with a polyester mesh fastened to it by sutures. The weight of the pieces was recorded to enable compensation for slight differences of size. The pieces were placed in 6-well plates (TPP AG, Trasadingen, Switzerland) and 7 mL of assay medium was added to each well.

To study the general growth characteristics of bacteria on the graft pieces, overnight culture of the bacterium was diluted in Krebs-Ringer, and 70 μL was added to the EVAR piece in the well, achieving a final well-concentration of $10^2$ colony forming units (CFUs) per mL [18,19]. Culturing was used to measure the starting concentration. The 6-well plate with graft pieces and bacteria was then incubated for 15 minutes to achieve adhesion to the graft pieces, after which the pieces were washed once is saline and moved to new assay medium for continued incubation. To measure the CFU on the graft pieces, they were removed from the medium, dipped three times in sterile 0.9% saline to remove bacterial sediment not attached to the graft pieces [18,19], and placed in sterile 50 mL Falcon tubes. The tubes were filled with sterile 0.9% saline to the 50 mL marking and sonicated using a Branson 5210 sonicator (Branson Ultrasonics, USA) to dislodge attached bacteria. The tubes were centrifuged (10 minutes, 3720 G) to concentrate dislodged bacteria in the bottom. The 45 topmost millilitres were then carefully removed by pipetting. The remaining solution including the graft piece was vortexed, and the suspension diluted in a dilution series, 100 μL was plated on blood agar plates and incubated. The colonies were counted the next day to determine CFU/mL in the solution, and the value was adjusted for the weight of the EVAR graft pieces to produce CFU/g.

To test the time dependence of bacterial attachment to EVAR graft pieces with a bacterial concentration similar to bacteraemia [20,21], the pieces were challenged with $10^2$ CFU/mL of bacteria. After 5, 15, 30, or 60 minutes, the pieces were removed from the assay medium, washed once in saline, and transferred to new assay medium. The presence or absence of growth was then assessed after 24 hours of incubation. This was done with graft pieces consisting of both metal, polyester mesh, and the sutures fastening the mesh to the metal, and with pieces taken from the end of the grafts, consisting of only metal.

### *Antibiotic exposure assay*

Determination of antibiotic tolerance of the bacteria attached to the graft pieces was prepared by adding $10^8$ CFU/mL bacteria to the pieces in assay medium. After a 15-minute attachment phase, the pieces were moved to new assay medium after being washed once in saline. After 0, 4, 18, or 24 hours of incubation in the new assay medium, the pieces were washed once in saline and transferred to new assay medium with antibiotic at 10 × MIC and incubated for 24 hours. The pieces were then washed thrice in saline and sonicated. The solution was plated on blood agar, and the CFU/g was determined.

### *Scanning electron microscopy*

Scanning electron microscopy (SEM) was performed at the Core Facility for Integrated Microscopy (CFIM) at the Faculty of Health and Medical Science, University of Copenhagen. Bacteria at a concentration of $10^8$ CFU/mL were added to EVAR pieces as described above, incubated according to the protocol, and washed three times in saline.

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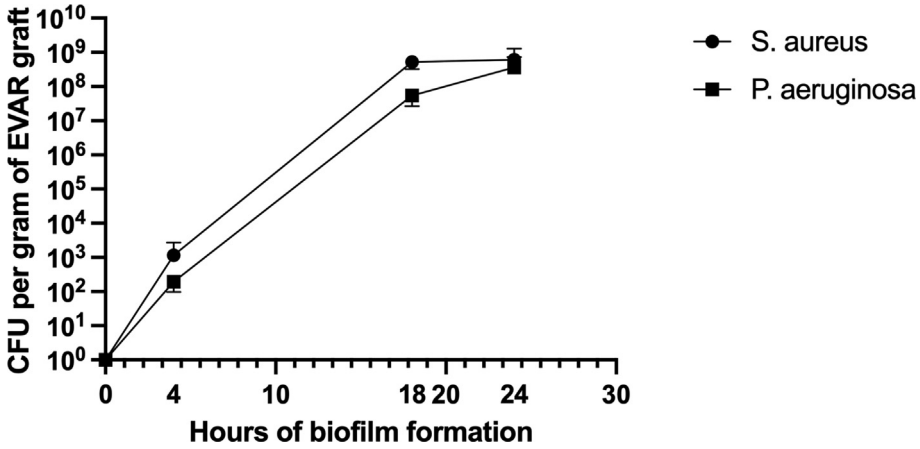


**Fig. 1.** EVAR pieces were infected with $10^2$ CFU/mL *S. aureus* or *P. aeruginosa*. After 15 min, the pieces were washed once and moved to new assay medium for 0-, 4-, 18-, or 24-h incubation. The EVAR pieces were washed three times and sonicated. Bacteria per gram of EVAR piece are shown as detected by quantitative bacteriology. Means and standard deviations are shown. CFU, colony forming unit; EVAR, endovascular aneurysm repair.

Specimens were fixed in 2% glutaraldehyde in 0.05 M sodium phosphate buffer, pH 7.4. After three rinses in 0.15 M sodium phosphate buffer (pH 7.4) specimens were post-fixed in 1% OsO4 in 0.12 M sodium cacodylate buffer (pH 7.4) for 2 hours. After a rinse in distilled water, the specimens were dehydrated to 100% ethanol according to standard procedures and critical point dried (Balzers CPD 030) with $CO_2$. The specimens were subsequently mounted on stubs using double adhesive carbon tape (Ted Pella) as an adhesive and sputter coated with 6 nm gold (Leica ACE 200). Specimens were examined with a FEI Quanta 3D scanning electron microscope (Eindhoven, The Netherlands) operated at an accelerating voltage of 2 kV.

## Statistics

Statistics were performed using GraphPad Prism 9 (Dotmatics, Boston, USA). Wilcoxon's signed rank test was used for comparisons of antibiotic tolerance. For other calculations, Fisher's exact test (two-tailed) was used.

## Results

### *Bacterial attachment to EVAR pieces*

Bacterial exposure of $10^2$ CFU/mL for 15 minutes established biofilms on all prostheses fragments. The CFU count per gram EVAR graft increased significantly over time to a final level of approximately $10^9$ CFU/g for both *S. aureus* and *P. aeruginosa*. Median CFU/g increased from 30 (range <5–2270 CFU/g) to $5.2 \times 10^8$ (range $1.15 \times 10^8$–$1.1 \times 10^9$) for *S. aureus*, and 63 (range <5–260 CFU/g) to $9.1 \times 10^7$ (range $3.5 \times 10^7$–$6.25 \times 10^8$) for *P. aeruginosa* at 0 and 4 vs. 18 and 24 hours, respectively, p 0.029 for both bacteria. These bacteria remained attached to the EVAR pieces despite repeated washing in saline (Fig. 1 and Table 1).

### *SEM evaluation of colonized EVAR grafts*

With SEM we were able to confirm bacterial attachment to the EVAR graft pieces and relate the findings to the development of antibiotic tolerance. The SEM pictures are suggestive of an increase in bacterial density consistent with the CFU/g as seen by culturing. Already after 4 hours of incubation, extracellular strands were seen connecting individual *P. aeruginosa* to each other and to the surface, something that was observed more frequently at 18 and 24 hours including a growth of bacteria in dense clusters.

*S. aureus* was also observed to grow in clusters after 4 hours, but the extracellular matrix was detected only after 18 hours and in smaller quantities than for *P. aeruginosa* (Fig. 2). Both *S. aureus* and *P. aeruginosa* formed complexes on the suture knots, which fasten the polyester mesh to the metal. *S. aureus* adhered in larger numbers to the polyester mesh as compared with the metal, whereas *P. aeruginosa* seemed to adhere equally to metal and polyester (Fig. 3).

### *Culture evaluation of colonized EVAR grafts*

Both *S. aureus* and *P. aeruginosa* attached in all repeats to graft pieces containing all components at exposure times of 15 minutes and more, but not always at exposure times of 5 minutes. *S. aureus* required markedly less time to attach to whole EVAR graft pieces than to the metal part, something not seen in *P. aeruginosa*. The difference between attachment to metal only compared with pieces containing all components was statistically significant for *S. aureus* at 15 and 30 minutes of exposure (Table 1). The reduced

**Table 1**
Comparison of colonized EVAR subunits

| Exposure time | *P. aeruginosa* | | | *S. aureus* | | |
|---|---|---|---|---|---|---|
| | Whole EVAR piece | Metal part only | p (whole piece vs. metal only) | Whole EVAR piece | Metal part only | p (whole piece vs. metal only) |
| 5 min | 2/6 | 0/6 | 0.45 | 3/6 | 0/6 | 0.18 |
| 15 min | 6/6 | 6/6 | 1.0 | 6/6 | 0/6 | <0.01 |
| 30 min | 6/6 | 6/6 | 1.0 | 6/6 | 1/6 | 0.02 |
| 60 min | 6/6 | 6/6 | 1.0 | 6/6 | 2/6 | 0.06 |

Whole EVAR pieces or metal part only were infected with $10^2$ CFU/mL *S. aureus* or *P. aeruginosa*. After 5, 15, 30, or 60 min of incubation, the EVAR pieces were washed once in saline and moved to fresh assay medium. The pieces were then incubated for 24 h and the presence or absence of bacteria was assessed after sonication and culture. CFU, colony forming unit; EVAR, endovascular aneurysm repair.

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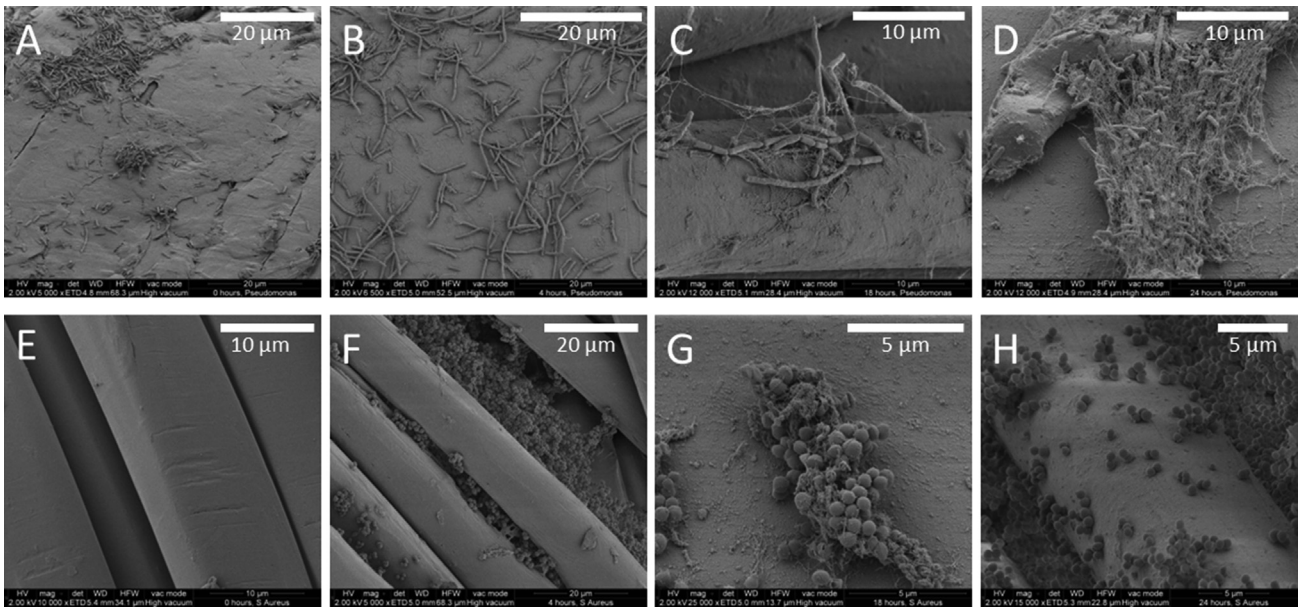


**Fig. 2.** SEM of *S. aureus* (a–d) and *P. aeruginosa* (e–h) on EVAR. An inoculum of $10^8$ CFU/mL was used. (a) and (e) were taken directly after the 15-min exposure of EVAR pieces to the bacterial inoculum, followed by washing the pieces three times in saline. (b) and (f) were recorded after 4 h of incubation, (c) and (g) after 18 h of incubation, and (d) and (h) after 24 h of incubation. Magnifications from 5000 times to 25 000 times. Scale indicated by white bars. CFU, colony forming unit; EVAR, endovascular aneurysm repair; SEM, scanning electron microscopy.

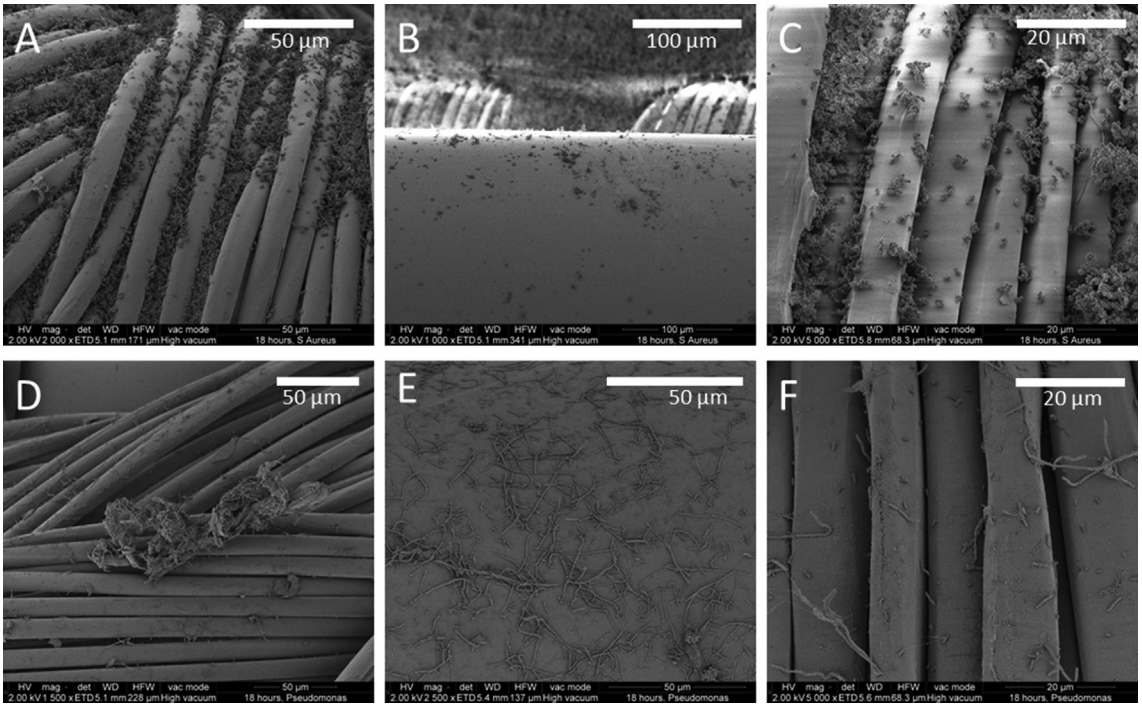


**Fig. 3.** SEM of *S. aureus* (a–c) and *P. aeruginosa* (d–f) on EVAR. Inoculum of $10^8$ CFU/mL was used, with images recorded after 18 h of incubation. (a) and (d) display the suture knot fastening the polyester mesh to the metal. (b) and (e) display the metal. (c) and (f) display bacteria on the polyester mesh. Magnifications from 1000 times to 5000 times. Scale indicated by white bars. CFU, colony forming unit; EVAR, endovascular aneurysm repair; SEM, scanning electron microscopy.

probability to attach to metal only for *S. aureus* as compared with *P.* aeruginosa was also statistically significant at 15 and 30 minutes ($p < 0.01$ and p 0.02).

*Antibiotic tolerance development*

The antibiotic exposure assay was used to determine if there was a development of antibiotic tolerance over time, as the bacteria had more time to develop biofilm-like properties on the graft pieces. For both *S. aureus* and *P. aeruginosa*, the effect of the tested antibiotics at ten times the MIC was markedly decreased the longer the bacteria had grown on the graft pieces. The bactericidal effect, measured by reduction of CFU/g compared with control, was reduced between 10 and 1000 times when comparing graft pieces with bacteria having had 24 hours to grow after the first 15 minutes attachment phase, as compared with graft pieces that had been challenged with

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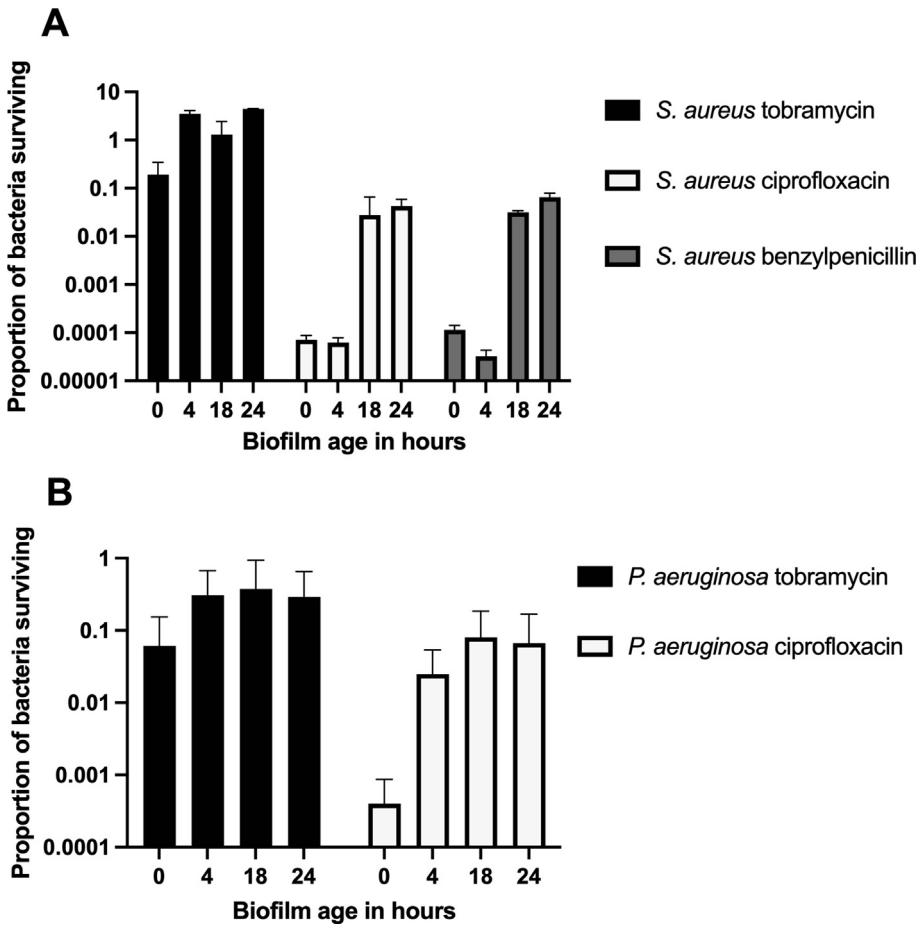


**Fig. 4.** The ratio between the CFU/g for antibiotic treated and the related pretreatment control of EVAR pieces ($CFU_{\text{after antibiotics at biofilm age x}}/CFU_{\text{before antibiotics at biofilm age x}}$). (a) shows values for *S. aureus* ($n = 2$ replicates). (b) shows values for *P. aeruginosa* ($n = 4$ replicates). The X-axis shows the age of the biofilm at the time of starting antibiotic exposure. All experiments used ten times the MIC values of the antibiotics tested. For *S. aureus* MICs were: 0.03125 mg/L of penicillin G, 0.125 mg/L of tobramycin, and 0.25 mg/L of ciprofloxacin. For *P. aeruginosa* MICs were: 0.5 mg/L of tobramycin and 0.25 mg/L of ciprofloxacin. 24 h of antibiotic exposure time was used. Means and standard deviations are shown. CFU, colony forming unit; EVAR, endovascular aneurysm; MIC, minimal inhibitory concentration.

antibiotics directly after the first 15 minutes attachment phase (Fig. 4). Comparing the CFU/g ratio between antibiotic exposed bacteria (combining all antibiotic classes) who had grown for 24 vs. 0 hours, the difference was statistically significantly decreased for both *S. aureus* (p 0.03) and *P. aeruginosa* ($p < 0.01$). No changes of MIC were detected when bacteria were tested in planktonic phase, indicating that the antibiotic tolerance was physiological and not because of acquiring antimicrobial resistance mechanisms.

## Discussion

Summarizing our findings in light of previous research, we show that bacteria can attach to EVAR graft pieces, including using concentrations and exposure times similar to those encountered in clinical situations. The bacteria also resist washing and remain attached until exposed to sonication, which points towards them forming an actual mature biofilm. In addition, the antibiotic tolerance exhibited to all investigated antibiotics, without acquisition of phenotypic antibiotic resistance in planktonic phase (although no sequencing was performed, so genetic changes cannot be ruled out), is a strong indicator of the bacteria forming an actual biofilm. The verification of adhesion and aggregation of the bacteria to the EVAR graft pieces by SEM also strengthens the biofilm hypothesis. Antibiotic tolerance without the acquisition of specific antibiotic resistance mechanisms is considered to be one attribute of bacteria in biofilms. Adhesion of the bacteria to each other and to surfaces forming aggregates is also a core conceptual component of bacterial biofilms [18,19,22].

Limitations of this study were the use of only two bacterial species, the *in vitro* setting, the use of a medium that is different from the blood that the bacteria would encounter in an EVAR graft infection, the use of only one type of EVAR grafts, and using only three antibiotics not aimed to fully mimic clinical pharmacokinetics. *S. aureus* was chosen as the most frequent reported pathogen (with this wild-type isolate being similar to those clinical isolates that are penicillin-susceptible [23,24]), and *P. aeruginosa* as a representative Gram-negative rod. Other bacteria, antibiotics, and graft types require further study. The higher bacterial concentration of $10^8$ CFU/mL used for SEM and antibiotic exposure is higher than what is seen *in vivo* but ensured that antibiotic effects could be seen at all timepoints. It is important to keep the high concentration in mind when interpreting results. The meaning of our study as a proof of concept is that a representative and reproducible *in vitro* system is necessary to investigate and identify the best possible treatment strategies to handle these difficult-to-treat infections.

The short exposure time of 5–15 minutes needed for both *S. aureus* and *P. aeruginosa* to attach to EVAR graft pieces when all components were present has implications for the pathogenic process of *in vivo* EVAR graft infections, and for further research and clinical practice. Infections of EVAR prostheses can occur in

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different situations, such as during insertion, through subsequent bacteraemia, or from infections in surrounding organs [6,14]. This might indicate that endogenous substances covering parts of the EVAR grafts might play a role such as endothelial cells and blood components, although investigations on removed infected EVAR prostheses are needed to clarify this. Differences in attachment of bacteria to the distinct parts of the EVAR grafts were intriguing, especially as the risk profiles for attachment of *S. aureus* and *P. aeruginosa* were strikingly different *in vitro*. Further studies with different materials in the grafts, and with different blood components coating the grafts might help in elucidating this.

In conclusion, the model shows that bacterial adhesion to EVAR grafts can happen rapidly at clinically relevant bacterial concentrations and strongly indicate that biofilm forms. In this experimental setting, bacterial eradication was not achievable using antibiotic monotherapy even at ten times the MIC. The model might both be used to test other antibiotics, alone or in combination, and non-antibiotic adjunctive treatments such as hyperbaric oxygen or bacteriophages, as well as be expanded to include blood components to better mimic the *in vivo* situation.

## Author contributions

Conceptualization: CM, TS, NE, and KV. Methodology: TS, CM, FS, LC, TB, and KQ. Investigation: TS, FS, and KQ. Resources: CM, NE, KV, and TB. Visualization: TS, CM, and KQ. Project administration, supervision: CM. Funding acquisition: TS. Writing, original draft: TS and CM. Writing, review and editing: TS, FS, LC, TB, NE, KV, and CM. All authors approved the final version of the manuscript.

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