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DISSERTATION

IMPROVEMENT OF THE DIAGNOSIS OF IMPLANT-ASSOCIATED INFECTION

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BACKGROUND

ABSTRACT

Treatment of implant-associated infections essentially differs from the one of aseptic implant failure. Therefore, an accurate diagnosis of the underlying cause of implant failure is crucial. Conventional diagnostic methods such as culture or leukocyte count of synovial fluid have limited sensitivity and specificity. Therefore, novel, innovative strategies for improved (more accurate and faster) diagnosis of implant-associated infections are needed.

The aim of this work was to improve the diagnosis of implant-associated infections: (1) to evaluate the performance of D-lactate (bacterial metabolite) in synovial fluid as independent diagnostic marker; (2) to investigate the activity of chemical methods (i.e. ethylenediaminetetraacetic acid and dithiothreitol) and mechanical methods (i.e. sonication) for biofilm dislodgement and evaluate their potential role in the routine microbiological diagnosis; (3) to investigate the influence of the implants material type, such as polyethylene, titanium and cobalt-chromium alloy, on the biofilm formation.

Results showed that the optimal cut-off of synovial fluid D-lactate to differentiate periprosthetic joint infections (PJI) from aseptic failure (AF) was 1.3 mmol/L with sensitivity and specificity more than 94% and 88%, respectively, and was comparable to synovial fluid leukocyte count. The comparison of the ability of chemical and physical methods to dislodge bacterial biofilm in an established in vitro model of artificial biofilm showed that sonication dislodges significantly more bacteria compared to chemical method. The results were derived applying three independent methods: colony counts, isothermal microcalorimetry and scanning electron microscopy. Investigating the influence of implants' biomaterial on microbial adhesion, the results reviled that polyethylene showed larger biofilm burden compared to metal alloys (titanium and cobalt-chromium alloy), suggesting intrinsic differences in the ability of microorganisms to form biofilms on various biomaterials.

This work demonstrated that D-lactate had good performance for the diagnosis of PJI and might be used as a highly sensitive and specific bacterial specific biomarker. In the intraoperative stage, sonication is the most sufficient method for bacterial biofilm dislodgement and bacterial detection. Using sonication, polyethylene showed higher bacterial load compared to metal alloys, indicating that microorganisms have different adhesion affinity on different biomaterials in vivo. Sonication of polyethylene liners may be sufficient to diagnose implant-associated infections rather than using the whole prosthesis.

ABSTRAKT

Die Behandlung Implantat-assoziierter Infektionen unterscheidet sich wesentlich von der Behandlung eines aseptischen Implantatversagens. Daher ist eine genaue Diagnosestellung der zugrunde liegenden Ursache des Implantatversagens entscheidend. Herkömmliche diagnostische Verfahren wie die Kultur oder die Leukozytenzahl der Synovialflüssigkeit weisen eine begrenzte Sensitivität und Spezifität auf. Daher sind neuartige, innovative Strategien zur verbesserten (genaueren und schnelleren) Diagnosestellung Implantat-assoziierter Infektionen erforderlich.

Ziel dieser Arbeit war es, die Diagnostik Implantat-assoziierter Infektionen zu verbessern: (1) Bewertung der Aussagekraft von D-Laktat (bakterieller Metabolit) in der Synovialflüssigkeit als unabhängiger diagnostischer Marker; (2) Untersuchung der Effektivität chemischer Methoden (d.h. Ethylenediaminetetraacetyl-Säure und Dithiothreitol) und mechanischer Methoden (d.h. Sonikation) zur Ablösung von Biofilmen und Bewertung derer Rolle bei der routinemäßigen mikrobiologischen Untersuchung ; (3) Untersuchung des Einflusses des Materials des Implantates wie Polyethylen, Titan und Kobalt-Chrom-Legierung auf die Biofilmbildung und den mikrobiellen Nachweis im Sonikat.

Die Ergebnisse zeigten, dass der optimale Grenzwert für D-Laktat in der Synovialflüssigkeit zur Unterscheidung periprothetischer Infektionen (PPI) und aseptischer Lockerungen (AL) 1,3 mmol/L betrug mit einer Sensitivität und Spezifität von mehr als 94% und 88% . Der Vergleich der chemischen Methode und Sonikation zur Ablösung von Biofilmen zeigte, dass die Sonikation signifikant mehr Bakterien entfernen konnte. Die Untersuchung des Einflusses des Materials des Implantates auf die mikrobielle Adhäsion zeigte, dass Polyethylen eine größere Biofilmlast aufwies als Metalllegierungen (Titan und Kobalt-Chrom-Legierung).

Diese Arbeit zeigte, dass D-Laktat eine gute Performance für die Diagnosestellung von PPI aufweist und als sensitiver und bakterienspezifischer Biomarker verwendet werden kann. Bei der intraoperativen Diagnistik ist die Sonikation die effizienteste Methode zur Ablösung vom Biofilm und damit zum Bakteriennachweis. Die Sonikation des Polyethylens wies im Vergleich zu Metalllegierungen eine höhere Bakterienbelastung auf, was darauf hinweist, dass in vivo eine unterschiedliche Adhäsionsaffinität der Mikroorganismen an verschiedenen Biomaterialien besteht. Die alleinige Sonikation von Polyethyleninlays könnte deswegen für die Diagnostik von PPI ausreichend sein.

1. INTRODUCTION

Arthroplasty enormously improved the quality of life. The number of hip or knee replacements is continuously increasing worldwide. The annual increase ranges from 5.3-17% (1). However, implants carry an inherent risk of complications, including failing integration in the host or infection. Periprosthetic joint infection (PJI) represents one of the most complex complications occurs in 0.3–2% after primary and in 4% of revision arthroplasties (2-6). In addition to economic costs that increase 3–4-fold, PJI leads to relevant consequences such as removal of implant or development of chronic post-implantation osteomyelitis and permanent disability (7). Treatment of PJI essentially differs from the one of aseptic prosthesis failure (AF). Therefore, an accurate diagnosis of the underlying cause of prosthetic failure is crucial to plan adequate treatment, including open surgical intervention.

Synovial fluid analysis is the standard *preoperative* test routinely performed before prosthesis revision surgery. Conventional diagnostic methods (such as synovial fluid and periprosthetic tissue culture) have limited sensitivity reported to be 60-70% (8, 9). The culture sensitivity is especially limited in chronic infections, typically associated with low microbial burden, and in patients previously receiving antibiotics (10). The synovial fluid leukocyte count and differential (i.e. percentage of granulocytes) has high sensitivity (6), but synovial fluid leukocyte count may also be increased without infection in case of dislocations, periprosthetic fracture or within the first 6 weeks after surgery du to physiologic inflammatory healing process (2, 4, 6, 11). Other used diagnostic methods are insufficiently specific or associated with artifacts caused by implants (imaging) or require invasive procedure (surgical revision). Therefore, novel, innovative strategies for improved (more accurate and faster) diagnosis of implant-associated infections is needed. Novel biomarkers in synovial fluid such as alfa-defensin, leukocyte esterase and calprotectin (12-14) are abundantly present in neutrophils and therefore in patients with aseptic conditions, which are associated with high synovial fluid leukocyte count, such as crystal-induced inflammation and other conditions that induce aseptic inflammation, and therefore are not be applicable for the diagnosis of PJI. D-lactate is a pathogen-specific metabolite, produced nearly exclusively by bacteria (15). This biomarker was previously evaluated in primarily sterile body fluids, including synovial (16, 17) and cerebrospinal fluid (18). L-rotatory and D-rotatory isomers of lactate are both products of intracellular metabolism, however, mammalian cells contain only the enzyme L-lactate dehydrogenase (LDH) and can produce almost exclusively L-lactate. Consequently, the serum concentration of D-lactate in humans is very low (nanomolar to micromolar range) as a product of a minor off-shoot pathway of glycolysis. In contrast, bacterial species possess both D-LDH

and L-LDH enzymes and, therefore, produce measurable amounts of D-lactate and L-lactate. Consequently, they are detectable in body fluids in the millimolar range (19, 20). In the *intraoperative* diagnostic the considerable challenge represents the investigation of explanted implants. The detection of bacteria is restricted by biofilm formation on the implant surface (21, 22). To isolate and identify microorganisms embedded in a polymeric matrix attached to the device surface, the dislodgment and dispersion of the sessile community as an add-on procedure to routinely conducted microbiological analysis represent the first step before plating the specimen on culture media (23). To improve biofilm removal from implant surface, different approaches had been investigated. Among others, sonication is based on mechanical biofilm dislodgement. Cavitation caused by ultrasound waves creates a jet of liquid in the vicinity of the implant. These shear forces disaggregate biofilm, enrich the resulting sonication fluid with dislodged bacteria thereby improving microbial detection in patients with implants (8). The ability of chemical dislodgement such as metal-chelating agent ethylenediaminetetraacetic acid (EDTA) and the strong reducing agent dithiothreitol (DTT) was proposed as an alternative method to sonication. The ability of EDTA to chelate and potentiate the cell walls of bacteria and destabilize biofilms by sequestering calcium, magnesium, zinc, and iron suggests its use to be suitable for the biofilm detachment (24). DTT denatures proteins by reducing their disulfide bonds and therefore might disaggregate biofilm by chemical interactions (25).

Most commonly isolated microorganisms in patients with orthopedic implant infections (PJI, fracture fixation-associated and spinal implant-associated infections) are coagulase-negative staphylococci (30-45%) and *Staphylococcus aureus* (12-23%), followed by streptococci (9-10%), enterococci (3-7%), gram-negative bacilli (3-6%) and anaerobes (2-4%) (4, 26, 27). The formation of bacterial biofilm on the implant surface is differentiated in two phases: the primary adherence phase, followed by the accumulation phase. The microbial adhesion is usually mediated by production of numerous factors, i.e. a variety of adhesins and extracellular enzymes. In the accumulation phase further extracellular components (such as polysaccharides, DNA, proteins and lipids) foster microbial proliferation and colonization at the abiotic surface and stimulate the development of biofilm (28). Experimental animal studies revealed that various types of materials have different affinity for microbial adherence and biofilm formation (29-31). In clinical studies investigating different biomaterials of hip and knee prosthesis was shown that polyethylene has the highest bacterial loads compared to metal (32). Whereas it is well known that the first step in the bacterial biofilm formation is the interaction of microorganisms with the implant surface and their adhesion to it (33), it is indispensable to investigate the adhesion mechanisms between bacteria and biomaterial surface,

and to determine ways in which implant surface properties can counteract and modulate this adhesion process.

Taken together, the combination of different diagnostics approaches improves conventional methods and shows the great effort needed to optimize the diagnostic of implant-associated infections. By discovering new bacterial-specific biomarkers, investigating bacterial-biomaterial interactions and evaluating novel diagnostics approaches, the diagnosis of biofilm-associated infections could be significant improved in the upcoming years.

2. AIM OF THE THESIS

The main aim of this work is to improve the diagnosis of implant-associated infections by developing novel approaches, which either increase sensitivity and/or specificity, provide results faster or are easier to perform or less expensive. Therefore, the main objectives are:

1. To evaluate the performance of D-lactate in synovial fluid as an independent diagnostic marker for the diagnosis of PJI.

(Study A: Karbysheva et al., Performance of synovial fluid D-lactate for the diagnosis of periprosthetic joint infection: A prospective observational study. Journal of Infection (2019); Study B: Karbysheva et al., Synovial Fluid D-Lactate – A novel pathogen-specific biomarker for the diagnosis of periprosthetic joint infection. Journal of Arthroplasty (2020));

2. To investigate the activity of chemical methods (i.e. EDTA and DTT) and mechanical methods (i.e. sonication) for biofilm dislodgement and to evaluate their potential role in the routine microbiological diagnostic of implant-associated infections.

(Study C: Karbysheva et al., Comparison of sonication with chemical biofilm dislodgement methods using chelating and reducing agents: implications for the microbiological diagnosis of implant associated infection. PLOS ONE (2020));

3. To investigate the influence of the material type, such as polyethylene, titanium and cobalt-chromium alloy, on the biofilm formation and the microbial detection in sonication culture of retrieved prostheses components.

(Study D: Karbysheva et al., Influence of retrieved hip- and knee-prosthesis biomaterial on microbial detection by sonication. European Cells and Materials (2019));

3. MATERIALS AND METHODS

3.1. Diagnostic of periprosthetic joint infection

PJI was defined using both, the more widely accepted Musculoskeletal Infection Society (MSIS) definition criteria for PJI from the year 2013 (34-36) and the institutional PJI definition criteria, which are summarized in the Table 1. The definition criteria include visible purulence, sinus tract, serum C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), synovial fluid leukocyte count and percentage of granulocytes, histopathology and cultures. Acute infection was diagnosed if the infection occurred within 4 weeks after surgery or if the patient reported new onset of symptoms lasting not longer than 4 weeks. Infections that occurred more than 4 weeks after the last surgery and were symptomatic for more than 4 weeks were defined as chronic infections. Furthermore, based on the interval between last revision surgery or primary implantation and time of aspiration, all infections were classified into early (i.e. ≤ 3 months) and delayed or late (i.e. > 3 months) infections.

Table 1 Definition criteria of periprosthetic joint infection.

Musculoskeletal Infection Society (MSIS) criteria (PJI is defined by presence of ≥ 1 major or ≥ 3 minor criteria)	**Institutional criteria** (PJI is defined by presence of ≥ 1 of any of the following 4 criteria)
Major criteria: • Two positive periprosthetic cultures (phenotypically identical organisms) • Sinus tract communicating with the joint **Minor criteria**: • Elevated CRP[1] or ESR (> 30 mm/hour) • Elevated synovial fluid leukocyte count[2] or positive leukocyte esterase strip test (++ or +++) • Elevated synovial fluid percentage of granulocytes[3] • A single positive culture • Positive histological analysis of periprosthetic tissue[4]	• Purulence around the prosthesis or sinus tract • Increased synovial fluid leukocyte count[5] • Positive histopathology[6] • Significant microbial growth in synovial fluid, periprosthetic tissue[7] or sonication culture[8]

[1] > 10 mg/l in chronic infections or > 100 mg/l in acute infections.

[2] > 3,000 leukocytes/µl in chronic infections or > 10,000 leukocytes/µl in acute infections.

[3] > 80% in chronic infections or > 90% in acute infections.

[4] Defined as > 5 neutrophils per high-powered field in 5 high-power fields observed on periprosthetic tissue at 400x magnification.

[5] Leukocytes > 2000/µl or > 70% granulocytes; synovial fluid leukocyte count was not considered diagnostic within the first 6 weeks after surgery, in inflammatory joint disease and in case of periprosthetic fracture or luxation. In these situations, the leukocyte count can be increased also in the absence of an infection (37).

[6] Defined as a mean of > 23 granulocytes per 10 high-power fields (type II or type III, according to Krenn et al. (38)).

[7] Periprosthetic tissue culture was considered positive if ≥ 1 specimen was positive in highly virulent organisms (or ≥ 2 specimens showed microbial growth of a low-virulent pathogen).

[8] Sonication was considered positive by growth of > 50 colony-forming units (CFU)/mL sonication fluid, except for *Staphylococcus aureus*, streptococci and gram-negative rods, for which any growth (*i.e.* ≥ 1 CFU/mL) was considered positive (8).

(Study B: Table S1 in Karbysheva et al., Journal of Arthroplasty (2020)).

3.1. Retrieval and investigation of synovial fluid

Synovial fluid was aspirated under sterile conditions preoperatively in the outpatient department or during revision surgery before opening the joint capsule (Study A and B).

D-lactate was determined spectrophotometrically from the optical density of the prepared sample. One 1 mL of synovial fluid was transferred to a native vial for determination of D-lactate using a commercial kit (D-lactam Kit; VL-Diagnostics, Leipzig, Germany). Aliquots for D-lactate determination were stored at 4 °C ±1 °C and analyzed within 48 h after aspiration. The tests were performed according to the manufacturer´s instructions. The determination is based on spectrophotometric method with a standard microplate absorbance reader at 570 nm (DYNEX Technologies MRX, Chantilly VA, USA), requiring 50 µl of synovial fluid. In the assay D-lactate dehydrogenase (D-LDH) catalyzes the oxidation of D-lactic acid to pyruvate, along with the concomitant reduction of nicotinamide adenine dinucleotide (NAD+) to NADH. NADH reacts with the fluorescent substrate to yield coloration of the mixture.

For microbiological analysis, each sample of synovial fluid was inoculated in 0.1 mL aliquots on Columbia Blood Agar and thioglycolate broth for aerobic and anaerobic culture, and into blood culture pediatric bottles (BacTec PedsPlus/F, Beckton Dickinson, Shannon, Ireland). All

culture media were incubated at 35 °C for 14 days. Identification and susceptibility testing of isolated microorganisms were performed using an automatic bacteriological analyzer VITEK 2 (bioMérieux, Marcy L'Etoile, France).

For determination of leukocyte count and percentage of granulocytes, 1 mL of synovial fluid was collected in a vial containing ethylenediaminetetraacetic acid (EDTA). Clotted specimens were treated with 10 µl hyaluronidase (Sigma-Aldrich Chemie, Taufkirchen, Germany) for 10 minutes at room temperature. The test was performed by flow-cytometry using an automated haematology analyzer (XE-2100, Sysmex, Norderstedt, Germany).

3.2. Retrieval and investigation of explanted prostheses

Prosthetic components retrieved at revision surgery were separately placed into sterile containers (Study A, B and D). Then, the explanted material was transported within 4 h to the laboratory for sonication. Hundred mL sterile 0.9% NaCl was added to each container. The implants were totally submerged in the fluid. Then, the samples were vortexed for 30 sec, sonicated at 40 kHz at intensity 0.1 Watt/ cm^2 (BactoSonic, BANDELIN electronic, Berlin, Germany) for 1 min and vortexed again for 30 sec. Then 0.5 mL aliquots were plated on Columbia Blood Agar and into thioglycolate broth (Sigma-Aldrich, St. Louis, MO, USA). Inoculations were done before sonication (i.e. vortexing culture) and after sonication (i.e. sonication culture). All culture media were incubated at 35 °C for 14 days. Identification and susceptibility testing of isolated microorganisms were performed using an automatic bacteriological analyzer VITEK 2 (bioMérieux, Marcy L'Etoile, France). Cultures were quantified by counting the number of colonies that grew on the plate and adjusting to the number of CFU (CFU/mL).

3.3. Storage and culture of bacterial strains

Stocks of *S. epidermidis* ATCC 35984, *S. aureus* ATCC 43300, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 53278 were stored in cryovial bead preservation system at -80 °C (Study C). Bacterial strains were cultured on tryptic soy agar with 5% sheep blood for 24 h at 35 °C (Sigma-Aldrich, St. Louis, MO, USA) in an ambient air incubator.

3.4. Biofilm growth conditions

As a model to grow the bacterial biofilm porous glass beads (diameter 4 mm, pore sizes 60 µm, ROBU®, Hattert, Germany) were used (Study B). To form biofilms, beads were placed in 2 mL of brain heart infusion broth (BHIb, Sigma-Aldrich, St. Louis, MO, USA) containing $1x10^8$ CFU/mL inoculum of *S. epidermidis*, *S. aureus*, *E. coli* or *P. aeruginosa* and incubated at 35°C.

After 24 h, beads were re-incubated in fresh BHIb and biofilms were statically grown for further 72 h at 35°C. After biofilm formation, beads were washed six times with 2 mL 0.9% NaCl to remove planktonic bacteria.

3.5. Biofilm dislodgement by chemical methods (EDTA or DTT) or sonication

To define the minimal chemical concentration and treatment duration for biofilm dislodging, washed beads were placed in 1 mL of EDTA at concentrations 12, 25 and 50 mM or DTT at concentrations 0.5, 1 and 5 g/L and exposed for 5, 15 and 30 min. Untreated beads incubated with 0.9% NaCl were used as negative control. To evaluate the sonication effect, each bead was inoculated in 1 mL 0.9% NaCl, vortexed for 30 sec, sonicated at 40 kHz at intensity 0.1 Watt/cm^2 (BactoSonic, BANDELIN electronic, Berlin, Germany) for 1 min and vortexed again for 30 sec. One-hundred microliter of serial dilutions of the resulting sonication fluid or the solution obtained after chemical treatment with DTT or EDTA were plated onto Tryptic Soy Agar (TSA) (Sigma-Aldrich, St. Louis, MO, USA). After 24 h of incubation at 35°C, the CFU/mL number was counted.

3.6. Isothermal microcalorimetry analysis

To prove the dislodgement effect of previously described methods and reveal the presence of bacterial cells remained attached on the bead surface, treated beads were washed six times in 2 mL 0.9% NaCl to remove the dislodged biofilm and placed in 4 ml-glass ampules containing 3 mL of BHIb. The ampoules were air-tightly sealed and introduced into the microcalorimeter (TAM III, TA Instruments, Newcastle, DE, USA), first in the equilibration position for 15 min to reach 35°C and avoid heat disturbance in the measuring position. Heat flow (μW) was recorded up to 20 h. The calorimetric time to detection (TTD) was defined as the time from insertion of the ampoule into the calorimeter until the exponentially rising heat flow signal exceeded 100 μW to distinguish microbial heat production from the thermal background. Growth media without bacteria served as negative control.

3.7. Scanning electron microscopy (SEM)

Beads with biofilm were fixed with 2.5% (v/v) glutaraldehyde in natrium cacodylat buffer and the samples were dehydrated with increasing concentrations of ethanol for 2 min each. The samples were stored in vacuum until use. Prior to analysis by Scanning electron microscope (GeminiSEM 300, Carl Zeiss, OberkochenDSM 982 GEMINI, Zeiss Oberkochen, Germany), the samples were subjected to gold sputtering (Sputter coater MED 020, Balzer, BingenMED 020, BAL-TEC). All experiments were performed in triplicate.

4. RESULTS

This chapter contains key results published in Journal of Infection (2019) (Study A), Journal of Arthroplasty (2020) (Study B), PLOS ONE (2020) (Study C) and European Cells and Materials (2019) (Study D).

Performance of synovial fluid D-lactate for the diagnosis of prosthetic joint infection

In Study A and B we investigated the performance of synovial fluid D-lactate for the diagnosis of PJI. In Study A, 148 consecutive patients were included who were evaluated for a painful prosthetic hip, knee or shoulder joint and underwent a diagnostic joint aspiration before revision arthroplasty. Applying institutional PJI definition criteria, 44 (31%) were diagnosed with PJI and 104 (69%) with AF. The optimal D-lactate cut-off value was calculated at 1.26 mmol/l with sensitivity and specificity of 86.4% and 81.7%, respectively. As shown in Table 2, sensitivity of D-lactate for the diagnosis of infection was significantly higher compared to conventional tests (leukocyte count and granulocyte percentage).

Positive findings	**Aseptic failure(n=104)**	**PJI*** **(n=44)**	**AUC (%)** (95% CI)	**Sensitivity (%)** (95% CI)	**Specificity (%)** (95% CI)	**PPV (%)** (95% CI)	**NPV (%)** (95% CI)	**Accuracy (%)** (95% CI)
Non-microbiological tests								
Clinical features[a]	0	19	-	43.2 (29.5-56.8)	100	100	80.6 (77.0-84.6)	83.1 (79.1-87.2)
Synovial fluid D-lactate >1.26 mmol/l	19	38	90.3 (85.7-95.0)	86.4 (75.0-95.5)	81.7 (74.0-88.5)	66.7 (57.8-76.6)	93.5 (88.7-97.5)	83.1 (77.0-89.1)
Synovial fluid leukocyte count >2000/µl[b]	9	35	91.0 (85.1-96.8)	79.5 (68.2-90.9)	91.3 (85.6-96.2)	80.0 (69.4-90.2)	91.4 (86.8-96.0)	87.8 (82.4-92.6)
Synovial fluid granulocyte percentage >70%[b]	8	25	86.1 (79.4-92.9)	56.8 (40.9-70.5)	92.3 (86.5-97.1)	75.9 (62.9-88.9)	83.5 (78.8-88.3)	81.8 (75.7-87.2)
Leukocyte count or percentage of granulocytes[c]	9	35	-	79.5 (68.2-90.9)	89.4 (83.7-95.2)	76.2 (66.0-87.2)	91.3 (86.5-95.9)	86.5 (81.1-91.9)
Histopathology of periprosthetic tissue	0/43	25/34	-	73.5 (58.8-88.2)	100	100	82.7 (75.4-91.5)	88.3 (81.8-94.8)
Microbiological tests								

Synovial fluid culture	8	20	-	45.5 (31.8-61.4)	100	100	81.2 (77.6-86.0)	83.8 (79.7-85.5)
Periprosthetic tissue culture[d]	7/63	17/41	-	41.5 (26.8-56.1)	100	100	72.4 (68.8-77.8)	76.9 (71.2-82.7)
Sonication fluid culture[d]	5/49	17/39	-	43.6 (28.2-59.0)	100	100	69.0 (63.6-75.4)	75.0 (68.2-81.8)
Any culture specimen	19	23	-	52.3 (38.6-65.9)	100	100	83.2 (79.4-87.4)	85.8 (81.8-89.9)

Table 2 Performance of non-microbiological and microbiological tests for the diagnosis of infection according to institutional criteria.

Note: If denominator is shown, the test was not performed in all patients.

PJI, periprosthetic joint infection; AF, aseptic failure; PPV, positive predictive value; NPV, negative predictive value; 95% CI, 95% confidence interval.

[a] Eleven patients had visible purulence of the synovial fluid, 1 patient had sinus tract and 7 patients had both.

[b] In 12 of 148 patients, the leukocyte count (n = 9) or granulocyte percentage (n = 8) were increased but were not diagnostic for PJI because of concomitant crystal arthropathy (n = 1), recurrent dislocation (n = 2), rheumatologic joint disease (n = 3), early postoperative status (n = 2), trauma (n = 2), periprosthetic fracture (n = 1) or metallosis with crystals (n = 1).

[c] The false positive results were interpreted as positive for assessing performance. In 3 cases, leukocyte count and percentage of granulocytes were not elevated above the cut-off although defined as not interpretable.

[d] Growth of low-virulent microorganism in only one specimen was not sufficient for the diagnosis of PJI.

(Study A: Table 3 in Karbysheva et al., Journal of Infection (2019)).

The mean D-lactate concentration was significantly higher in patients with PJI than in AF, Figure 1.

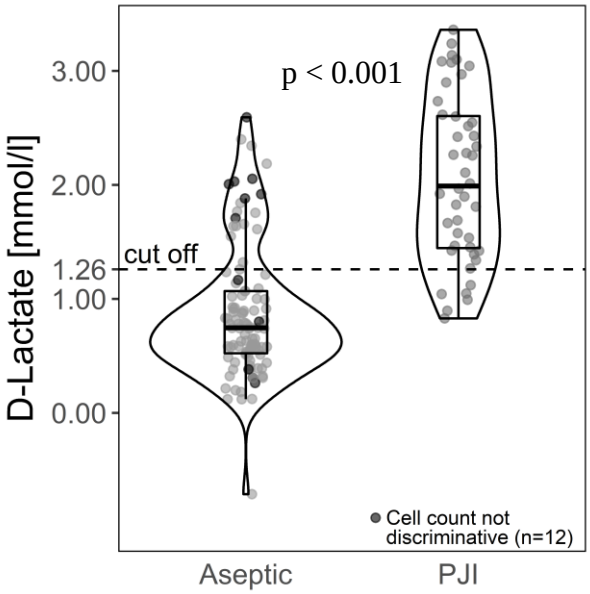


Figure 1 Distribution of D-lactate in patients with AF and PJI. Twelve patients with underlying inflammatory conditions and elevated leukocyte count or percentage of granulocytes above the threshold are presented with dark grey dots. (Study A: Figure 2 in Karbysheva et al., Journal of Infection (2019)).

No significant differences were observed between any pairwise comparisons of the area under the receiver operating characteristic curve (AUC) between investigated synovial fluid biomarkers (AUC D-lactate vs. AUC leukocyte count); Figure 2).

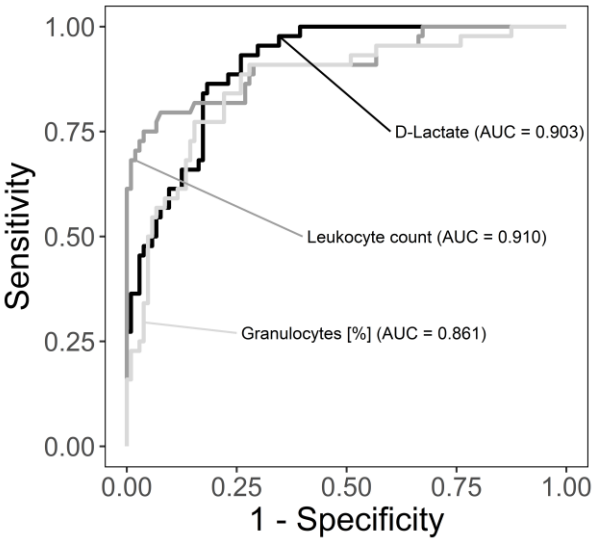


Figure 2 The receiver operating characteristic curve (ROC) curves of synovial fluid biomarkers for PJI. The area under the receiver operating characteristic curve (AUC) of D-

lactate, leukocyte count and percentage of granulocytes are 0.903, 0.910 and 0.861, respectively. (Study A: Figure 1 in Karbysheva et al., Journal of Infection (2019)).

In Study B we studied the performance of synovial fluid D-lactate in a larger prospective cohort using both, the more widely accepted MSIS definition criteria for PJI from the year 2013 (34-36) and the institutional PJI definition criteria. Of 224 included patients, 71 (32%) were diagnosed with PJI and 153 (68%) with AF using MSIS criteria, whereas 92 patients (41%) were diagnosed with PJI and 132 (59%) with AF applying institutional criteria. The study revealed that synovial fluid D-lactate showed the highest sensitivity and specificity at the cut-off of 1.3 mmol/l irrespective of applied diagnostic criteria for the diagnosis of PJI (MSIS or institutional criteria), Table 3. Applying MSIS criteria, synovial fluid D-lactate showed a sensitivity of 94.3% and specificity of 78.4% at cutoff of 1.3 mmol/l. Applying institutional criteria, the synovial fluid D-lactate had a sensitivity of 92.4% and specificity of 88.6% at cutoff of 1.3 mmol/l.

D-lactate, cut-off (mmol/l)	PJI	AF	AUC	Sensitivity, %	Specificity, %	PPV, %	NPV, %
			(95% CI)				
According to MSIS criteria							
>1.2	68/71	39/153	0.93 (0.89-0.96)	95.7 (88.1-99.1)	74.5 (66.8-81.2)	63.6 (53.7-72.6)	97.4 (92.7-99.4)
>1.3*	67/71	33/153	0.93 (0.89-0.96)	94.3 (86.2-98.4)	78.4 (71.1-84.7)	67.0 (56.9-76.1)	96.8 (91.9-99.1)
>1.4	63/71	32/153	0.93 (0.89-0.96)	88.7 (79.0-95.0)	79.4 (72.5-85.8)	67.0 (56.5-76.4)	93.8 (88.2-97.3)
According to institutional criteria							
>1.2	87/92	20/132	0.95 (0.93-0.98)	94.6 (87.8-98.2)	84.8 (77.6-90.5)	79.4 (70.5-86.6)	95.7 (90.3-98.6)
>1.3*	85/92	17/132	0.95	92.4	88.6	85.0	94.4

			(0.93-0.98)	(84.9-96.9)	(81.9-93.5)	(76.5-91.3)	(88.7-97.7)
>1.4	79/92	15/132	0.95 (0.93-0.98)	85.8 (77.0-92.2)	88.6 (76.7-89.7)	84.0 (75.0-90.8)	90.0 (83.5-94.6)

Table 3 Performance of synovial fluid D-lactate in 224 patients, depending of the cut-off for the diagnosis of PJI diagnosed according to MSIS and institutional criteria.

Note: If denominator is shown, the test was not performed in all patients

PJI, periprosthetic joint infection; AF, aseptic failure; AUC, area under the receiver operating characteristic curve; PPV, positive predictive value; NPV, negative predictive value; 95% CI, 95% confidence interval.

* The proposed optimal cut-off to discriminate PJI from AF. (Study B: Table 3 in Karbysheva et al., Journal of Arthroplasty (2020)).

The median concentration of D-lactate was significantly higher in patients with PJI than in those with AF applying MSIS criteria (2.6 mmol/l [1.9 - 2.9 mmol/l] vs. 0.7 mmol/l [0.4 - 1.2 mmol/l], p <0.0001) and applying our institutional criteria (2.4 mmol/l [1.8 - 2.9 mmol/l] vs. 0.7 mmol/l [0.3-1.0 mmol/l], p <0.001, , Figure 3.

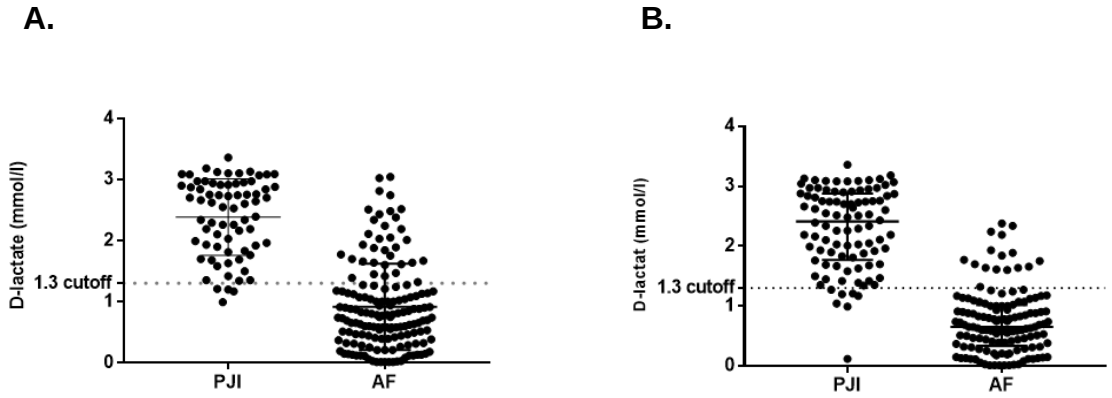


Figure 3 Distribution of D-lactate concentration in synovial fluid with corresponding receiver operation characteristic (ROC) curve. A - applying MSIS criteria. B - applying institutional criteria. (Study B: Figure 1 in Karbysheva et al., Journal of Arthroplasty (2020)).

Moreover in study B was shown that the concentration of D-lactate was significantly lower in patients with PJI caused by coagulase-negative staphylococci, typical low-virulent pathogen,

than in patients with PJI caused by highly virulent bacteria such as *S. aureus* ($p < 0.001$) or streptococci ($p = 0.016$). No statistically significant difference in D-lactate concentration was observed in those caused by low-virulent microorganisms and culture-negative PJI ($p = 0.050$), Figure 4.

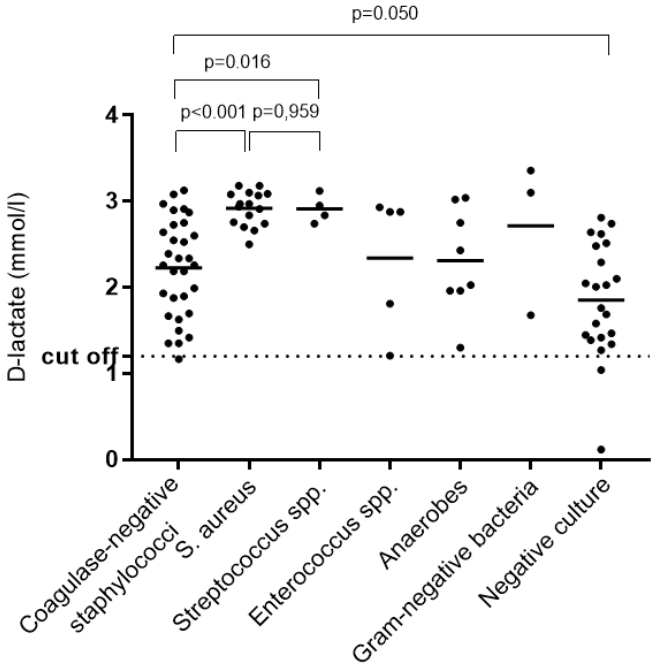


Figure 4 Synovial fluid D-lactate concentration stratified according to the isolated pathogen in patients with PJI. (Study B: Figure 2 in Karbysheva et al., Journal of Arthroplasty (2020)).

Activity of chemical methods (i.e. EDTA and DTT) and mechanical methods (i.e. sonication) for biofilm dislodgement

Study C revealed that sonication detected significantly higher CFU counts when bacterial biofilm of *S. epidermidis*, *S. aureus*, *E. coli* and *P. aeruginosa* were used at 7.5, 7.3, 6.2 and 6.5 $\log_{10}$ CFU/mL, respectively compared to treatment with chemical agents (EDTA 25 mM, 15 min: 6.3, 6.4, 5.2 and 5.1 $\log_{10}$ CFU/mL; DTT: 6.1, 6.3, 5.1 and 5.2 $\log_{10}$ CFU/mL, respectively ($p < 0.05$). The mean colony count obtained after use of chemicals was similar to those observed after treatment with 0.9% NaCl used as control (6.0, 6.3, 5.1 and 5.0 $\log_{10}$ CFU/mL, respectively), Figure 5.

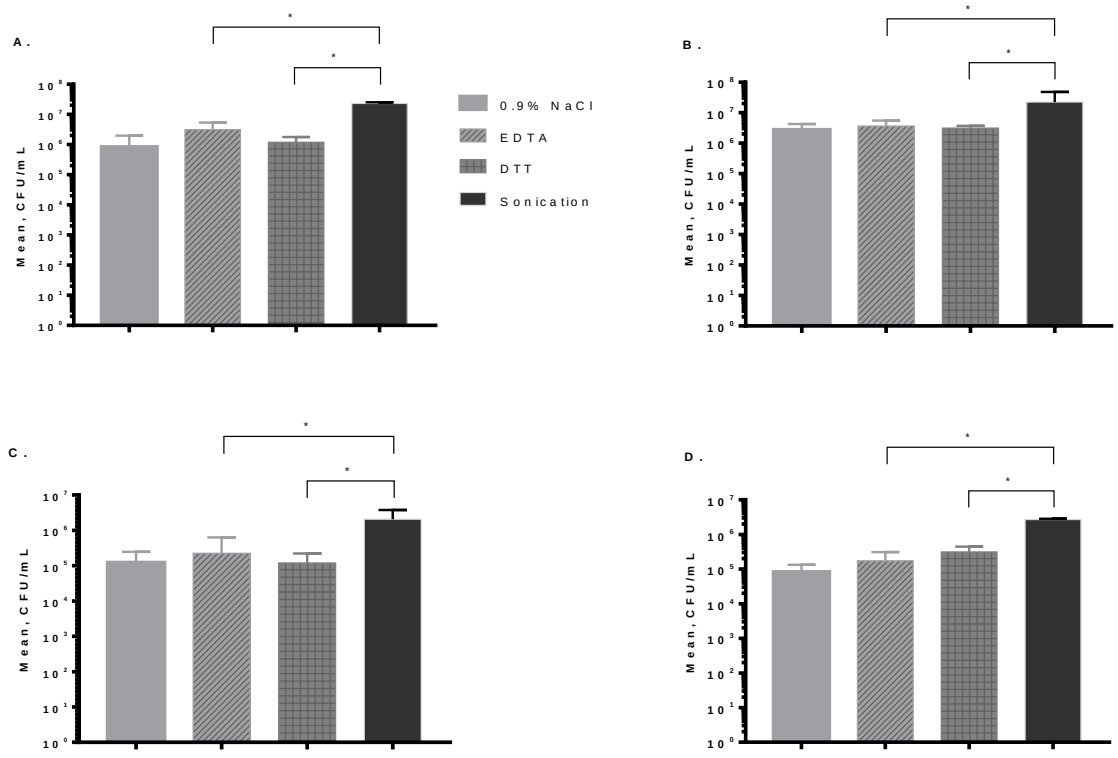


Figure 5 Comparison of quantitative analysis of biofilm dislodging methods. (A) *S. epidermidis* biofilm. (B) *S. aureus* biofilm. (C) *E. coli* biofilm. (D) *P. aeruginosa* biofilm. Mean values are shown, error bars represent standard deviation. * Statistically significant difference ($p < 0.05$). (Study C: Figure 2 in Karbysheva et al., PLOS ONE (2020)).

Isothermal microcalorimetry revealed that heat produced by samples containing sonicated glass beads with *S. epidermidis*, *S. aureus*, *E. coli* or *P. aeruginosa* biofilm was detected after 11, 12, 7.8 and 11 h, respectively. In contrast, heat production exceeding the threshold of 100 µW was observed earlier for the samples that were previously treated with EDTA (after 6.5, 6.1, 4.9 h) and DTT (after 6.4, 5.8 and 4.5 h), respectively, confirming the presence of a higher number of residual bacteria on beads treated with chemical methods in comparison to those after sonication. This time difference was statistically significant ($p < 0.05$). No difference in heat production was observed after treatment with chemicals and control (6.3, 4.6, 4.5 and 4.6 h, respectively) ($p = 0.3$), Figure 6.

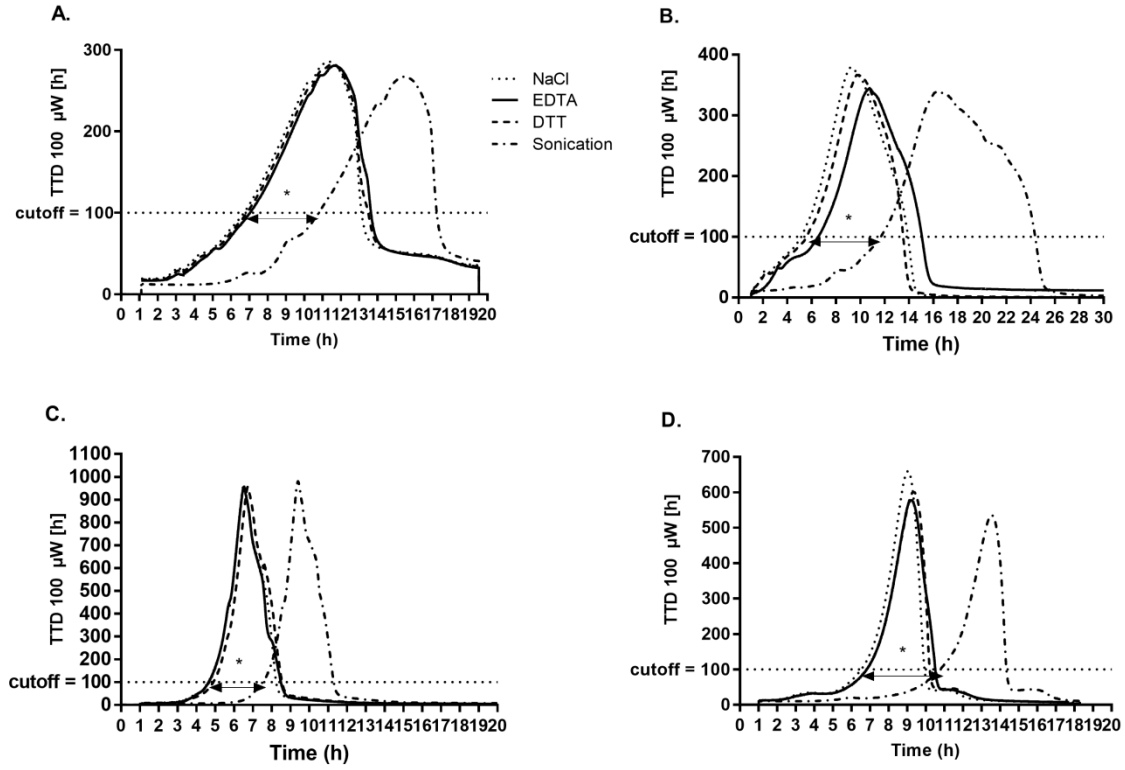


Figure 6 The microcalorimetric time to detection (TTD) of bacterial growth. (A) *S. epidermidis* biofilm. (B) *S. aureus* biofilm. (C) *E. coli* biofilm. (D) *P. aeruginosa* biofilm.
* Statistically significant difference ($p < 0.05$). (Study C: Figure 3 in Karbysheva et al., PLOS ONE (2020)).

The scanning electron microscope images showed substantial less biofilm biomass remaining on the beads for all microorganisms when sonication was applied compared to control as well as both chemical methods, Figures 7 – 10.

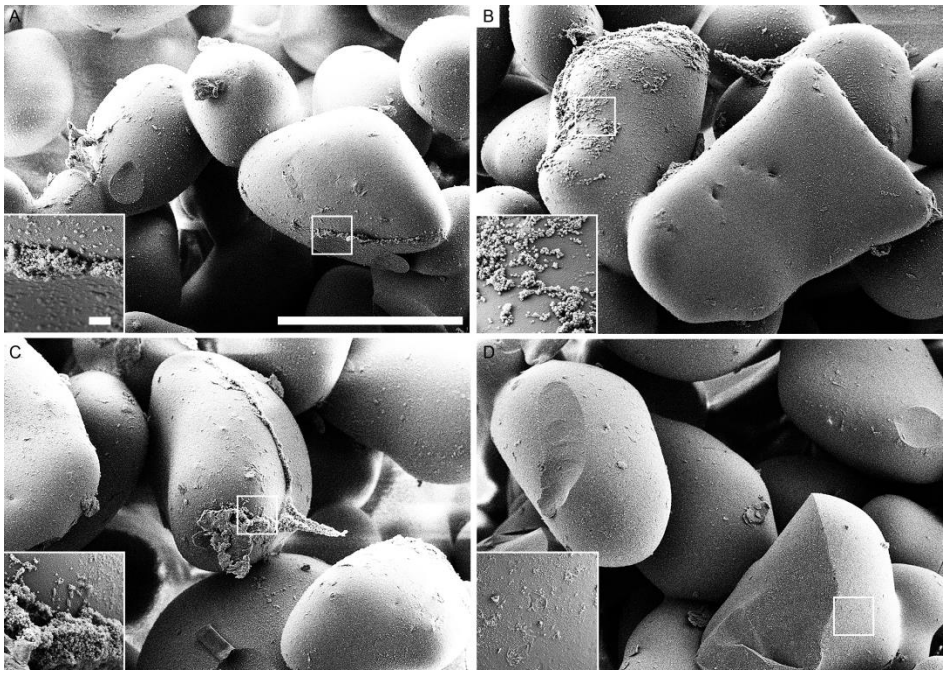


Figure 7 Scanning electron microscopy (SEM) of *S. epidermidis* biofilm. (A) beads after 0.9% NaCl treatment (control). (B) beads after EDTA treatment. (C) beads after DTT treatment. (D) beads after sonication treatment. Scale bars: 200 µm (inserts in the images represent 5 µm). (Study C: Figure 4 in Karbysheva et al., PLOS ONE (2020)).

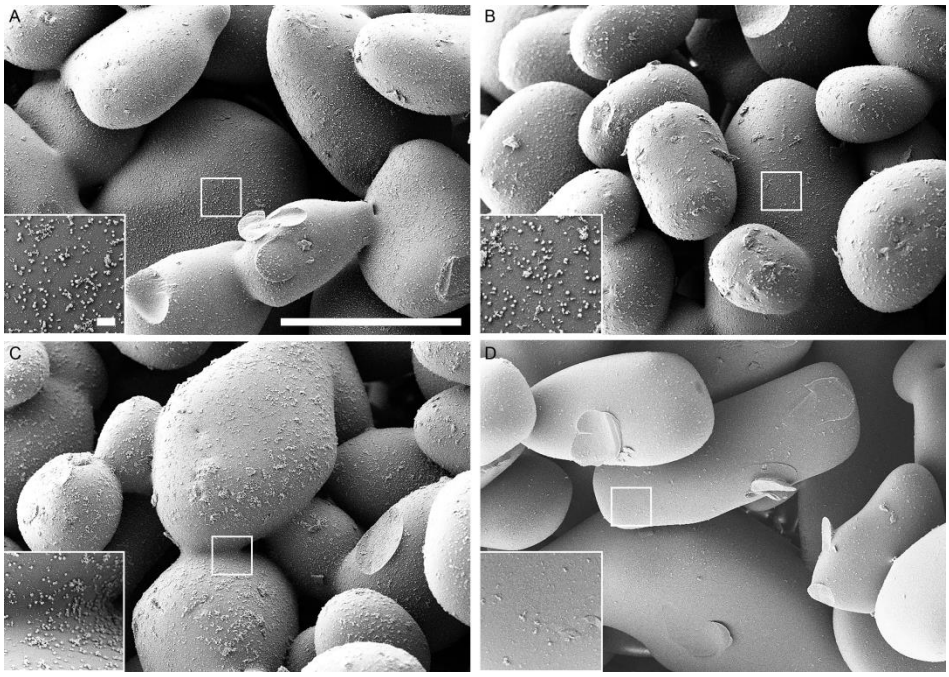


Figure 8 Scanning electron microscopy (SEM) of *S. aureus* biofilm. (A) beads after 0.9% NaCl treatment (control). (B) beads after EDTA treatment. (C) beads after DTT treatment. (D) beads after sonication treatment. Scale bars: 200 µm (inserts in the images represent 5 µm). (Study C: Figure 5 in Karbysheva et al., PLOS ONE (2020)).

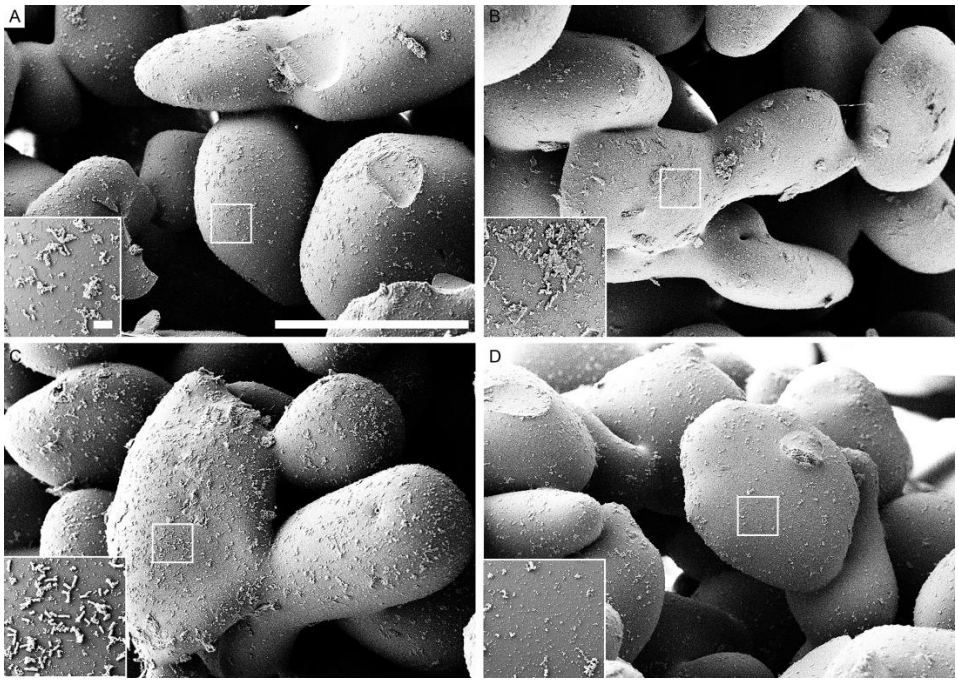


Figure 9 Scanning electron microscopy (SEM) of *E. coli* biofilm. (A) beads after 0.9% NaCl treatment (control). (B) beads after EDTA treatment. (C) beads after DTT treatment. (D) beads after sonication treatment. Scale bars: 200 µm (inserts in the images represent 5 µm). (Study C: Figure 6 in Karbysheva et al., PLOS ONE (2020)).

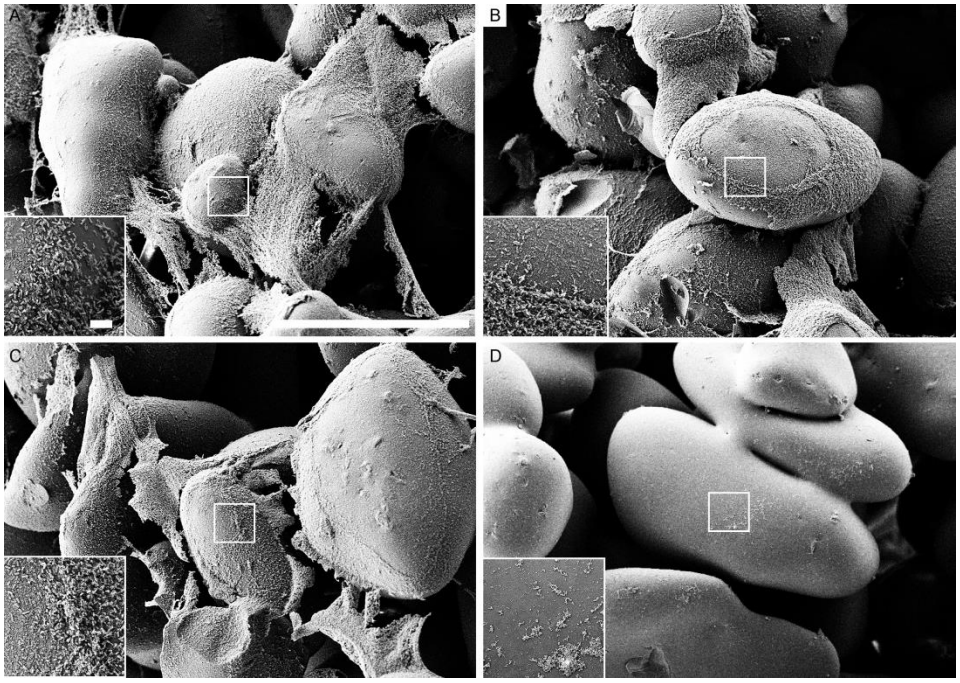


Figure 10 Scanning electron microscopy (SEM) of *P. aeruginosa* biofilm. (A) beads after 0.9% NaCl treatment (control). (B) beads after EDTA treatment. (C) beads after DTT treatment. (D)

beads after sonication treatment. Scale bars: 200 µm (inserts in the images represent 5 µm). (Study C: Figure 7 in Karbysheva et al., PLOS ONE (2020)).

Influence of the type of biomaterial of retrieved hip and knee prosthesis on microbial detection by sonication

Study D showed that applying sonication method, hip and knee polyethylene liners grew bacteria in 100%, followed by femoral components of the knee (90%) and acetabular cups (88%), while femoral head and stem both showed the lowest positivity rate of 50%, Figure 11.

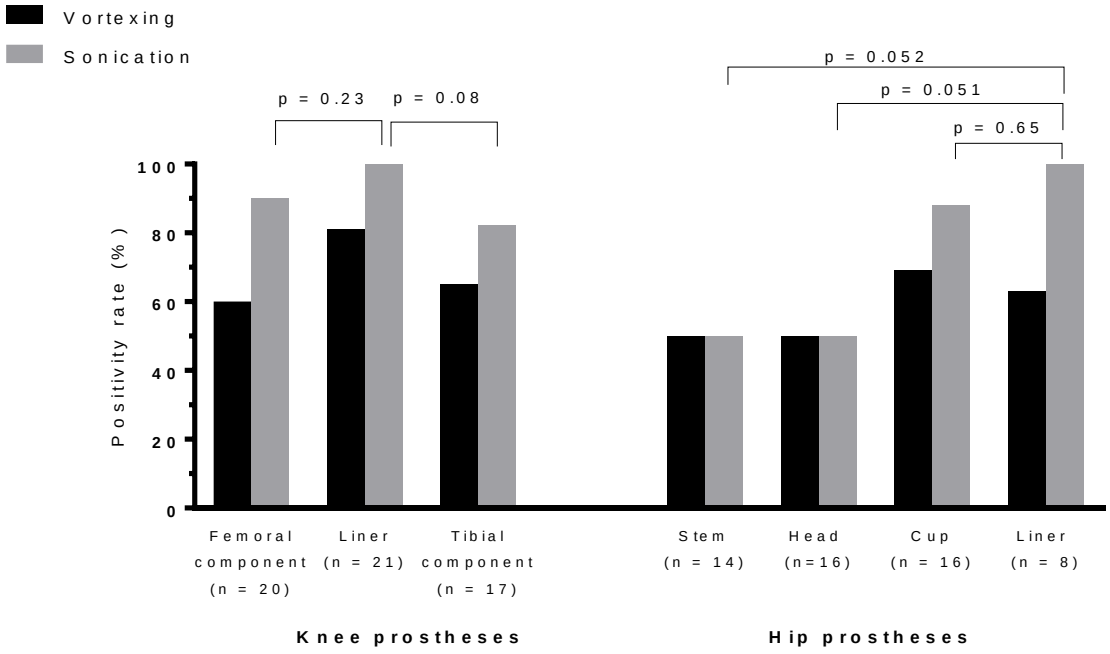


Figure 11 Positivity rate of cultures after vortexing and sonication according to the type of component in knee and hip prostheses. (Study D: Figure 1 in Karbysheva et al., European Cells and Materials (2019)).

Stratified according to the type of biomaterial bacteria in sonication fluid cultures grew in all polyethylene components, followed by titanium alloy (79%) and components made of cobalt-chromium (71%), Figure 12.

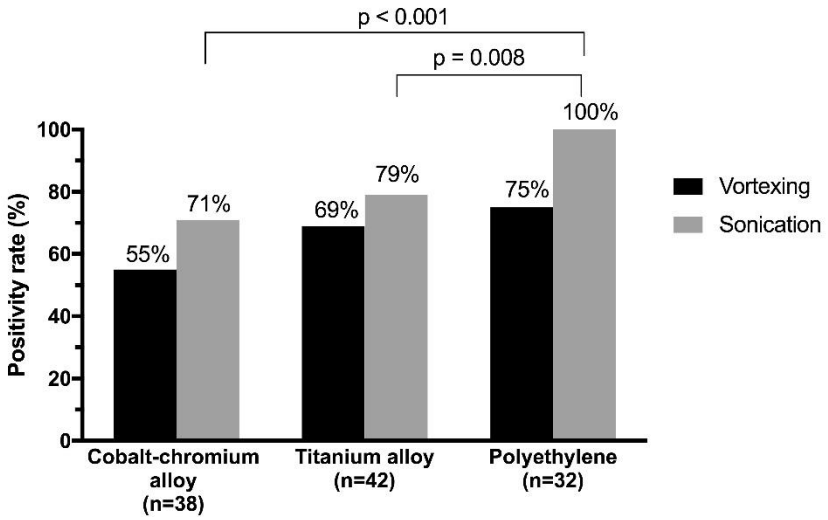
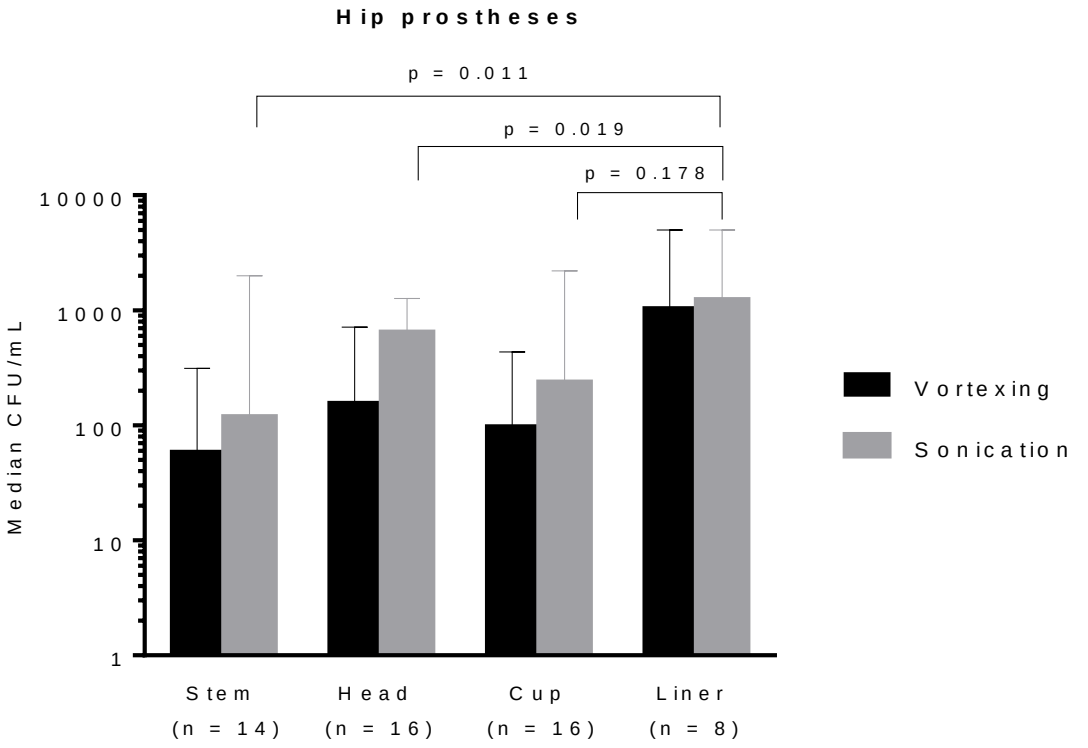


Figure 12 Positivity rate of cultures after vortexing and sonication according to the type of material. (Study D: Figure 2 in Karbysheva et al., European Cells and Materials (2019)).

Using sonication, significantly higher CFU/mL were found on hip liners, median (1250 CFU/mL), than on femoral stems, median (120 CFU/mL, $p = 0.011$) or heads, median (650 CFU/mL, $p < 0.019$), but not on cups, median (270 CFU/mL, $p = 0.178$). In knees, polyethylene liners showed significantly higher CFU/ml, median (2000 CFU/mL) than on femoral knee components, median (330 CFU/mL, $p = 0.021$), but not on tibial components, medial (800 CFU/mL, $p = 0.149$), Figure 13.


Hip prostheses
p = 0.011
p = 0.019
p = 0.178
10000
1000
100
10
1
Median CFU/mL
Stem (n = 14)
Head (n = 16)
Cup (n = 16)
Liner (n = 8)
Vortexing
Sonication

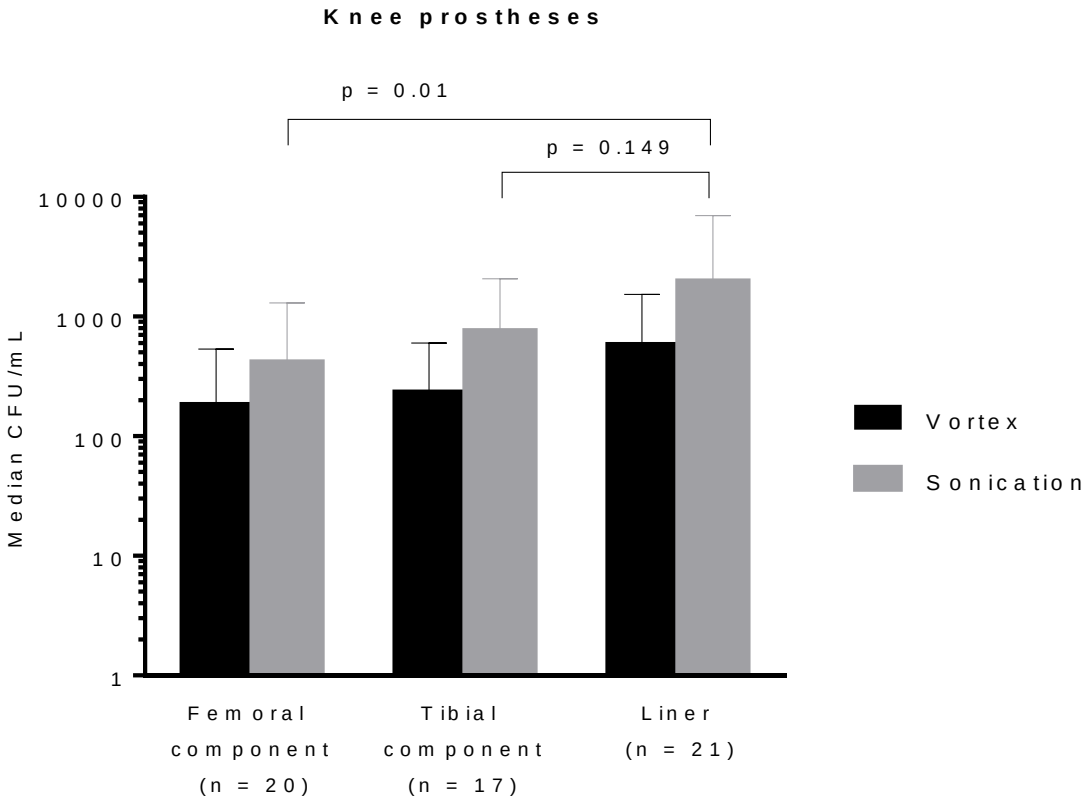

Knee prostheses
p = 0.01
p = 0.149
10000
1000
100
10
1
Median CFU/mL
Femoral component (n = 20)
Tibial component (n = 17)
Liner (n = 21)
Vortex
Sonication

Figure 13 Quantitative analysis of dislodged bacteria by vortexing and sonication (CFU/mL) in hip and knee prostheses according to the type of prosthesis component (median values are shown, error bars represent interquartile range). (Study D: Figure 3 in Karbysheva et al., European Cells and Materials (2019)).

The highest bacterial load was detected from polyethylene by using sonication, which was significantly higher than from any tested metal alloys (cobalt-chromium and titanium) by using sonication ($p < 0.05$), Figure 14.

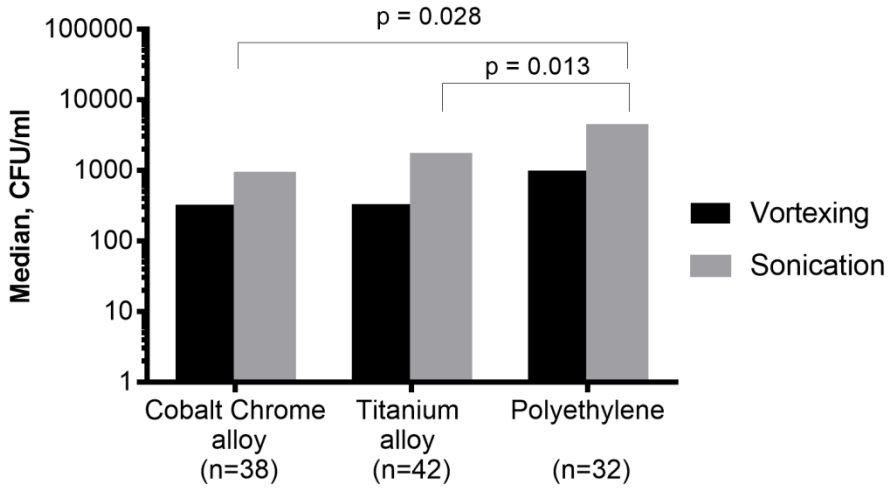


Figure 14 Quantitative analysis of dislodged bacteria by vortexing and sonication (CFU/mL) according to the type of material (median values are shown, error bars represent interquartile range). (Study D: Figure 4 in Karbysheva et al., European Cells and Materials (2019)).

5. DISCUSSION

The accurate diagnosis of implant-associated infections is based on the combination of clinical signs and laboratory tests (39). However, particularly chronic infections, are clinically difficult to distinguish from AF as patients often present with only discrete clinical symptoms. None of the routine blood tests such as leukocyte count, ESR, CRP or procalcitonin (PCT) have sufficient sensitivity or specificity to diagnose or exclude PJI. In PJI caused by low-virulence pathogens systemic inflammatory markers are often normal (40-42). Biomarkers in synovial fluid such as leukocyte count, alfa-defensin, leukocyte esterase and calprotectin nowadays used for the diagnosis of PJI are host-specific and normally elevated after surgery and in patients with crystal-induced inflammation and other conditions inducing aseptic inflammation. Therefore in Study A and B we evaluated the performance of D-lactate (bacterial specific

metabolite) in synovial fluid as independent diagnostic marker for the diagnosis of PJI. Although this method is known for many years, currently only a few colorimetric and fluorimetric assays are available and in use only for research and development purposes. Therefore, before the implementation of such kits in clinical practice, it is important to define the optimal synovial fluid D-lactate cut-off to discriminate septic from aseptic prosthetic failure. In our study the optimal synovial fluid D-lactate cut-off was 1.3 mmol/l with high sensitivity and specificity independent of the applied definition criteria (MSIS or institutional criteria). Synovial fluid D-lactate showed good performance for the diagnosis of PJI, at least comparable to synovial fluid leukocyte count which is routinely used as a gold standard. Furthermore, we found that the concentration of D-lactate reflects the virulence of the bacterial species and its microbial burden, which explains the observed differences between different species of bacteria. Advantages of the D-lactate test are low volume of synovial fluid required (50 µl) and the low expense, comparable to conventional tests (bacterial culture and leukocyte count) (calculated on actual production costs). However, both colorimetric and fluorimetric assays require complex instrumentation (a spectrometer or an optical reader) to estimate the concentration of D-lactate in real samples, which takes up to 2 h to get the final result. The good diagnostic performance of D-lactate test showed in our studies makes it attractive for future research developing a rapid point-of-care test for PJI. The main limitation of our studies is the lack of patients with rheumatic joint disease or crystal-induced arthritis, making conclusions about the value of synovial fluid D-lactate in aseptic inflammatory joint conditions impossible. However, rheumatic diseases are today less common in patients with prosthetic joints; this situation is more relevant in acute arthritis of native joints. In our study, 5 patients with AF had underlying rheumatologic joint disease, among whom in one D-lactate was positive (1.3 mmol).

In Study C and D we focused to improve the intraoperative diagnostic of implant-associated infections using microbiological investigation of explanted devices. The detection of bacteria from these samples is hampered due to biofilm formation on the implant surface. In order to isolate and identify the microorganism responsible for the infection, the dislodgment and dispersion of this sessile community represent the first step before plating the specimen on culture medium (23).

In Study C we investigated the ability of different approaches for biofilm dislodgement, including mechanical (sonication) and chemical dislodgement using EDTA or DTT. The ability of EDTA and DTT to disaggregate biofilm by chemical interactions was proposed to be suitable for the biofilm detachment (24, 25, 43). Our study revealed that mean colony count obtained after treatment of bacterial biofilms with EDTA and DTT was similar to those observed after

treatment with 0.9% NaCl used as control. In contrast, sonication was superior to the chemical method for dislodgement of bacterial biofilms. Significantly higher CFU counts of *S. epidermidis, S. aureus, E. coli* and *P. aeruginosa* biofilm were detected after sonication compared to both chemical agents. Our results derived from colony counting of dislodged bacterial cells were confirmed by two additional independent techniques, namely isothermal microcalorimetry and SEM imaging. Isothermal microcalorimetry showed a significant delay in the detection of bacterial metabolism-related heat production from the beads pretreated with sonication compared to chemical treatments (EDTA and DTT), suggesting that significantly less bacteria remained attached to the beads after sonication. To visualize the bacteria remaining in the biofilms on the glass beads surface after treatment with either chemicals or sonication methods, the SEM was used. Significantly less biofilm remaining on the beads when sonication was applied compared to chemical methods was visualized by SEM. Recently published *ex vivo* studies showed that treatment of explanted prosthesis with DTT may be superior to sonication for the diagnosis of periprosthetic joint infection (43-45). The different types of biomaterial used for *ex vivo* biofilm studying may in part explain the discordance of the results. There are several limitations of our study. First, anaerobes (e.g. *Cutibacterium spp.*) were not tested. It is possible that anaerobes may show better results with chemical methods due to their lower susceptibility to sonication. Second, only laboratory strains were used for biofilm formation. Typically, the use of clinical strains in the evaluation of new methods is restricted due to large variability. Third, we used only porous glass beads for biofilm formation. Due to a high volume-to-surface ratio this model is probably more suitable for biofilm investigation than smooth materials.

Given the evidence in previous studies that various types of materials have different affinity for microbial adherence and biofilm formation (32, 46), we aimed to investigate in Study D the influence of the material type of the implants, such as polyethylene, titanium and cobalt-chromium alloy, on the biofilm formation and to evaluate bacterial burden on the implant surface using sonication as the most efficient method for biofilm dislodgement and bacterial detection as was shown in Study C. The removed biofilm from explanted implants was quantitatively and qualitatively evaluated and extrapolated back to the total biofilm biomass originally present on the prosthesis surface. Quantitative analysis showed that bacterial count was significantly higher after sonication of polyethylene than of metal alloys. Qualitative assay revealed that bacteria grew in all polyethylene components, followed by titanium alloy (79%) and components made of cobalt-chromium (71%). Our findings were consistent with results published by Patel *et al.*, observing that the highest level of bacterial adherence was detected

on highly cross-linked polyethylene, followed by titanium, stainless steel, and trabecular metal, with the lowest level of bacterial adherence occurring on the cobalt-chromium alloy (47). The adhesion of bacteria to the implant surface depends on many factors related to the biomaterial's intrinsic properties, particularly its chemistry and physical properties (e.g. roughness and surface charge) (48, 49). Alam *et al.* showed that 85% of bacteria adhering to polyethylene surfaces remained alive and in active proliferation. This could explain the faster growth of bacteria on the polyethylene surface compared to metal, which induces various adverse effects against bacterial adhesion and proliferation, caused by the release of ions (50). On the other hand, polyethylene components of prosthetic joints may begin degradation under physiological conditions more rapid than metal components, increasing the roughness and thereby bacterial biofilm formation. These results suggest that sonication of retrieved polyethylene liners may be sufficient for the detection of the infecting pathogen in patients with chronic PJI, rather than submitting the whole prosthesis for sonication. The limitation of our study was that surface features were not analyzed. A further limitation was the inability to calculate the exact component surface in relation to the fluid used for sonication.

Taken together, the evolution in the diagnostic of implant-associated musculoskeletal infection represents a major milestone in the regenerative therapy and contributes to a successful surgical and antimicrobial therapy of PJI and other implant-associated infections. The reported findings of this project suggest that the application of new diagnostics methods in the *preoperative* stage, such as bacterial specific biomarkers (D-lactate) in the synovial fluid, in combination with novel approaches in the *intraoperative* diagnostic, using sonication of polyethylene components of the implants, may provide paramount improvements for the diagnosis of biofilm-associated infections.

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STATUTORY DECLARATION

"I, Svetlana Karbysheva, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic "Improvement of the diagnosis of implant-associated infection", independently and without the support of third parties, and that I used no other sources and aids than those stated.

All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me."

Date Signature

DECLARATION OF SHARE IN PUBLICATION

Svetlana Karbysheva had the following share in the following publication:

Publication 1. Katsiaryna Yermak[1], **Svetlana Karbysheva[1]**, Carsten Perka, Andrej Trampuz, Nora Renz. Performance of synovial fluid D -lactate for the diagnosis of periprosthetic joint infection: A prospective observational study. Journal of Infection (2019); 123-129, doi: https://doi.org/10.1016/j.jinf.2019.05.015.

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Contribution in detail:

- Planning and organization of all experiments in consultation with Mr. PD Dr. Trampuz.
- Execution of the experiments, in detail: processing of spectophotometric measurements, impact of various factors on precision of the spectophotometric measurements, creating formulas, determination of the D-lactate cutoff .
- Participation in experiments to accumulate a portion of the data and evaluation of all data, interpretation of all results and participation in the implementation of the data analysis.
- Writing the manuscript and contributing to the final version of the paper.

Publication 2. **Svetlana Karbysheva**, Katsiaryna Yermak, Ludmila Grigoricheva, Nora Renz, Carsten Perka, Andrej Trampuz. Synovial Fluid D-Lactate – a novel pathogen-specific biomarker for the diagnosis of periprosthetic joint infection. Journal of Arthroplasty (2020); 2223-2229, doi: https://doi.org/ 10.1016/j.arth.2020.03.016

Contribution in detail:

- Planning and organization of all experiments in consultation with Mr. PD Dr. Trampuz.
- Carrying out all experiments, in detail: processing of spectophotometric measurements, evaluation of synovial fluid D-lactate concentration stratified according to isolated pathogen, creating formulas, determination of the D-lactate cutoff.
- Processing and evaluation of all data, collecting demographic data of the patients and infection characteristics, interpretation of all results and participation in the

interpretation of the results. From my evaluation, tables 1 and 2, figures 1 and 2 were created.

- Writing the manuscript and contributing to the final version of the paper.

Publication 3. **Svetlana Karbysheva**, Mariagrazia Di Luca, Maria Eugenia Butini1, Tobias Winkler, Michael Schütz, Andrej Trampuz. Comparison of sonication with chemical biofilm dislodgement methods using chelating and reducing agents: implications for the microbiological diagnosis of implant associated infection. PLOS ONE (2020); e0231389, doi: https://doi.org/ 89. https://10.1371/journal.pone.0231389

Contribution in detail:

- Planning and organization of all experiments in consultation with Mr. PD Dr. Trampuz.
- Carrying out all experiments, in detail: Formulation of different concentrations of the chemical substances (EDTA and DTT) for biofilm removal from the implants.
- Processing and evaluation of all data, interpretation of all results and participation in the interpretation of the results. From my evaluation, figures 1, 2, 3, 4, 5, 6 and 7 were created.
- Writing the manuscript and contributing to the final version of the paper.

Publication 4. **Svetlana Karbysheva**, Ludmila Grigoricheva, Vadim Golnik, Sergey Popov, Nora Renz, Andrej Trampuz. Influence of the type of biomaterial of retrieved hip and knee prosthesis on microbial detection by sonication. European Cells and Materials (2019); 16-22, doi: https://doi.org/10.22203/eCM.v037a02.

Contribution in detail:

- Planning and organization of all experiments in consultation with Mr. PD Dr. Trampuz.
- Carrying out all experiments, in detail: Evaluation of the formation of a biofilm on various types of implant material such as polyethylene, titanium and cobalt-chromium alloy. The quantitative determination of bacteria in ultrasound cultures.
- Processing and evaluation of all data, interpretation of all results and participation in the interpretation of the results. Tables 1, 2 and Figures 1, 2, 3 and 4 were created on the basis of my statistical evaluation.
- Writing the manuscript and contributing to the final version of the paper.

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Signature, date and stamp of the supervising University teacher

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Signature of the doctoral candidate

SELECTED PUBLICATIONS

Publication 1 (Study A): Performance of synovial fluid D-lactate for the diagnosis of periprosthetic joint infection: A prospective observational study

Katsiaryna Yermak[1], **Svetlana Karbysheva[1]**, Carsten Perka, Andrej Trampuz, Nora Renz

[1]authors equally contributed as first authors

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Impact factor (2018): 5.099

Publication 2 (Study B): Synovial Fluid D-Lactate – a novel pathogen-specific biomarker for the diagnosis of periprosthetic joint infection

Svetlana Karbysheva, Katsiaryna Yermak, Ludmila Grigoricheva, Nora Renz, Carsten Perka, Andrej Trampuz

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Publication 3 (Study C): Comparison of sonication with chemical biofilm dislodgement methods using chelating and reducing agents: implications for the microbiological diagnosis of implant associated infection

Svetlana Karbysheva, Mariagrazia Di Luca, Maria Eugenia Butini1, Tobias Winkler, Michael Schütz, Andrej Trampuz

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Publication 4 (Study D): Influence of the type of biomaterial of retrieved hip and knee prosthesis on microbial detection by sonication

Svetlana Karbysheva, Ludmila Grigoricheva, Vadim Golnik, Sergey Popov, Nora Renz, Andrej Trampuz

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CURRICULUM VITAE

My curriculum vitae does not appear in the electronic version of my thesis for reasons of data protection.

COMPLETE LIST OF PUBLICATIONS

Published peer-reviewed articles

1. Katsiaryna Yermak[1], **Svetlana Karbysheva[1]**, Carsten Perka, Andrej Trampuz, Nora Renz (2019). Performance of synovial fluid D-lactate for the diagnosis of periprosthetic joint infection: A prospective observational study
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2. **Svetlana Karbysheva**, Katsiaryna Yermak, Ludmila Grigoricheva, Nora Renz, Carsten Perka, Andrej Trampuz (2020). Synovial Fluid D-Lactate – a novel pathogen-specific biomarker for the diagnosis of periprosthetic joint infection
   Journal of Arthroplasty; 2223-2229
   doi: https://doi.org/ 10.1016/j.arth.2020.03.016
   Impact factor (2018): 3.524
3. **Svetlana Karbysheva**, Mariagrazia Di Luca, Maria Eugenia Butini1, Tobias Winkler, Michael Schütz, Andrej Trampuz (2020). Comparison of sonication with chemical biofilm dislodgement methods using chelating and reducing agents: implications for the microbiological diagnosis of implant associated infection
   PLOS ONE; e0231389
   doi: https://doi.org/10.1371/journal.pone.0231389
   Impact factor (2018): 2.776
4. **Svetlana Karbysheva**, Ludmila Grigoricheva, Vadim Golnik, Sergey Popov, Nora Renz, Andrej Trampuz (2019). Influence of the type of biomaterial of retrieved hip and knee prosthesis on microbial detection by sonication
   European Cells and Materials; 16-22
   doi: https://doi.org/10.22203/eCM.v037a02
   Impact factor (2019): 3.682
5. Mariagrazia Di Luca, Anna Koliszak, **Svetlana Karbysheva**, Anuradha Chowdhary, Jacques F. Meis, Andrej Trampuz (2019). Thermogenic Characterization and Antifungal Susceptibility of *Candida auris* by Microcalorimetry
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Manuscript under preparation

1. **Svetlana Karbysheva**, Sabrina Cabric, Anna Koliszak, Andrej Trampuz. Performance of MicroDTTect for the diagnosis of prosthetic joint infections compared with sonication method.

Abstracts at international conferences

1. **Svetlana Karbysheva**, Sabrina Cabric, Anna Koliszak, Andrej Trampuz (2018). Performance of MicroDTTect for the diagnosis of PJI compared with sonication method. Poster presentation at 28th European Conference of Clinical Microbiology and Infectious Diseases (21-24 April 2018), Madrid, Spain
2. **Svetlana Karbysheva**, Michael Schütz, Andrej Trampuz (2018). D-lactate, a bacterial specific marker for the accurate diagnosis of prosthetic joint infection. Poster presentation at 28th European Conference of Clinical Microbiology and Infectious Diseases (21-24 April 2018), Madrid, Spain
3. **Svetlana Karbysheva**, Mariagrazia Di Luca, Maria Eugenia Butini, Michael Schütz, Andrej Trampuz (2018). Comparison of sonication and chemical methods for biofilm detection, including chelating and reducing agents. Oral presentation at 28th European Conference of Clinical Microbiology and Infectious Diseases (21-24 April 2018), Madrid, Spain
4. **Svetlana Karbysheva**, Ludmila Grigoricheva, Michael Schütz, Andrej Trampuz (2018). Performance of synovial fluid D-Lactate for the diagnosis of low-grade and culture negative PJI. Oral presentation at 19th European Congress of European Federation of National Associations of Orthopaedics and Traumatology (30 May-01 June 2018), Barcelona, Spain
5. **Svetlana Karbysheva**, Ludmila Grigoricheva, Michael Schütz, Andrej Trampuz (2018). D-lactate, a bacterial specific marker for the diagnosis of prosthetic joint infection and septic arthritis. Oral presentation at 37th Annual Meeting of the European Bone and Joint Infection Society (6-8 September 2018), Helsinki, Finland
6. **Svetlana Karbysheva**, Mariagrazia Di Luca, Maria Eugenia Butini, Michael Schütz, Andrej Trampuz (2018). Comparison of sonication and chemical methods for biofilm detection, including chelating and reducing agents. Oral presentation at 37th Annual Meeting of the European Bone and Joint Infection Society (6-8 September 2018), Helsinki, Finland

7. **Svetlana Karbysheva**, Yermak K, Trampuz A. (2018). Synovial fluid D-lactate, a bacterial-specific marker for infection of prosthetic joints. Poster presentation at 9th BSRT Symposium (28-30 November 2018), Berlin, Germany
8. **Svetlana Karbysheva**, Sabrina Cabric, Anna Koliszak, Andrej Trampuz (2019). Comparison of chemical biofilm dislodgement (MicroDTTect) and sonication for the diagnosis of periprosthetic joint infection. Oral presentation at 20th European Congress of European Federation of National Associations of Orthopaedics and Traumatology (5-7 June 2019), Lisbon, Portugal
9. **Svetlana Karbysheva**, Ludmila Grigoricheva, Andrej Trampuz (2019). Influence of biomaterial type of retrieved hip and knee prosthesis on microbial detection by sonication. Oral presentation at 20th European Congress of European Federation of National Associations of Orthopaedics and Traumatology (5-7 June 2019), Lisbon, Portugal

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