PhD THESIS

DIAGNOSIS OF SEPTIC ARTHRITIS IN HUMAN SYNOVIUM SAMPLES USING DSC

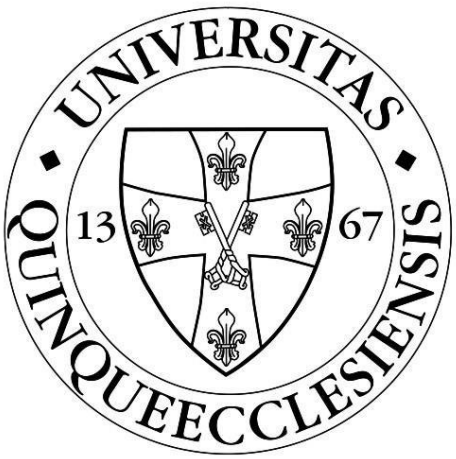
ÁRPÁD LEVENTE DANDÉ MD.

Doctoral School of Interdisciplinary Medical Sciences D93

Leader of Doctoral School: Balázs Sümegi PhD, DSc

Head of the Program: Miklós Nyitrai PhD, DSc

Supervisors: Lőrinczy Dénes PhD, DSc, Norbert Wiegand MD, PhD



University of Pécs, Medical School

Department of Traumatology and Hand Surgery

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I. Introduction

I.1. Literature review

In addition to major advances in medicine, the unprecedented development of biochemistry, biophysics and electronics during the twentieth century has led to the development of novel, cutting-edge medical devices, medicinal products and technologies, which become indispensable for modern therapies. With the advancement of information technology and nanotechnology, exceptional results and discoveries have been made in many fields of research over the past decades around the globe. Parallel to this, state-of-the-art medical diagnostic tools and advanced surgical equipment have become part of the daily practice in primary care and in hospitals with advanced clinical background.

Significant advances have also been made in the rehabilitative surgery of the musculoskeletal system and in the reconstruction of degenerative joint diseases by the continuous improvement of implants used in locomotor surgery and by the implementation of modern surgical techniques. Despite the minimal invasive surgical methods, state-of-the-art implants, and the aseptic modern operating environment, infections at the area of implanted prostheses continue to pose a serious challenge.

Following the isolation and purification of penicillin in 1940, and the identification of its chemical structure, the pharmaceutical industry has created the opportunity for the commercial production of synthetic penicillin. Used in wound healing as early as World War II, penicillin has made the allied troops successful for being able to heal infected wounds, and thus preventing amputations and cases of death.

After the purification of penicillin in 1940 and the subsequent synthesis of its derivatives, a number of broad spectrum antibiotics have been successfully used for the treatment of bacterial infections of various pathologies. Despite initial success, the resistance to penicillin detected in certain hospital strains of *Staphylococcus aureus* in 1942 has risen to 80% over two decades. Following the introduction of methicillin in 1961, methicillin-resistant *S. aureus* (MRSA) strains [1], which are now found worldwide along with the rapid rise of antibiotic resistance, have gradually emerged.

Microorganisms (bacteria, fungi, and viruses) can cause severe conditions in the musculoskeletal system, including the joints, which may endanger not only the integrity of joint and bone, but also the life of the patient. Timely recognition and appropriate treatment of the pathologies are urgent issues and constitute one of the most important tasks for healthcare professionals in the field of musculoskeletal disorders (MSDs). It is important to collaborate with other professionals involved in MSDs and to extend their knowledge through appropriate training.

The analysis of synovial fluid samples (composition, microbiological assays) is paramount in the diagnosis and treatment of septic arthritis. From a therapeutic point of view, a novel procedure which is faster than microbiological culture assays would be extremely useful.

While diagnosis is based on isolation of bacterium species from synovial fluid samples, patient history, clinical appearance, laboratory results, and image analysis are also important. Quick recognition, immediate aggressive antibiotic therapy, and appropriate surgical treatment are essential for a good prognosis. However, even with a quick diagnosis and treatment, mortality rates remain high [2]. Depending on the clinical picture and the primary diagnostic results, arthroscopic joint dranaige, lavage, extended joint exploration, synovectomy, or necrectomy may be considered during emergency care. In many cases, a belated surgical intervention [3] results in extensive tissue necrosis (necrosis of synovia and the stabilising ligaments, or softening, detachment, and necrosis of the cartilage surface).

In many cases, without isolating the bacterium, and in the presence of a masked clinical picture, the patient does not receive appropriate treatment, which can lead to severe joint damage, usually resulting in permanent lesions. In contrast, *Gonococcal* arthritis is often successfully treated with antibiotic therapy, with a very low complication rate and excellent prognosis for complete return to normal joint function [2].

Although a number of international comprehensive studies address the diagnostic criteria of periprosthetic and joint infections, we can conclude that a single confirmatory test for an infection does not provide a conclusive diagnosis.

With the rapid advancement of biotechnology, medical diagnostic procedures and equipment, technologies that are partly forgotten, and other untapped potentials could bring success stories in the fight against microorganisms.

Every year, 20,000 septic arthritis cases are reported in the United States, with a prevalence of 7–8 cases per 100,000 average population, which is close to the European figure, with an estimated incidence of 6 cases per 100,000 people [12].

In recent years, the prevalence of *Pneumococcal* arthritis has decreased, and at the same time, the proportion of Gram-negative, non-group A *Streptococcus* and anaerobic pathogens has increased. Other than *Gonococcus*, the most commonly identified pathogens in the synovial fluid of the adult population are various *Staphylococcus* strains.

I.2. Diagnostic options and criteria

Joint and major joint purulent periprosthetic infection and subsequent ulceration, indicated by an array of physical examinations and imaging methods (X-ray, ultrasound, CT, MRI, scintigraphy) and supported by laboratory tests, undoubtedly require immediate attention. In many cases, however, in the complete or partial absence of symptoms in a patient receiving immunosuppression, specific laboratory diagnostic procedures become essential. In the clinical practice, effective treatment can be achieved based on the combined use of serological markers, synovial tests, and microbiological inoculations [14].

According to some studies, it is important for microbiological sampling that patients should not receive systemic antibiotic treatment before surgery [15 - 18], and that they receive therapeutic or prophylactic antibiotic doses during surgery only, after samples for the microbiological test have been collected [14]. If immediate treatment with antibiotics is required, it is recommended that at least joint puncture be performed prior to administration of the antibiotic. It is important that, wherever possible, the pathogen is identified before the planned surgery, so that treatment optimised for the patient and the pathogen can be selected.

At least 3-5 sites should be sampled during the surgical procedure. It is worth collecting samples from well-defined locations, which should always include that

surface of the bone which is in contact with the prosthesis. In case of an infection around the hip joint prosthesis, the joint capsule, the area around the prosthetic shaft, and the region of the joint socket appear to be suitable for sampling. For infection around the knee prosthesis, the synovial membrane, the femoral component, the tibial component, the patella, and the dorsal region of the capsule appear to be appropriate sites for sampling [19, 20, 21]. If samples intended for microbiological examination are processed within 24 hours, it is advisable to submit separate tissue samples in sterile tubes without transport medium to facilitate the isolation of the pathogens.

To avoid false-negative inoculations, Trampuz et al. recommend sonication [22], whereby following removal of the prosthesis, the bacteria are recovered from the biofilm in an ultrasonic sonicator, after the removed prosthesis is placed under sterile conditions (in a plastic airtight container) and transferred to the laboratory. Isolated bacteria are then examined by microbiological methods [23].

When performing a histological examination, the specimen is informative only if it comes from a site around the prosthesis (membrane around an infected prosthesis). The diagnosis of periprosthetic infection can be made if the periprosthetic tissue sample is full of infection-related cell types such as granulocytes, CD15-positive cells and macrophages, and microabscesses [24, 25].

We need to distinguish between degenerative joint diseases and inflammatory joint infections caused by pathogens. Gout-induced arthropathies usually develop and are more common in middle-aged men. In more than half of the cases, gout attacks start at the first metatarsophalangeal joint, which can be triggered by joint trauma. It usually kicks off at night and the inflammatory signs are pronounced: pain, swelling and redness. Arthritis caused by untreated gout gradually leads to chronic destructive polyarthritis. Acute gout attacks are accompanied by fever, elevated CRP, and moderately accelerated erythrocyte sedimentation rate, whereas serum uric acid levels are generally high.

Septic arthritis is usually accompanied by a sudden onset of septic fever, and is localized to a single joint. However, in older patients, in patients with an immunosuppressed state, and sometimes in the presence of a joint prosthesis, fever and other inflammatory signs may be totally absent. Acute monoarthritis cases should

be considered bacterial or septic arthritis until proven otherwise. Among the inflammatory laboratory parameters, CRP is generally markedly elevated, erythrocyte sedimentation rate is accelerated, but leukocytosis may be absent. Similar to bacterial arthritis, crystalline arthritis may also have a fulminant appearance. If crystalline arthropathy is suspected, an immediate crystal analysis of the aspirate is required.

Gonococcal arthritis is often characterised by a sudden onset. It presents as mono- or oligoarthritis, usually affecting the upper limbs. Arthritis is often associated with tenosynovitis or periarthritis. Characteristics of *Gonococcal* arthritis include pustular skin lesions and transient joint pain.

Cases of viral arthritis usually present as mild poly- or oligoarthritis, characterised by a sudden onset, benign, and self-limiting. Parvovirus B19 is a common cause of adult arthritis or arthralgia. Arthritis is most often associated with rubella and arbovirus infection, which is easily recognisable by characteristic rashes. Arboviral arthritis is associated with skin rashes and pruritus. During viral arthritis, erythrocyte sedimentation rate and CRP levels are only mildly abnormal, and the synovial fluid usually contains mononuclear cells.

Borrelia arthritis may be a late manifestation of Lyme disease, in addition to the neurological syndrome and carditis. Fever, headache, myalgia and lymphadenopathy are usually present at the onset of the disease. Arthritis usually manifests as recurrent mono- or oligoarthritis [55]. Early diagnosis is essential for a favorable outcome.

In recent years, there is an urgent need to develop a rapid and reliable laboratory test to reduce the incidence of purulent periprosthetic joint infections. To this end, a research team in Philadelphia led by Deirmengian and Parvizi has aimed at developing a variety of biomarkers. The team has studied 16 peptides derived from synovial fluid, which may play a role in the detection of periprosthetic infections. Of these, alpha defensin has been shown to be the most promising candidate and has therefore been proposed for further scrutiny in the diagnosis of periprosthetic joint infection [27]. An easy-to-use rapid test (Synovasure® Alpha Defensin Test) is now available in the clinical practice, which clearly demonstrates αD positivity after 10 minutes [109].

Despite the high sensitivity and specificity of biomarkers, antibiotic susceptibility and resistance remain an open question; given that isoperibolic calorimetric tests

(DSC) are suitable for detecting these, we found that this method is duly substantiated [26].

I.3. Algorithms for the diagnosis of periprosthetic infections

The currently available diagnostic options are only capable of detecting infections with 96-98% accuracy in the best case scenario [56].

There are now clear recommendations for the verification of periprosthetic infections, due to the efforts of various professional societies, including IDSA (Infection Disease Society of America), AAOS (American Academy of Orthopedic Surgeons), and MSIS (Musculoskeletal Infection Society) [57, 14, 58].

MSIS has adopted, based on international consensus, the standard criteria for the diagnosis of periprosthetic joint infections [51], which has been used in numerous comprehensive studies [14, 52, 53, 54].

One of the following should hold true for periprosthetic joint infection:
1. Fistula leading to the joint
2. Pathogens growing from two separate tissue or fluid samples taken from the site around the prosthesis
3. Of the following six conditions, if four are met: *a. Increased* erythrocyte sedimentation rate *(We) & CRP (We>30 mm/hour; CRP>10 mg/L)* *b. Increased white blood cell (WBC) count in synovial fluid (WBC>3000 cell/μL)* *c. Increased proportion of neutrophils in synovial fluid (>65%)* *d. Presence of pus in the affected joint* *e. Successful isolation of a pathogen from a periprotetic tissue or fluid* *f. During histological examination of periprosthetic tissue with a magnification greater than x400, the number of neutrophils is greater than 5 in 5 fields of view (>5 neutrophils/ field of view)*

Table 1. Determination criteria of periprosthetic joint infection by the MSIS team.

Internationally recognized experts at the Philadelphia Conference in 2014 have reached a consensus with regards to the algorithm for the diagnosis and treatment of

periprosthetic joint infections, which is still considered to be decisive [50], despite emerging novel diagnostic options would provide other opportunities for an established diagnosis [9, 62, 26, 60, 68, 69, 70, 71, 72, 75, 76, 77, 78].

Based on the diagnostic algorithm for joint and periprosthetic infections, it is important that the diagnosis be made as soon as possible, and thereafter the progression of the infection and the time course of the infection be judged, on the basis of which it is recommended to develop a therapeutic plan and start the treatment.

I. 4. DSC, a neglected method

DSC (Differential Scanning Calorimetry) is a highly sensitive instrument for measuring structural and conformational changes in biological and biochemical processes, which can be used in the temperature range of -20°C to 100°C. The device operates on the principle of measuring the heat flux. A heat sink block contains the measuring and the reference cells. The block can be heated/cooled using a program. The measured output parameters are the heat flux as a function of time or temperature. To execute the program, the control signal is provided by the temperature differences between the heat sink, the sample holder, and the sample-reference cell. The sample is loaded in a way that the heat capacity of the two cells is approximately the same (Figure 1). Thus, during programmed heating, the temperature of the two vessels changes in the same way, their temperature difference being zero (the reference is the sample buffer) until some process (endo/exothermic) occurs in the measuring cell containing the macromolecular system (Figure 2).

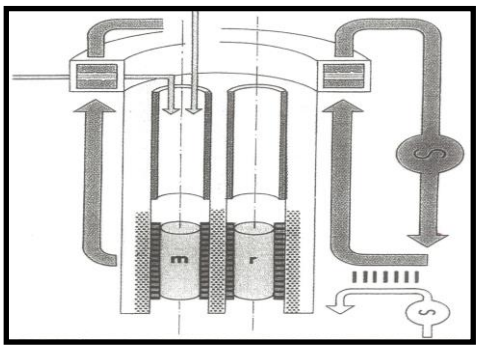

Figure 1. Schematic arrangement of the cells of a SETARAM Micro DSC-II calorimeter.

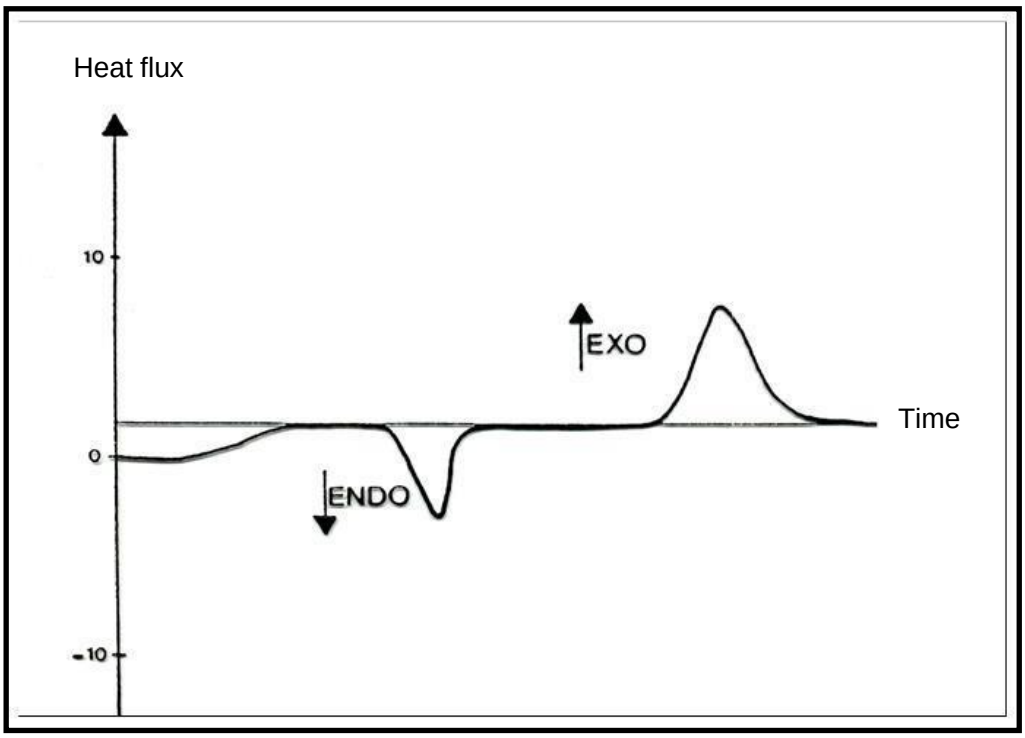

Figure 2. Measurable output signal of the SETARAM Micro DSC-II.

Depending on the polarity of the temperature difference and its magnitude, power must be supplied to either the measuring or the reference cell to maintain thermal balance between the original heating program and the cells. The system records this energy (heat flux, e.g. mW) as an output signal either as a function of time or the actual temperature. Therefore, the information directly obtained from the process is the area (integrate) below the output signal, which is the so-called calorimetric enthalpy (since the cells are under constant pressure due to the complete seal). This is the energy that is required to transform the sample from one conformation to another macromolecular structure, or that is released during e.g. crystal formation. From the graphical processing of the data, the heat capacity of the sample at constant pressure can easily be obtained (since the system is inhomogeneous), which indicates the difference between the native and denatured states.

II. Aims

The aim of this dissertation is to map diagnostic procedures for the acceleration of the timely recognition and treatment of septic periprosthetic joint inflammatory processes, and to implement them in the clinical practice.

To confirm the low sensitivity of the hypothesized conventional microbiological inoculations, a retrospective clinical study covering the years 2012-2016 was conducted to assess the reliability of arthritic and periprosthetic major joint microbiological inoculations.

The aim of this work was to evaluate the distribution of bacteria present in patients treated with septic joint and periprosthetic inflammatory conditions at our clinic during the 5-year period indicated above, as well as the diagnostic specificity of conventional laboratory and microbiological inoculations used in clinical practice, based on the retrospective study.

With the permission obtained from the Regional Research Ethics Committee, we investigated the denaturation of synovial fluid and the thermal changes of the proliferation of various bacterial strains recorded by differential scanning calorimetry (DSC) in synovial media by establishing an *in vitro* assay model at the Department of Biophysics, University of Pécs (UoP).

In light of the results gained by the experimental model that we developed, we investigated the benefits and possibilities of translating the calorimetric procedure into the clinical practice.

III. Materials and methods

III.1. Retrospective clinical study

Based on the main profiles of the Department of Traumatology and Hand Surgery, University of Pécs, following obtaining permission from the Regional and Institutional Research and Ethics Committee (Doc. No. 6737), we undertook to analyse the relationship between the aetiology of early and late joint or periprosthetic infections registered during primary major joint or reconstruction surgeries, as well as the sensitivity of conventional microbiological assays.

Following the authorisation of my request for statistical and scientific data collection (UoP, 56301/2017), I obtained data from the e-Med Solution system of the UoP Clinical Center, as well as the UoP Prosthesis Waiting List System (2912 UoP, 03 Traumatology) for statistical analysis. Patient data from between 1 January 2012 and 31 December 2016 from the Department of Traumatology and Hand Surgery (UoP) was collected and processed. An annual summary of primary or revision surgeries performed according to elective and acute major joint endoprostheses was made, and the distribution of bacterial strains causing primary small and major joint and periprosthetic infections was analysed.

Prior to the implantation of a major joint prosthesis performed at the scheduled time, the patients underwent a series of focal examinations (otorhinolaryngology, oral surgery, gynecology, cardiology, urology, proctology, pulmonology exams) in all cases.

The inflammatory processes around the septic joints and major joint prostheses were identified by comparing the electronic medical records (X-ray, CT, MRI, laboratory test results, microbiological inoculation results) and the surgical records.

III.2. Factors influencing synovial fluid composition

It is known that pathologies that promote increased production of synovial fluid may be of inflammatory or non-inflammatory origin. In addition to the general rheumatologic classification, bacterial, viral and fungal arthritis should be highlighted, which does not only lead to an increase in synovial fluid volume, but its qualitative composition also changes. Normal synovial fluid is viscous and its consistency is provided by hyaluronic acid polymers. During inflammatory conditions, mucin polymers become fragmented, while in acetic acid, mucin polymers and fragments are denatured in different forms.

Using the Kellgren-Lawrence and modified Kellgren-Lawrence radiology grading systems (Table 2), synovial fluid was collected from osteoarthritic patients with non-inflammatory synovial fluid production with varying degrees of severity (Grade I-IV); patients either had traumatic cartilage lesions, or required osteoarthritis treatment [26].

Grading	Kellgren-Lawrence	Modified Kellgren-Lawrence
Grade 0	No osteoarthritic changes	No osteoarthritic changes
Grade 1 **(Gr-I.)**	Joint space narrowing suspected, osteophytic "labrum formation" possible	Osteophyte questionable
Grade 2 **(Gr-II.)**	Osteophytes and possible joint space narrowing	Clear sign of osteophytes
Grade 3 **(Gr-III.)**	Moderate, multiple osteophytes, clear joint space narrowing, possible signs of sclerosis and deformity of epiphyses	Joint space narrowing
Grade 4 **(Gr-IV.)**	Large osteophytes, extensive joint space narrowing, severe sclerosis, extensive deformity of epiphyses	Virtual loss of joint cavity ("bone on bone")

Table 2. Description of the Kellgren-Lawrence and modified Kellgren-Lawrence grading systems [61].

By considering the inclusion criterion, we compared the different DSC curves of patients with different degrees of arthritis generated by thermal denaturation of synovial fluid samples free of exogenous impacts and inflammatory processes.

III.3. Collection and storage of synovial samples

Synovial fluid sampling was performed after the patient or their relatives have been informed and have given their informed consent, according to the ethical guidelines of the 2003 Helsinki Declaration and with the permission of the Regional and Institutional Research and Ethics Committee (Permit No. 6737).

Adult patients with acute or chronic knee arthritis (symptoms include pain, lack of movement, and joint inflammation) have been included in our study, who underwent knee joint hyaluronic acid treatment, arthroscopy, or knee joint surface reconstruction prosthesis implantation at our specialist practice. On the patient's side, participation in the study did not require any specific interventions. During conservative or surgical

intervention, 2 to 5 mL of synovial fluid drained during knee puncture by the physician treating the patient under sterile conditions was placed in pre-sterilized **B230 BSM470** 10-mL sealed native test tubes. The data necessary for sample identification were recorded on the test tube.

After processing the synovial samples (centrifugation at 3,500 rpm for 15 minutes, then transfer of the pure synovia into another sterile tube under aseptic conditions), samples were analysed by DSC at the Department of Biophysics, UoP, either immediately or after deep-freezing and storage at -70°C (in a standard freezer).

III.4. Denaturation of synovial samples and DSC analysis

Why did we choose the thermal analytical test?

We aimed to address whether the composition of the synovial fluid may have a major influence on the growth of bacteria.

After processing the synovial samples derived from patients meeting the inclusion criteria, their thermal denaturation was analysed by DSC [62]. For the DSC analysis, samples were stored at -70/-20°C until the start of the assays. Samples were analysed by a SETARAM Micro DSC-II calorimeter. All assays were performed at 0 to 100°C. In all cases, the heating rate was 0.3 K/min. For denaturation measurements, conventional Hastelloy cells (V = 1 mL) and an average sample volume of 950 µL was used; the weight of the samples used for the measurement was 950 mg (for liquid samples). For “solid” samples, 100-200 mg of sample was used.

Double-distilled water or physiological saline was used as reference. The reference and sample cells were weighed to an accuracy of ± 0.1 mg, so correction for the heat capacity of the reference and sample cells was not required. Calorimetric enthalpy was calculated from the area under the heat absorption curve using two-point fitting SETARAM integration. Based on the measured denaturation temperatures of the different samples and the calculated calorimetric enthalpy values, we could infer possible structural differences during various stages of the disease/treatment (Figure 3). Thermal parameters for denaturation are as follows:

- start (T_1) and end (T_2) of denaturation
- energy required for denaturation (ΔH = calorimetric enthalpy)

- maximum temperature of denaturation (T_m= 50% of the sample transforms into another conformation)
- half-width ($T_{1/2}$: if small, the interaction/cooperativity is very strong within the given thermal unit; if large, interaction/cooperativity is very weak. This means that the new state formed during denaturation is either more rigid or loose.)
- the difference between the baselines of the native and denatured states gives the difference between the heat capacity (ΔC_p) of the two states.

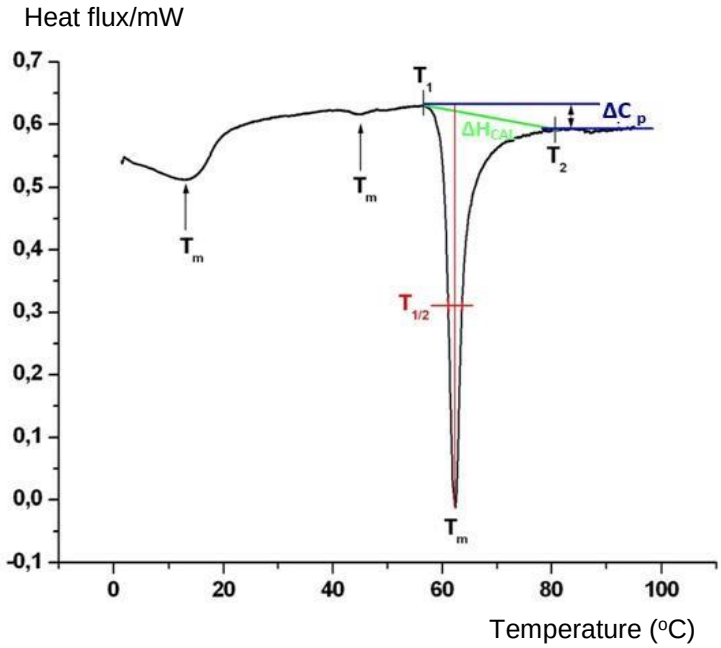

Figure 3. A representarive denaturation curve and its main parameters.

III.5. Examination of bacterial strains in the clinical practice

In addition to the necessary radiological and general laboratory diagnostic examination of patients with suspected septic arthritis, whenever possible, microbiological inoculation was performed, and the laboratory and biochemical quality composition of synovial fluid samples or secretions obtained from major joint punctures was determined under sterile conditions. The antibiotic sensitivity of the isolated pathogens was also studied using microbiological assays in each case, which produced a final result after 4 or 5 days.

In the case of confirmed joint or prosthetic infection, a broad spectrum combined antibiotic therapy was initiated as part of the surgical preparation, which was adjusted

as necessary to reflect the antibiotic sensitivity as determined by the microbiological assay.

III.6. Calorimetric assay of bacterial strains

Based on the preliminary clinical study and validated published results in the literature, an experimental model was developed with the permission of the Regional and Institutional Research Ethics Committee.

At the DSC laboratory of the Department of Biophysics, UoP, using sterile human synovial fluid samples under experimental conditions, we started to record the growth of bacterial strains which are more common in clinical practice and may cause serious diagnostic and treatment problems [26].

Bacterial strains derived from special cultures at the Department of Microbiology (UoP) were examined as follows: *Staphylococcus aureus* ATCC 29213; MRSA (Methicillin resistant *Staphylococcus aureus*) #4262 (# means being isolated from a pyogenic clinical specimen); *Staphylococcus epidermidis* ATCC 14990; *Streptococcus pyogenes* NCCP 11610; *Escherichia coli* ATCC 25922; *Pseudomonas aeruginosa* ATCC 27853. Plates of test bacteria growing on Mueller-Hinton agar (OXOID Ltd., UK) were used, from which bacterial suspension was prepared in Mueller-Hinton broth (OXOID Ltd., UK) at 10^3 or 10^5 colony forming units (CFU). The resulting bacterial suspensions of various colony forming units (10^3, 10^5 CFU/mL) were assayed by photometry (Perkin Elmer photometer, 600 nm E = 0.100) and culturing. The study protocol was prepared in accordance with the ethical guidelines of the 2003 Helsinki Declaration.

Specific amounts of the samples with adjusted colony forming units were placed in specially designed mixing vessels (Figure 4) of the differential scanning calorimeter (DSC) under aseptic conditions in the laboratory of the Department of Biophysics, UoP.

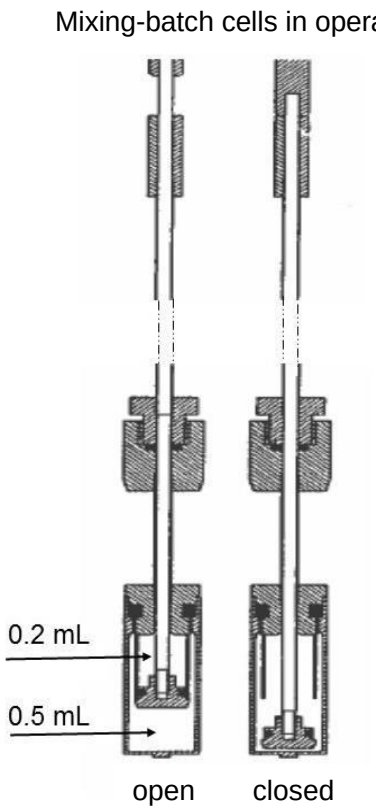

Figure 4. Specially designed double walled, sealable, sterile mixing vessel.

Under aseptic conditions, the mixing vials were filled in such a way that 0.2 mL of the bacterial suspension with the required colony forming units (10^3 and 10^5 CFU/mL) was added to the top of the vial, and 0.5 mL of Mueller-Hinton bouillon was added to the larger, bottom part of the vial. The mixing vessel filled with the bacterial sample was incubated in parallel with reference samples which contained Mueller-Hinton bouillon only (0.2 mL was added to the top of the vial and 0.5 mL was added to the lower portion). After the heat equilibrium has been reached, the cells of the mixing vessel were connected and 5 minutes after the mixing effect, the temperature characteristics were monitored with a SETARAM Micro DSC-II calorimeter. The proliferation curves of the initial (T_1) and thermal endpoints (T_2) and the maximum proliferation (T_m) were determined using the SETARAM software.

Calorimetric enthalpy change (ΔH_{cal}) was calculated from the area under the curve between the endpoints by two-point SETARAM integration, and the data were compared with calorimetric assays performed on different bacterial strains [26].

IV. Results

IV.1. Results of the retrospective clinical study

Based on the 5-year retrospective clinical study, a total of 439 TEP hip implant and 554 HEP hip implant surgeries were performed between January 2012 and the end of December 2016. Of the 181 hip joint revision surgeries, 36 had to be resolved by removal of the hip joint prosthesis. Knee TEP implant surgeries were performed in 238 cases for degenerative knee joint lesions and following post-traumatic arthrosis. Based on the cumulative results, during the 5-year period studied, in case of the 22 knee replacement surgery operations, 5 interventions were completed with removal of the prosthesis (Figure 5).

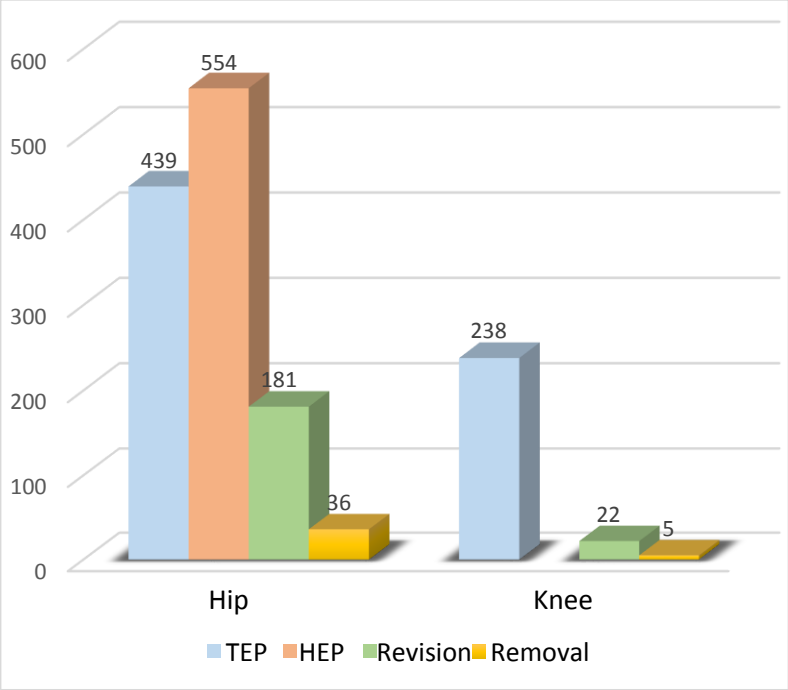

Figure 5. Summary of prosthetic surgeries performed at the Department of Traumatology and Hand Surgery, UoP, between 2012-2016.

According to our study, more than fifty percent of the primarily implanted hip prostheses in our clinic were preceded by a femoral neck fracture, where no prior detailed focal search has been performed before the intervention. In the majority of cases (for hip prosthesis implantation following femoral neck fracture), according to the rules of the profession, hemi-endoprosthesis (HEP) implantation has been performed, adapted to the needs and operability of patients with poor general condition, which is still characterised by a much higher morbidity with regards to both general and septic

complications (Figure 10) [26]. With the increasing frequency of degenerative lesions of the major joints, the number of primary hip and knee prostheses implanted as per patient requirements is increasing exponentially from year to year (Figure 6).

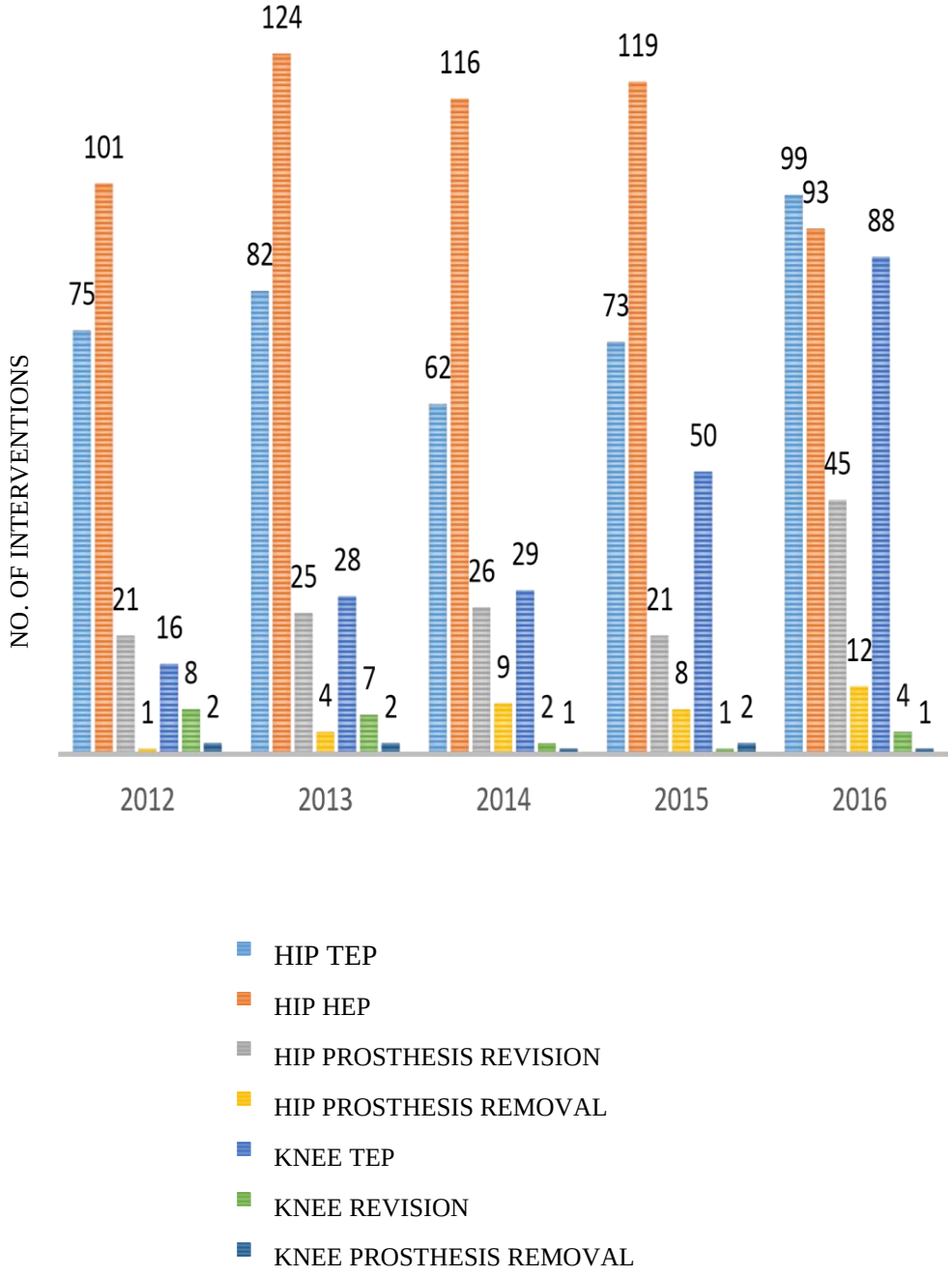

Figure 6. Distribution of prosthesis surgeries between 2012-2016.

Following predominantly acute injuries (post femoral neck fracture), there is an increasing tendency for permanent Girdlestone status as a result of purulent septic periprosthetic hip lesions.

As a result of microbiological assays on samples derived from the patients during treatment, 137 cases of joint and periprosthetic infections were found by conventional culture. In case of patients treated with infections, 65 (47.45%) patients were identified with infections at small joints of the hand or foot, and 72 (52.55%) patients with major joint infections. The majority of small joint infections occur after external trauma or injury, while some of the major joint infections are due to prior medical interventions and constitute one of the early or late complications following major joint prosthesis implantation surgery [26].

With regards to assessing the reliability of conventional microbiological studies, the 5-year retrospective clinical study has revealed that of the 137 confirmed cases of purulent joint and periprosthetic lesions, primary inoculations failed to detect any pathogens in 30 cases (21.88%).

According to our results (Figure 7), different *Staphylococcus* strains are still the most prevalent in case of primary inoculations: *Staphylococcus aureus*, 43 (22.16%); *Staphylococcus coagulase negative*, 22 (11.34%); *Staphylococcus epidermidis*, 3 (1.54%); *methicillin resistant Staphylococcus aureus (MRSA)*, 4 (2.06%).

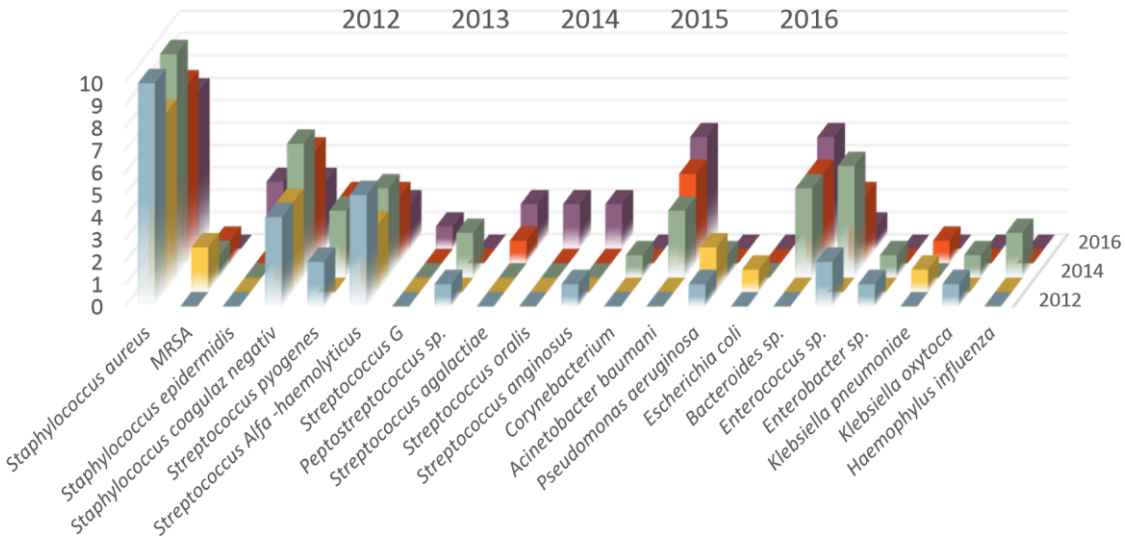

Figure 7. Distribution of identified bacterial strains.

Based on our retrospective survey on the period between January 2012 and December 2016 at the Department of Traumatology and Hand Surgery, UoP, we prepared a summary on the most common joint and periprosthetic infections [26] (Table 3).

Bacterial strains	Hip joint	Knee joint	Ankle joint	Shoulder joint	Elbow joint	Small joint of foot	Small joint of hand
Staphylococcus aureus	8	12	0	2	1	4	16
MRSA	2	1	0	0	1	0	0
Staphylococcus epidermidis	2	0	0	0	0	1	0
Staphylococcus coagulase negative	12	4	1	1	0	0	3
Streptococcus pyogenes	0	2	0	0	0	2	5
Streptococcus Alfa -haemolytic	0	1	1	0	0	1	13
Streptococcus Beta- haemolytic	0	0	0	0	0	1	0
Streptococcus G	1	0	0	0	0	0	0
Peptostreptococcus sp.	1	0	0	0	0	0	2
Streptococcus agalactiae	2	0	0	0	0	0	2
Streptococcus oralis	0	0	0	0	0	1	1
Streptococcus pneumoniae	0	1	0	0	0	0	0
Streptococcus anginosus	2	1	0	0	0	0	0
Corynebacterium	0	0	0	0	0	0	1
Acinetobacter baumani	2	0	0	0	0	0	0
Pseudomonas aeruginosa	8	2	0	0	0	2	0
E. coli	2	0	0	0	0	0	2
E. hemolyticus	0	0	0	0	0	0	1
Bacteroides sp.	0	0	0	0	0	0	1
Enterococcus sp.	10	0	0	0	0	1	2
Enterobacter sp.	2	0	0	1	0	3	5
Klebsiella pneumoniae	0	0	0	1	0	0	1
Klebsiella oxytoca	0	0	0	0	0	0	2
Haemophylus influenza	0	0	0	0	0	0	1
Morganelli morgani	0	0	0	0	0	1	0
Sum of positive results	**54**	**24**	**2**	**5**	**2**	**17**	**58**
Primary negative result	**1**	**3**	**0**	**3**	**0**	**0**	**23**

Table 3. Distribution of bacteria isolated in case of joint infections between January 2012 and December 2016 at the University of Pecs, Department of Traumatology and Hand Surgery.

The beneficial effects of pre-surgery focal search performed prior to the increasing number of primary surgeries has been observed, which resulted in a decresase in the incidence of septic complications.

IV.2. Results of denaturation assays of synovial samples

Synovial fluid samples taken from different degrees of severity of arthrosis were assays by a SETARAM Micro DSC-II calorimeter at a heating rate of 0.3 K min^{-1} at

temperatures between 37°C and 90°C [62]. Following denaturation of the samples, the calorimetric results of patients with different degrees of arthrosis were compared (Figure 8).

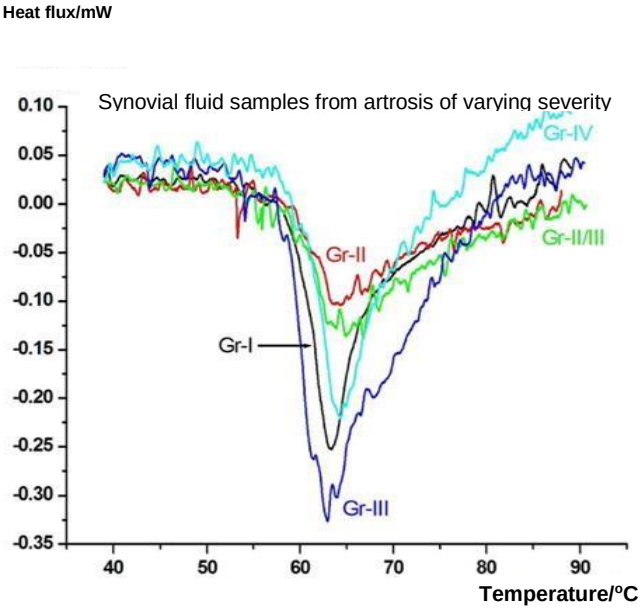

Figure 8. Comparison of denaturation curves of synovial fluid samples derived from arthrosis states of varying severity.

There is a significant change associated with the progression of arthrosis as evident by the change in the half-width ($T_{1/2}$) of the denaturation curves, which is the temperature range of the structural transformation at 50% of the maximum heat flux. Low values indicate strong structural cooperation, while the larger figures indicate weaker interaction between the potential components. For Gr-II, Gr-II / III and Gr-III samples, these data refer to the possible “loosening” of the samples, i.e. the structure is less orgasnised at different severity levels (see Table 4).

Severity	T_m/ °C	$T_{1/2}$/ °C	ΔH_{cal}/Jg^{-1}
I.	63.4	4	0.46
II.	64.3	11.5	0.32
II-III.	63.7 ± 0.6	12.4 ± 0.4	0.54 ± 0.06
III.	63.5	10.9	0.58
IV.	64.2 ± 0.7	8.2 ± 0.3	0.4 ± 0.05

Table 4. Parameters of the denaturation of synovial fluid samples.

IV.3. Calorimetric results of the proliferation of bacterial strains

Under experimental conditions, the growth of bacterial strains commonly encountered during the clinical practice was investigated with the SETARAM Micro DSC-II and III calorimeters. After equilibration between the special mixing cell filled with

the bacterial strain of the test bacterium and the reference cell, and after combining the contents of both cells at the same time, the heat production of the proliferation process was recorded as a function of time. At the concentrations tested (10^3 CFU/mL and 10^5 CFU/mL), the data was characteristic to the bacterium species, and had different course for different strains (Figures 9–11).

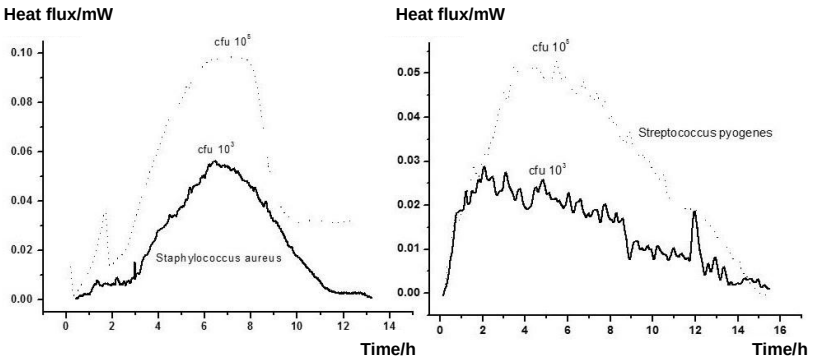

Figure 9. Thermal changes registered during the proliferation of *Staphylococcus aureus* and *Streptococcus pyogenes* at 37°C.

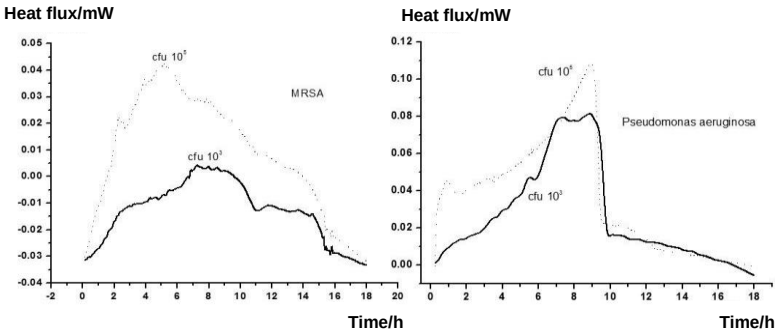

Figure 10. Thermal changes registered during the proliferation of MRSA and *Pseudomonas aeruginosa* at 37°C.

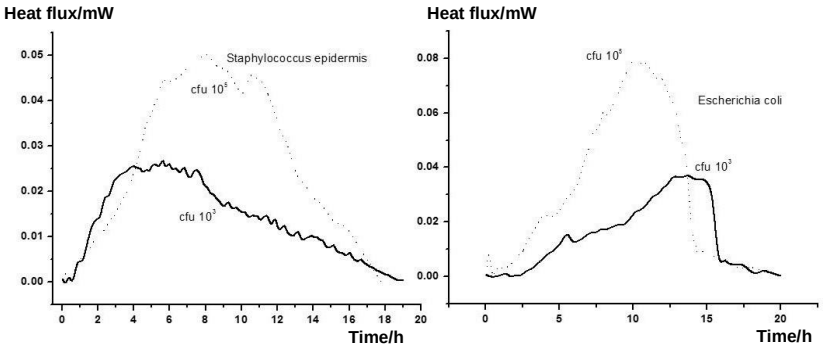

Figure 11. Thermal changes registered during the proliferation of *Staphylococcus epidermidis* and *Escherichia coli* at 37°C.

In case of the proliferation of the clinically most infectious *S. aureus* (Figure 9), the effect was clearly visible. At 10^5 CFU for *S. aureus*, the pre-phase of growth was approximately 1.5 hours (but more pronounced than at 10^3 CFU). At both CFU values, the heat flux of growth reached its peak within 6–7 hours and the process was completed after 13 hours. For *S. pyogenes*, the initial phase of proliferation could be recorded after 1 and 2 hours, depending on the CFU, with a maximum heat flux of about 2.5 hours at 10^3 CFU/mL, and 6 hours at 10^5 CFU/mL. The process was completed after 16 hours, and a highly fluctuating heat flux at 10^3 CFU/mL was recorded.

The proliferation kinetics of MRSA and *P. aeruginosa* (Figure 10.) are also bacterium specific. For MRSA, total heat production comprises at least three different growth phases, suggesting different growth kinetics. In case of 10^3 CFU, the heat flux peaked at 7-8 hours, while in case of 10^5 CFU/mL, the peak was detected at approx. 5 hours 31 minutes, and the end point was at 18 hours. For *P. aeruginosa*, at both CFU values, the growth rate was significantly reduced after the peak at 9 hours. At the 10^3 CFU/mL value for *P. aeruginosa*, at least three different proliferation kinetics could be distinguished.

Interestingly, *S. epidermidis* was the only bacterium where the rate of proliferation was showing the opposite CFU dependence (Figure 11). Achieving the maximum heat flux at 10^3 CFU/mL was faster than in case of 10^5 CFU/mL. For *E. coli,* also significantly reduced bacterial growth rate was detected at 10^3 CFU/mL. The end of the process is the longest in case of both CFUs, 20 hours.

Table 5 shows the most important thermal parameters to summarise our results. The end of the growth phase, the time to reach the maximum heat flux, and the calorimetric enthalpy values enable the identification of individual bacteria, and can be used to draw diagnostic conclusions. The determination of the thermal parameters t_m and ΔH_{cal} is much faster compared to the identification of bacteria by the classical microbiological methods; therefore, the appropriate antibiotic therapy becomes

available sooner by thermal analysis, and the empirical therapy used up to now can be changed to a targeted, specific antibiotic therapy [26].

Bacterial strains studied	Thermal parameters			
	t_o	t_e	t_m	$\Delta H_{cal}/Jg^{-1}$
Staphylococcus aureus				
CFU 10^3	26 min	13 h 22 min	6 h 27 min	-1.48
CFU 10^5	13 min	13 h 30 min	7 h	-1.98
Streptococcus pyogenes				
CFU 10_3	10 min	15 h 30 min	2 h	-0.64
CFU 10^5	12 min	15h 50 min	5 h 30 min	-2.45
MRSA				
CFU 10^3	15 min	18 h	7 h 16 min	-0.91
CFU 10^5	8 min	18 h	5h 5 min	-3.67
Pseudomonas aeruginosa				
CFU 10^3	16 min	18 h	8 h 50 min	-2.48
CFU 10^5	16 min	17 h 50 min	9 h	-3.32
Staphylococcus epidermidis				
CFU 10^3	8 min	19h	5h 37 min	-0.66
CFU 10^5	10 min	17 h 50 min	8h 20 min	-1.58
Escherichia coli				
CFU 10^3	8 min	20h	13 h	-1.56
CFU 10^5	9 min	19 h 35 min	9 h 55 min	-3.22

Tabe 5. Thermal characteristics of bacteria frequently causing joint infections (t_o = start point, t_e = end point, t_m = time of maximal growth rate, ΔH_{cal} = calorimetric enthalpy of growth [exotermic process]; data are mean, n = 3). The time of growth maximum heat flux and calorimetric enthalpy are bacterium specific parameters.

The denaturation properties of synovial samples taken from different severities of arthrosis examined primarily in our experimental model were determined to be non-discriminatory in bacterial growth and detection.

V. Discussion

Given the severity of the damage to the body due to bacterial infections, special care has been taken during the clinical practice in each case for the appropriate treatment of any patient who is suspected of having a joint infection. The retrospective study shows that with the increase in the frequency of major joint degenerative lesions, the number of primary implanted hip and knee prostheses increases exponentially each year, to meet the demand of patient needs (Figure 6). With the increasing wear and loosening of hip prostheses implanted years and decades ago, the number of hip prosthesis revisions also increases every year, along with the increasing tendency of

permanently abandoned Girdlestone status due to septic purulent hip joint periprosthetic infections (Figure 6).

Based on available data, the incidence of septic complications is 12–15% after hip prosthesis revisions, and 23–25% after knee prosthesis revisions in the United States and the United Kingdom, and the cost of treatment is several times higher than that of primary uncomplicated major joint prosthesis implantations [63-65]. Infections related to prosthesis revision, beyond the burden posed on the patient, also significantly increases hospital costs. In a French study, the cost of hospitalisation for a purulent periprosthetic infection caused by prosthesis revision is in the realm of EUR 32,000, which is 2.6-times the cost of the revision surgery, and 3.6-times the cost of the original primary prosthetic surgery [66]. Supported by statistical data, there is an urgent need to find solutions for the management of periprosthetic infections and to quickly determine the use of effective antibiotic therapy available.

According to previous studies [62, 26, 67], in order to get a quick diagnosis and the possibility of a targeted antibiotic therapy, the application of isoperibolic calorimetry in case of suspect septic arthritis or periprosthetic infection may be advantageous compared to conventional microbiological cultures due to its significantly shorter duration [68-70]. Calorimetric assays have been previously used to identify various bacterial strains based on changes in the thermal properties caused by bacterial metabolism [9, 62, 26, 71, 72]. Based on previous studies, using a calorimetric experimental model for periprosthetic joint infection, a 5-sample calorimeter can provide an accurate response to pathogen antibiotic susceptibility or resistance within a few hours [69], by adding different broad spectrum antibiotics separately to the infected specimen being tested, and recording the release or absence of a growth heat flux using a calorimeter.

According to our study (Table 3), the Gram-positive *Staphylococcus aureus* was the most common pathogen in case of septic complications of knee and hip prostheses, which corroborates published data [73, 74]. In economic terms, with the increase in the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) and other multidrug-resistant strains amongst *Staphylococcus* infections, there is a significant increase in the cost of care, which places a greater economic burden on the healthcare system.

According to a recent study, monitoring serum D-dimer in PJI is a pathologic marker, with 89% sensitivity and 93% specificity over both ESR and serum CRP [75]. Furthermore, PCT proved to be more sensitive than CRP in case of identifying septic arthritis [76]. In addition to measuring general inflammatory laboratory parameters (CRP, WBC, ESR, PCT), monitoring serum D-dimer following a cost-effective screening, the costly but also more sensitive and specific biomarkers and MALDI-TOF mass spectrometry analysis can provide rapid and reliable results for identifying pathogens, compared to microbiological culturing [74, 77, 78].

VI. Conclusions and new findings

VI.1. Conclusions

According to our results, we can conclude that in the case of septic arthritis of major joints, conventional diagnostic methods in daily practice (as determined from blood and synovial fluid) are not sufficient.

The 5-year retrospective clinical study to assess the reliability of conventional microbiological assays has revealed that in 21.88% of the confirmed purulent joint and periprosthetic infections, pathogens could not be detected, which proved to be too high in the fight against septic processes. Performing multiple microbiological inoculations of samples taken from multiple sites is essential. Based on the outcome of the clinical study, there is a much higher rate of septic complications in case of prosthetic implantations for hip and periprosthetic fractures, as well as prosthetic revisions, compared to planned primary prosthetic implantations designed for the given degenerative joint disease.

With the worldwide increase in the number of primary major joint prostheses, we should consider both the number of primary prosthetic revisions, and revisions triggered by periprosthetic fractures, which may increase the likelihood of septic complications.

Based on the clinical study, the sensitivity of microbiological inoculation, previously considered as the gold standard, in the case of joint and periprosthetic infection is low, which may also influence the efficiency of the focal examination conducted prior to the implantation of the elective prosthesis, considering the margin

of error of the method. From the aggregated data, we can conclude that conventional microbiological sampling alone is not sufficient to identify the pathogen. In the clinical practice, in addition to the routine use of serum laboratory tests (CRP, WBC, ESR, PCT, D-dimer), as well as the rarely used and costly analytical procedures, differential scanning calorimetry can prove to be a rapid, sensitive, alternative tool.

Calorimetric assays, applied for the diagnosis of bacterial infections and in the antibiotic susceptibility testing of isolated bacterial strains, can generate results in as few as 4-5 hours, which is currently unsurpassed by any other assays [26].

VI.2. New findings

- We can conclude that based on the data of our clinical study, prosthetic implantations necessitated by hip fracture and periprosthetic fracture have a much higher septic complication rate compared to primary prosthetic implantations designed for the given degenerative joint disease.
- In the case of septic arthritis of major joints, conventional diagnostic methods (routine blood and synovial fluid assays) used in daily practice are inadequate.
- In addition to the routinely used serum laboratory tests (CRP, WBC, ESR, PCT, D-dimer) and the very rarely used and costly analysis assays, differential scanning calorimetry (DSC) can be a fast, sensitive, and cost-effective alternative tool.
- The method is capable of providing data on antibiotic susceptibility testing and resistance assays of the isolated bacterial strains within 4-5 hours, in contrast to other assays.

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VIII. List of publications

VIII.1. Publications releated to the thesis

- Dandé Á, Nőt LG, Wiegand N, Kocsis B, Lőrinczy D. DSC analysis of human synovial fluid samples in the diagnostics of non-septic and septic arthritis. J. Therm Anal Calorim. . 2017:130;1249-52.
- Dandé Á, Nőt LG, Bűcs G, Wiegand N, Kocsis B, Lőrinczy D. Examination of typical bacterial strains in septic arthritis by isoperibol calorimeter. Journal of Therm Analysis and Calorimetry. 2017;https://doi.org/10.1007/s10973-0176859-2.
- Dandé Á, Nőt LG, Bűcs G, Kocsis B, Lőrinczy D, Wiegand N. Efficacy of microbiological culturing in the diagnostics of joint and periprosthetic infections. Injury 2018. *Mansucript under revision.*

VIII.2. List of presentations on conference, related to the thesis

- Dandé Á, Lázár I. Saroktáji csontcisztába terjedő szeptikus folyamat szanálása. XX. Dél- Magyarországi Traumatológus Kongresszus, Harkány- Pécs, 2015.
- Dandé Á, Nöt LG, Wiegand N, Lőrinczy D. Isoperibolic calorimetric- analysis of human synovial fluid samples in the diagnostics of septic arthritis. 35th Annual Meeting of the European Bone and Joint Infection Society in Oxford, United Kingdom, 1-3 September 2016.
- Dandé Á, Wiegand N, Naumov I, Nőt LG, Máthé T, Patczai B. Diagnosztikus nehézségek pathológiás töréskezelés esetén. PTE, Szakosztály, Tanulságos esetek fóruma, Pécs, 2017.
- Dandé Á, Nőt LG, Wiegand N, Kocsis B, Lőrinczy D. Differencial scanning calorimetry analysis of human synovial fluid in the diagnostics of septic arthritis. 1 st Journal of Thermal Analysis and Calorimetry Conference and 6th V4 (Joint Czech-Hungarian-Polish-Slovakian) Thermoanalytical Conference June 6-9, 2017 Budapest, Hungary.
- Dandé Á, Nöt LG, Bűcs G, Wiegand N, Kocsis B, Móricz O, Till J, Farkas P, Lőrinczy D. Az izoperibolikus kalorimetria és a differenciál pásztázó kalorimetria használata a traumatológiában. MTT 2017. évi Közös Kongresszusa, Pécs.
- Dandé Árpád, Nőt László G., Kocsis Béla, Wiegand Norbert, Lőrinczy Dénes. Humán synoviális minták termikus vizsgálata a szeptikus arthritis XI. Köszönetnyilvánítás

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