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Rīga Stradiņš University

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Clinical Course, Treatment Outcome, Cellular, and Molecular Biology Findings in Cases of Infective Endocarditis Caused by Various Microorganisms Among Cardiac Surgery Patients

Summary of the Doctoral Thesis for obtaining the scientific degree "Doctor of Science (*PhD*)"

Sector Group – Medical and Health Sciences
Sector – Clinical Medicine
Sub-Sector – Surgery

Riga, 2024

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Abbreviations used in the Thesis

BCNE	Blood culture-negative endocarditis
BCPE	Blood culture-positive endocarditis
BNP	B-type natriuretic peptide
CNS	Central nervous system
CP	Calprotectin
CRP	C-reactive protein
CT	Computed tomography
DAB	3,3-diaminobenzidine
DNA	Deoxyribonucleic acid
ECMO	Extracorporeal membrane oxygenation
EDD	End diastolic diameter
ESD	End systolic diameter
FFPE	Formalin-Fixed Paraffin-Embedded
GP1b	Glycoprotein Ib
HACEK	*Haemophilus parainfluenzae, Haemophilus aphrophilus, Haemophilus paraphrophilus, Haemophilus influenzae, Actinobacillus actinomycetemcomitans, Cardiobacterium hominis, Eikenella corrodens, Kingella kingae* and *Kingella denitrificans*
HIV	Human immunodeficiency virus
H&E	Haematoxylin and Eosin
OR	Odds ratio
IABP	Intra-aortic balloon pump
IE	Infective endocarditis
EF	Ejection fraction of the left ventricle
IgG	Immunoglobulin G
ICU	Intensive care unit

IVS	Interventricular septum
CoNS	Coagulase-negative staphylococci
BMI	Body mass index
LAVI	Left atrial volume index
LFA-1	Leukocyte function-associated antigen-1
MANOVA	Multivariate analysis of variance
MAPK	Mitogen-activated protein kinase
MPO	Myeloperoxidase
MRI	Magnetic resonance imaging
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Neutrophil elastase
NET	Neutrophil leukocyte extracellular traps
NGS	Next-generation sequencing
NBTE	Nonbacterial thrombotic endocarditis
NVE	Native valve endocarditis
PECAM-1	Platelet endothelial cell adhesion molecule-1
PI3K	Phosphoinositide 3-kinase
PCR	Polymerase chain reaction
PSGL-1	P-selectin glycoprotein ligand-1
PSCUH	Pauls Stradiņš Clinical University Hospital
PVE	Prosthetic valve endocarditis
rRNA	ribosomal ribonucleic acid
ROS	Reactive oxygen species
RR	Relative risk
SEM	Scanning electron microscopy
SD	Standard deviation
Syk	Spleen tyrosine kinase
TAPSE	Tricuspid annular plane systolic excursion

TOE	Transoesophageal echocardiography
TEM	Transmission electron microscopy
TLR	Toll-like receptors
HBV	Hepatitis B virus
HCV	Hepatitis C virus
95 % CI	95 % confidence interval

Introduction

Infective endocarditis (IE) is a life-threatening infectious disease that affects the inner layer of the heart (endocardium), including the heart valves. IE is associated with a high risk of mortality and complications. In-hospital mortality for these patients is 15–30 % (Fernández-Hidalgo et al., 2008). The diagnosis of endocarditis is often challenging due to its nonspecific symptoms and variable clinical presentations. The clinical course can be acute, progressive, or slow, with nonspecific symptoms, leading to a wide range of possible differential diagnoses (Habib et al., 2015a).

Currently, IE is diagnosed based on modified Duke criteria (Durack et al., 1994; Li et al., 2000; Fowler et al., 2023; Delgado et al., 2023), where imaging demonstrating endocardial involvement in the disease process and blood culture with identification of the microorganism and antibacterial sensitivity form the basis of the IE diagnosis. However, in up to 31 % of cases or more, it is not possible to initially identify the causative microorganism (Philippe Brouqui & Raoult, 2006). This situation is known as blood culture-negative infective endocarditis (BCNE), often attributed to prior antibiotic therapy. However, the causative agent can also be a fungal infection, obligate intracellular bacteria, or bacteria that do not grow in standard culture media (Kupferwasser & Bayer, 2001). In such cases, serological analyses or molecular diagnostic methods may be necessary (Fournier et al., 2010). Molecular diagnostic methods based on the amplification and sequencing of ribosomal RNA (rRNA) genes, such as 16S rRNA for bacteria and 18S rRNA for fungi, have been applied to excised valve tissues since the 1990s to identify the pathogenic microorganism (Goldenberger et al., 1997). Several studies have demonstrated the utility of molecular diagnostic methods and their impact on antibiotic therapy, considering their inclusion in Duke criteria for IE diagnosis (Breitkopf et al., 2005;

Gauduchon et al., 2003; Millar et al., 2001; Shrestha et al., 2015; Fowler et al., 2023).

The disease develops when microorganisms enter the bloodstream and adhere to damaged or inflamed endocardium, most commonly the surfaces of heart valves. The endothelial cell layer covering the normal heart and valve surfaces is resistant to bacterial and fungal infections (Durack et al., 1973). Experiments with animals have shown that repeated endothelial damage and transient bacteraemia, representing the constant periodic entry of bacteria into the blood through the entry point play a crucial role in the pathogenesis of endocarditis.

Infection can destroy valves, spread beyond the endocardium into the heart, cause embolization, and result in serious consequences such as cerebral infarction or death. Generally, patients with IE are most at risk due to uncontrolled systemic or local infection, systemic or pulmonary vegetation embolism, and heart valve damage leading to heart failure.

Untreated IE is almost 100 % fatal. The prognosis for IE patients is most influenced by factors such as the patient's clinical condition, the presence or absence of IE complications, the microorganism causing IE (e.g. higher mortality with *S. aureus*), and echocardiographic findings. The prognosis varies depending on the presence or absence of these factors.

This study is currently relevant because IE is a disease with much unknown in its pathogenesis. It is known that many inflammatory cells, primarily neutrophilic leukocytes, participate in the infectious process. The involvement of neutrophil extracellular traps (NET), a relatively recently discovered structure, in the IE process has also been demonstrated (Brinkmann et al., 2004). Bacteria induce an intense immune reaction, resulting in the formation of NET, which is one of the manifestations of the body's defence against infection. NETs are web-like structures released by activated immune cells, especially neutrophils. These

structures consist of deoxyribonucleic acid (DNA), histone proteins, and other proteins, capable of capturing and inactivating microorganisms. However, there are also studies suggesting that NETs could contribute to tissue damage and exacerbation of the inflammatory process (S. U.-S. Huang & O'Sullivan, 2022). To understand the role of neutrophilic leukocytes and NETs in the development of IE, it is necessary to thoroughly investigate the presence of these structures, as well as the significance of NETs in IE pathogenesis, molecular and cellular level mechanisms. The results of this study could provide insights into the role of neutrophilic leukocytes, their activation, and NETs in the pathogenesis of IE caused by various triggers.

Aim of the Thesis

To identify risk factors associated with unfavourable outcomes in IE caused by various microorganisms and to assess cellular and molecular biology findings in excised valve material.

Tasks of the Thesis

The tasks set to achieve the goal of the doctoral thesis are as follows:

1. To collect and analyse data on the clinical course, laboratory, and imaging diagnostic results from the histories of operated patients. Determine which microorganisms are associated with higher mortality and complications.
2. To identify risk factors associated with in-hospital mortality.
3. To evaluate and compare the outcomes of positive and negative blood cultures in IE in both the short-term and long-term.
4. To conduct microbiological cultures of excised valve material during heart surgeries, as well as an examination using 16S rRNA Next generation sequencing (16S rRNA NGS) technology. Assess its significance in diagnosing infectious endocarditis pathogens.

5. To conduct morphological examination of excised valve material obtained during cardiac surgeries to analyse the presence of neutrophil leukocytes, their activation markers, and neutrophil extracellular traps in cases of IE caused by various microorganisms.

Hypotheses of the Thesis

IE caused by various microorganisms is associated with different clinical outcomes, complications, and prognosis in cardiac surgery patients.

Novelty of the Thesis

In the conducted research of this work, the impact of the causative microorganism of IE and the lack of its identification on the outcome were analysed. By utilizing Next-generation sequencing technology and analysing surgical materials, it is possible to more frequently detect the pathogenic microorganism. In the recently released 2023 guidelines from the European Society of Cardiology and the European Association for Cardio-Thoracic Surgery on the management of IE, this method has emerged as an innovation, and there is limited information on its aspects in the context of IE. It is recommended to consider this method for diagnostic challenges, such as in cases of blood culture-negative IE. This work includes a morphological analysis of surgical material, ranging from macroscopic levels to electron microscopy. For the first time, the involvement of neutrophil activation markers and extracellular traps in the pathogenesis of *Bartonella spp.* endocarditis was analysed.

1 Material and methods

1.1 Patients' characteristics

Within the framework of the doctoral thesis, various studies were conducted at different time intervals, analysing current issues related to IE. In the studies "Risk factors associated with mortality in the IE patients requiring cardiac surgery", "Comparison of outcome between blood culture positive and negative IE patients undergoing cardiac surgery", and "Differences in the clinical course and outcome of IE caused by the most common microorganisms in cardiac surgery patients" a retrospective analysis of the aforementioned issues was performed. On the other hand, the studies "Application of 16S rRNA Next-generation sequencing in excised valve samples from IE patients" and "Comparison of morphological findings and neutrophil leukocyte activation in IE caused by *Bartonella spp.* and non-*Bartonella spp.*" involved a prospective investigation.

1.1.1 Characterization of patients included in the retrospective part of the study

The studies were conducted at various time intervals from 2015 to 2022. All the studies included patients who underwent surgery at Pauls Stradiņš Clinical University Hospital (PSCUH) due to IE. The research focused on the adult population, with all patients being at least 18 years old at the time of inclusion. The surgical indications were determined according to the 2015 guidelines from the European Society of Cardiology on the management of IE (Habib et al., 2015b), summarised in Table 1.1. Throughout the study period, the hospital did not have a recommended "endocarditis team" as per the guidelines. PSCUH is the only hospital in Latvia providing cardiac surgery, making it reasonable to consider that the study included all nationally operated patients with IE of heart valves. In the retrospective part of the studies, all patients

operated during the respective study period were included based on specific inclusion criteria for each study.

1.1.2 Characterization of patients included in the prospective part of the study

The studies were conducted from October 2019 to June 2022. These studies included patients who underwent surgery at PSCUH due to IE, with the same indications as mentioned in the retrospective part of the study previously. In contrast to the retrospective part, this section included randomly selected patients, and thus, the prospective part's patient cohort does not represent the entire population of patients operated during the respective period. Patients signed an informed consent form to participate in the study. The informed consent form was approved by the Central Medical Ethics Committee of Latvia. In cases where the patient himself/herself was unable to confirm participation before the operation, the consent was obtained from the patient's relatives. Analysis and examination data were obtained from patients' medical histories. The material obtained during the operation was sent for morphological studies and examination using 16S rRNA NGS technology. Additional details regarding the examination of the surgical material are provided in the following sections.

Table 1.1

Indications and urgency for surgery in IE of the left-sided valve (native valve endocarditis and prosthetic valve endocarditis)

Indication for surgery	Urgency of surgery[a]	Class[b]	Level[c]
Aortic or mitral native valve endocarditis or prosthetic valve endocarditis with severe acute regurgitation, obstruction, or fistula causing refractory pulmonary oedema and cardiogenic shock	Emergency	I	B
Aortic or mitral native valve endocarditis or prosthetic valve endocarditis with severe regurgitation or obstruction causing symptoms of heart failure or poor hemodynamic tolerance with echocardiographic signs	Urgent	I	B
Locally uncontrolled infection (abscess, pseudoaneurysm, fistula, vegetation enlargement)	Urgent	I	B
Fungal or multi-resistant organism-induced infection	Urgent / elective	I	C
Persistently positive blood cultures despite appropriate antibiotic therapy and adequate control of septic metastatic foci	Urgent	IIa	B
Valve prosthetic endocarditis caused by staphylococci or gram-negative bacteria other than the HACEK group	Urgent / elective	IIa	C
Aortic or mitral native valve endocarditis or prosthetic valve endocarditis with vegetation > 10 mm after one or more episodes of embolism despite appropriate antibiotic therapy	Urgent	I	B
Aortic or mitral native valve endocarditis with vegetation > 10 mm associated with severe valve stenosis or regurgitation and low surgical risk	Urgent	IIa	B
Aortic or mitral native valve endocarditis or prosthetic valve endocarditis with an isolated very large vegetation (> 30 mm)	Urgent	IIa	B
Aortic or mitral native valve endocarditis or prosthetic valve endocarditis with an isolated large vegetation (> 15 mm) and no other indications for surgery[d]	Urgent	IIb	C

[a] Urgent surgery: surgery is performed within 24 hours; emergent surgery: within a few days; elective surgery: after at least 1–2 weeks of antibiotic therapy.
[b] Class of recommendation.
[c] Level of evidence.
[d] Surgery may be preferable if a procedure preserving the native valve is possible.

1.2 Molecular biology methods

During cardiac surgery, a small vegetation sample was obtained from excised tissues for 16S rRNA NGS analysis. The sample was placed in a sterile container (Sarstedt AG & Co. KG, , Nümbrecht, Germany), immediately frozen, and stored at −20 °C. It was then transported to the laboratory.

1.3 Acquisition and preparation of surgical material for morphological studies

In this study, specimens utilised for morphological investigations were procured during cardiac surgeries. The valve and vegetation sample, if sufficiently large and suitable, was forwarded for histopathological, histochemical, immunohistochemical, as well as electron microscopical examinations. This comprehensive approach ensured a thorough morphological assessment of the collected surgical material.

For histopathological and histochemical investigation valvular leaflets with macroscopically visible vegetation were fixed in 10 % neutral formalin. Tissue samples were embedded in paraffin on the cut side from the free edge of the leaflet through the vegetation structure to the base of the leaflet. Conventional 4–5 µm-thick tissue sections were cut off and mounted on SuperFrost Plus slides (Gerhard Menzel GmbH, Braunschweig, Germany) (Gerhard Menzel GmbH, Braunschweig, Germany). The sections were routinely stained with haematoxylin and eosin (H&E). Additionally, the reticulin silver plating kit, according to Gordon & Sweets (Merck KGaA, Darmstadt, Germany, 1002510001), was used to visualise reticular fibres within the leaflet tissue and, simultaneously, to better identify bacteria using the advantage of the silver impregnation technique [34].

For immunohistochemical investigation formalin-fixed and paraffin-embedded (FFPE) samples were processed conventionally. Blocking of endogenous peroxidase activity was performed using 3 % H2O2 in methanol.

For specific antigen retrieval, sections were boiled in citrate buffer (pH 6) or TRIS/EDTA buffer (pH 9) as recommended by manufacturer protocols, and incubation with primary antibodies was conducted thereafter. For the recognition of various neutrophilic leukocyte activation markers, a broad spectrum of primary antibodies, including anti-myeloperoxidase (anti-MPO, Abcam, ab208670, 1:1000), anti-histone H3 (anti-Histone H3, Abcam, ab5103, 1:50), anti-calprotectin (anti-CP, Abcam, ab22506, 1:1000), anti-neutrophil elastase (anti-NE, Abcam, ab131260, 1:1000), and anti-P-selectin, CD62P (anti-Ps, Abcam, ab182135, 1:500), were used. Primary antibody amplification and visualization were performed using the HiDef Detection HRP Polymer system (No 954D-30, Cell Marque, Rocklin, CA, USA) and the 3,3 diaminobenzidine (DAB) tetrahydrochloride kit (No 957D-30, Cell Marque, Rocklin, CA, USA). Cell nuclei were counterstained with Mayer's haematoxylin. Primary antibodies were omitted in the negative controls of immunohistochemical reactions. The reaction results were assessed by two independent observers. Microphotographs were obtained as captures using a Glissando Slide Scanner (Objective Imaging Ltd., Cambridge, UK). The expression of antigens in the valve leaflet and vegetation was assessed in ten properly oriented microscopic fields for each region of interest and scored as follows: 0 – negative; 1 – weakly positive; 2 – strongly positive expression.

2 Statistical data analysis

2.1 Risk factors associated with mortality in the IE patients requiring cardiac surgery

Descriptive statistics for categorical variables were expressed as relative frequencies (percentages). Mean values (standard deviation – SD) and median (interquartile range – IQR) were utilised for the description of quantitative variables, respectively, for data with and without a normal distribution. Kaplan-Meier method was employed to generate patient survival curves. Cox proportional hazards models were constructed to identify factors associated with mortality risk. Two models were developed – one based on laboratory parameters and another on preoperative parameters.

Given the asymmetric distribution of most laboratory indicators (non-normal distribution), a logarithmic transformation was applied before their inclusion in the model. Continuous variables that did not meet the assumptions of the Cox proportional model were categorised (age and body mass index). Statistical significance was attributed to p-values below 0.05. All statistical analyses were conducted using IBM SPSS version 26.

2.2 Comparison of outcome between blood culture positive and negative IE patients undergoing cardiac surgery

Categorical values were expressed as relative frequencies (percentages). Mean values (standard deviation – SD) and median (interquartile range – IQR) were used for the description of quantitative variables, respectively, for data with and without a normal distribution. Visual tools were employed to assess data normality. For categorical variables, the Chi-square test or Fisher's exact test, according to assumptions, were utilised to compare differences between groups. For quantitative variables, Student's t-tests or Mann-Whitney U tests, based on test assumptions, were used to compare groups.

Single-factor and multi-factor logistic regressions were applied to examine the associations between potential factors affecting in-hospital and long-term survival. P-values below 0.05 were considered statistically significant. Statistical analyses were conducted using IBM SPSS version 26. RStudio version 1.3.1073 was used for survival analysis representation.

2.3 Differences in the clinical course and outcome of IE caused by the most common microorganisms in cardiac surgery patients

Statistical analyses were performed using RStudio version 1.4.1717. Descriptive statistics used mean values (SD) and median (IQR) for data with and without a normal distribution, respectively. The applied inferential statistical methods included the Kruskal-Wallis rank sum test, Pearson's Chi-square test, and Fisher's exact test with simulated p-values (based on 2000 iterations) for group comparisons according to their assumptions.

Survival analysis visualization utilised the Kaplan-Meier method, and group comparisons employed the log-rank test and the Peto and Peto modified Gehan-Wilcoxon test. P-values below 0.05 were considered statistically significant.

2.4 Application of 16S rRNA Next-generation sequencing in excised valve samples from IE patients

Statistical analyses were conducted using the gtsummary v.1.7.0 package in the R environment. Descriptive statistics included frequency and percentages for categorical variables, and mean, median, standard deviation, and interquartile range for continuous variables. Multivariate analysis of variance (MANOVA) was employed for inferential statistics to assess differences between *Bartonella spp.* positive and *Bartonella spp.* negative patients. P-values below 0.05 were considered statistically significant.

2.5 Comparison of morphological findings and neutrophil leukocyte activation in IE caused by *Bartonella spp.* and non-*Bartonella spp.*

Statistical analysis, as well as the creation of diagrams and graphs, was performed using Prism 9 software for MacOS (GraphPad Software, LLC, San Diego, CA, USA), JMP 16 (SAS, Cary, NC, USA), and Jamovi software version 2.4.8. Descriptive statistical analysis included calculating frequency and percentage for categorical variables and determining mean, median, standard deviation (SD), and interquartile range (IQR) for continuous variables.

For the dispersion of variable quantities between *Bartonella spp.* positive and negative patients, a multivariate analysis of variance (MANOVA) was conducted using the built-in statistics package in R. The comparison of intra- and extracellular expression of the examined immunohistochemical markers was analysed using the Kruskal-Wallis test, followed by the Benjamini, Krieger, and Yekutieli two-stage linear step-up procedure as a post-hoc correction method when necessary. In cases where the assumptions of the Kruskal-Wallis test were not met, the Mann-Whitney U rank sum test was used.

To explore possible correlations between clinical and immunohistochemical data, Spearman's rank correlation analysis was employed. Correlation matrices and correlation clustering were used to provide a clear overview of relationships between the investigated intracellular and extracellular immunohistochemical variables. Partial correlation analyses were performed to reduce the potential impact of covariates on specific associations.

Additionally, to identify patterns within the collected data concerning the studied individuals, a hierarchical clustering method was used. Alluvial diagrams were created to visually represent associations between variable quantities. Statistical significance between variables was determined at a threshold of $p < 0.05$.

3 Results

3.1 Risk factors associated with mortality in the IE patients requiring cardiac surgery

The study analysed the medical history data of 242 patients from January 1, 2015, to January 1, 2019, who underwent heart surgery at Pauls Stradiņš Clinical University Hospital due to IE. The indications for surgery were determined based on the 2015 European Society of Cardiology guidelines for the management of IE (see Table 1.1). Nine patients underwent surgery due to IE recurrence. The average age of patients was 55.42 (14.48) years, with the youngest being 20 years old and the oldest – 80 years old. Most surgeries were performed for left-sided IE, while right-sided endocarditis was found in 5.78 % of cases, often associated with intravenous drug use. Combined left and right-sided IE was observed in 4.97 % of patients. The most common comorbidities were chronic kidney disease and chronic obstructive pulmonary disease. Perioperative characteristics of IE patients are summarised in Table 3.1.

Table 3.1

Perioperative characteristics of IE patients

Age, years, mean	55.42 (14.48)
Sex, male, %	74.00
BMI, mean (kg/m^2)	25.74 (5.05)
Prosthetic valve endocarditis, %	21.07
Left heart side IE, %	89.25
Right heart side IE, %	5.78
History of intravenous drug use, %	7.85
Critical preoperative condition, %	12.81
EuroSCORE II risk, mean, %	6.49

The most frequently infected was the aortic valve, followed by the mitral valve. Both valves were involved in the infection process in 22.31 % of cases.

On average, patients had large vegetations, measuring 15.54 (8.90) mm. Other echocardiographic data are summarised in Table 3.2. Overall, the incidence of both subclinical and clinically detectable embolisms was 26.86 %. The most frequently observed embolism occurred in the central nervous system (CNS) and spleen. Other IE complications are summarised in Table 3.3.

Table 3.2

Preoperative echocardiographic parameters in IE patients

Vegetation size, mean, mm	15.54 (8.90)
Signs of locally uncontrolled infection, %	19.42
Aortic valve IE, %	39.26
Mitral valve IE, %	28.10
Aortic and mitral valve IE, %	22.31
Tricuspid valve IE, %	5.37
Aortic, mitral and tricuspid valve IE, %	1.65
EF of the left ventricle, mean, %	55.46 (8.97)

Table 3.3

Preoperative complications in IE patients

Perivalvular infection spread, %	19.42
Systemic embolism, %	26.86
CNS embolism, %	17.77
Spleen embolism, %	28.10
Kidney embolism, %	4.96

The most frequently detected pathogen was *S. aureus* in 17.36 % of cases, followed by *Streptococcus spp.* in 12.40 %, as well as *E. faecalis* in 12.40 %, and other microorganisms in 14.05 % of cases. In the study group, there was a very high incidence of blood culture-negative IE – 45.04 %. IE caused by multidrug-resistant microorganisms was observed in 4.13 % of cases. Among all microorganisms, *S. aureus* was associated with a 2.3 times

higher mortality rate in univariate analysis (HR 2.01; CI 0.98–4.09; p = 0.055), although statistical significance was not demonstrated in multivariate analysis (RR 2.01; CI 0.98–4.09; p = 0.055). Repeated surgical intervention for acute bleeding, early or late cardiac tamponade (including subxiphoid pericardial drainage), was necessary in 15.29 % of cases. Other complications are listed in Table 3.4.

Table 3.4

Most common postoperative IE complications

Reoperation for bleeding or cardiac tamponade, %	15.29
Intra-aortic balloon pump (IABP) use, %	10.33
Extracorporeal membrane oxygenation (ECMO) use, %	4.13
Haemodialysis for acute renal failure, %	9.09

In-hospital mortality was 11.16 % (27 patients). One-year mortality reached 21.70 % (Table 3.5). Kaplan-Meier survival curve for IE patients after cardiac surgery is depicted in Figure 3.1. The highest mortality was observed within the first year, followed by stabilization of the curve.

Table 3.5

Duration of hospital and intensive care unit stay, in-hospital mortality, one-year, and three-year survival

Hospital stay (days)	26.86 (14.69)
ICU stay (days)	4.54 (6.76)
In-hospital mortality (%)	11.16
One-year survival (%)	78.30
Three-year survival (%)	71.30

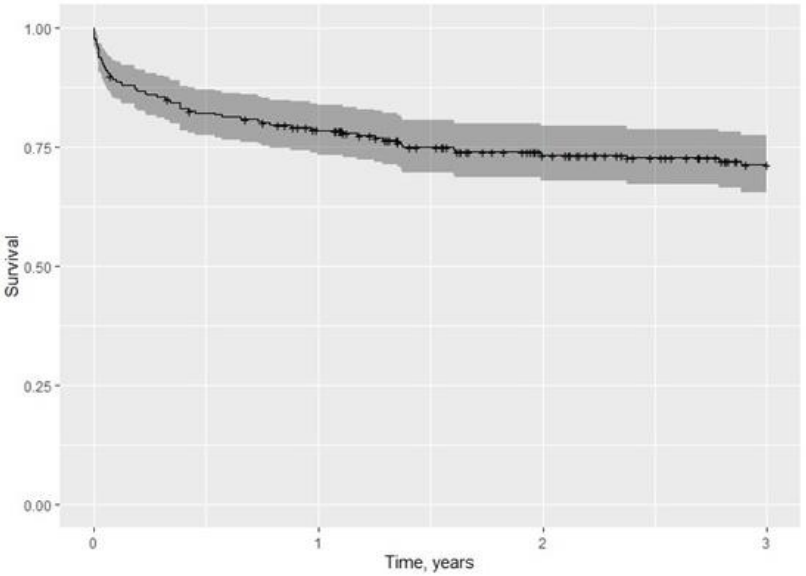

Figure 3.1 **Three-year follow-up survival of operated IE patients**

Risk factors for in-hospital mortality were determined. Elevated levels of C-reactive protein (CRO), B-type natriuretic peptide (BNP), and procalcitonin showed a statistically significant association with in-hospital mortality in univariate analysis, although this was not confirmed in multivariate analysis. In the multivariate analysis, there was a tendency for creatinine to be associated with in-hospital mortality, but it did not reach statistical significance (Table 3.6). Similarly, other risk factors were analysed (Table 3.7), where IE caused by *S. aureus*, perivalvular infection spread, and systemic embolism of vegetation were statistically significant factors for in-hospital mortality in univariate analysis. However, in multivariate analysis, only perivalvular infection spread was confirmed as an independent risk factor for in-hospital mortality (RR 1.99, CI 1.05–3.78; p = 0.035). There was also a tendency for the association with in-

hospital mortality in multivariate analysis for *S. aureus*-caused IE, but statistical significance was not achieved.

Table 3.6

Laboratory prognostic factors for in-hospital mortality in univariate and multivariate logistic regression analyses

Variable	HR (95 % CI)	p value	HR (95 % CI)	p value
log Leukocyte count, x 10^9/L	1.19 (0.91–1.57)	0.209	1.11 (0.67–1.84)	0.687
log CRP mg/L	1.2 (1.00–1.43)	0.050	0.91 (0.61–1.36)	0.636
log BNP, pg/ml	1.28 (1.04–1.57)	**0.020**	0.95 (0.67–1.36)	0.798
log Procalcitonin, ng/ml	1.2 (1.09–1.32)	< **0.001**	1.19 (0.95–1.49)	0.130
log Creatinine, µmol/L	1.39 (0.93–2.09)	0.110	2.35 (0.99–5.61)	0.054
log Glucose, mmol/L	1.07 (0.51–2.25)	0.856	0.76 (0.15–3.86)	0.739
Haemoglobin, g/l	0.99 (0.98–1.00)	0.086	1.00 (0.98–1.02)	0.865
Haematocrit, %	0.98 (0.94–1.02)	0.293	–	–
Platelet count, x 10^9/L	1 (1.00–1.00)	0.573	1.00 (1.00–1.00)	0.606
log Bilirubin, µmol/L	1.26 (0.84–1.89)	0.261	1.05 (0.56–1.96)	0.887

* Logarithmic data transformation was used to normalise certain parameter data.
** To avoid multicollinearity, haematocrit indicators were excluded in the multivariate analysis.

Table 3.7

Prognostic factors for in-hospital mortality in univariate and multivariate logistic regression analyses

Variable	HR (95 % CI)	p value	HR (95 % CI)	p value
Age 65 years or older	1.27 (0.78–2.06)	0.336	0.88 (0.48–1.61)	0.669
Male, sex	0.87 (0.52–1.46)	0.610	0.78 (0.42–1.45)	0.431
BMI above 25 kg/m^2	0.83 (0.51–1.35)	0.457	0.74 (0.43–1.27)	0.270
NVE	0.80 (0.46–1.39)	0.419	0.90 (0.46–1.76)	0.756
PVE	1.34 (0.78–2.31)	0.293	–	–
S. aureus infection	2.27 (1.36–3.80)	**0.002**	2.01 (0.98–4.09)	0.055

Table 3.7 continued

Variable	HR (95 % CI)	p value	HR (95 % CI)	p value
Streptococcus spp. infection	1.01 (0.50–2.02)	0.987	1.23 (0.53–2.87)	0.627
E. faecalis infection	0.83 (0.40–1.73)	0.616	1.48 (0.59–3.74)	0.408
Other microorganism infection	1.04 (0.53–2.03)	0.910	1.29 (0.53–3.18)	0.573
Multiresistant microorganism infection	1.30 (0.47–3.58)	0.607	1.40 (0.42–4.66)	0.581
EF of left ventricle	0.98 (0.95–1.00)	0.062	0.98 (0.95–1.01)	0.134
Size of vegetations	1.01 (0.98–1.04)	0.415	1.36 (0.62–3.00)	0.439
Perivalvular complications	1.98 (1.19–3.29)	**0.009**	1.99 (1.05–3.78)	0.035
Systemic embolism of vegetations	1.63 (1.00–2.64)	**0.048**	1.25 (0.70–2.25)	0.453

* To avoid multicollinearity PVE was excluded in multivariate analysis.

Summary of research results

1. In-hospital mortality for patients undergoing cardiac surgery due to IE was 11.2 %. One and three-year mortality rates were 21.7 % and 28.7 %, respectively.
2. Perivalvular infection spread is independently associated with increased in-hospital mortality. A similar trend was observed for IE caused by *S. aureus*.
3. None of the laboratory parameters were independently prognostic for in-hospital mortality, but elevated creatinine levels showed a trend in this direction.

3.2 Comparison of outcome between blood culture positive and negative IE patients undergoing cardiac surgery

This study examined the medical histories of patients who underwent surgery for IE between 2016 and 2019. Data were collected for a total of 207 patients, out of which 93 (44.9 %) had negative blood cultures. The identification of the causative microorganism from operative material was low in both groups: 13.2 % in the bacterial culture-negative endocarditis (BCNE) group and 5.4 % in the bacterial culture-positive endocarditis (BCPE) group. Preoperatively, all BCNE and 71 out of 93 BCPE patients were classified as definite IE cases based on the Duke criteria, with 22 patients classified as possible IE cases.

Patient characteristics and common comorbidities are summarised in Table 3.8. In the BCPE group, statistically more intravenous drug users were observed, along with a higher prevalence of type 1 diabetes and hepatitis C virus infection (HCV). The most frequently identified microorganisms in the BCPE group were *S. aureus* in 36 (31 %) cases, *Streptococcus spp.* in 27 (24 %) cases, *E. faecalis* in 24 (21 %) cases, and other microorganisms in 27 (24 %) cases. The frequencies of characteristic IE complications, such as embolisms, locally uncontrolled infections, hemodynamic instability, and the average vegetation size, did not significantly differ between the groups (Table 3.9). Overall, embolic events were observed in 60 (28.9 %) patients. In the laboratory parameters, the BCPE group showed significantly higher procalcitonin levels and lower haemoglobin and haematocrit levels (Table 3.10). Although the BCPE group exhibited a higher C-reactive protein (CRO) level, statistical significance was not reached.

Table 3.8

Characteristics and comorbidities of IE patients depending on blood culture status

Variable	Blood culture positive	Blood culture negative	p value
Age, mean (years)	57.17 (15.59)	53.61 (12.80)	0.073
Sex, male, %	78.07	65.59	**0.046**
BMI, mean (kg/m2)	25.45 (4.48)	25.91 (5.65)	0.531
PVE, %	21.51	19.30	0.694
Aortic valve IE, %	38.60	41.94	0.626
Mitral valve IE, %	32.46	22.58	0.116
Aortic and mitral valve IE, %	18.42	29.03	0.072
Left heart side IE, %	89.47	95.70	0.095
Intravenous drug injection history, %	12.28	4.30	**0.043**
Haemodynamically stable, %	88.60	92.47	0.348
Left ventricle ejection fraction, mean, %	55.24 (11.19)	55.77 (9.18)	0.714
EuroSCORE II risk, mean, %	7.35	6.80	0.595
Diabetes mellitus, type 1, %	5.56	0.00	**0.032**
Diabetes mellitus, type 2, %	9.26	8.79	0.909
HIV infection, %	6.42	1.10	0.074
HCV infection, %	15.74	5.49	**0.022**
HBV infection, %	0.00	1.10	0.457
Spondylodiscitis, %	5.26	1.08	0.132
Oncology, %	4.39	3.23	0.733
Arterial hypertension, %	4.59	12.09	0.720

Table 3.9

Occurrence of embolic events, locally uncontrolled infection, haemodynamic instability prior surgery and vegetation size in IE patients depending on blood culture status

Variable	Blood culture positive	Blood culture negative	p value
Embolic events prior to surgery, %	29.82	27.96	0.768
Locally uncontrolled infection, %	30.00	31.87	0.775
Haemodynamically unstable, %	11.40	7.53	0.348
Vegetation size, mm, mean	16.70 (9.95)	14.48 (7.69)	0.097

Table 3.10

Laboratory analyses in IE patients depending on blood culture status

Variable	Blood culture positive	Blood culture negative	p value
CRP, mg/L	37.90 (9.98–94.33)	25.00 (12.25–63.70)	0.206
Procalcitonin, ng/ml	0.45 (0.10–2.33)	0.10 (0.10–0.50)	**0.001**
BNP, pg/ml	560.45 (157.73–1580.05)	469.50 (178.35–1114.30)	0.424
Creatinine, µmol/L	85.50 (63.25–107.75)	87.50 (66.00–110.00)	0.734
Glucose, mmol/L	6.08 (1.70)	6.20 (1.56)	0.614
Bilirubin, µmol/L	8.00 (6.00–15.00)	8.00 (6.00–12.00)	0.592
Leukocyte count x 10^9/L	9.20 (4.15)	8.81 (3.73)	0.484
Haemoglobin, g/L	103.85 (23.00)	110.91 (21.09)	**0.024**
Haematocrit, %	32.12 (6.07)	34.06 (6.41)	**0.029**
Platelet count x 10^9/L	261.73 (129.83)	256. 58 (110.58)	0.763

Clinical outcomes, including hospitalization duration, intensive care unit (ICU) stay, frequency of reoperations due to bleeding, and in-hospital mortality, are summarised in Table 3.11. No statistically significant differences were observed in any of the groups.

Table 3.11

Duration of hospital and ICU stay, reoperation for bleeding and mortality rates in IE patients depending on blood culture status

Variable	Blood culture positive	Blood culture negative	p value
Length of stay, days, mean	25.00 (16.00–35.00)	23.00 (17.50–31.00)	0.378
ICU stay, days, mean	2.00 (1.00–5.00)	2.00 (1.00–3.00)	0.828
Reoperation for bleeding, %	17.54	12.90	0.358
Mortality, %	14.04	5.38	0.062

The detection of microorganisms and higher procalcitonin levels showed an association with in-hospital mortality in the univariate analysis, but it was not confirmed in the multivariate analysis (Table 3.12). In the positive blood culture IE group, among all microorganisms, *S. aureus* was independently associated with higher in-hospital mortality (Table 3.13). In the long term, the BCNE group exhibited better survival; however, statistical significance was not observed (p = 0.509). Survival curves are depicted in Figure 3.2.

Table 3.12

Predictors of hospital mortality by univariable and multivariable logistic regression analyses

Variable	Univariable OR (95 % CI)	p value	Multivariable adjusted OR (95 % CI)	p value
Microorganism detection	2.873 (1.011–8.167)	**0.048**	1.166 (0.319–4.272)	0.816
Age	1.024 (0.991–8.167)	0.155	1.027 (0.975–1.082)	0.319
Body mass index	1.034 (0.946–1.129)	0.466	1.034 (0.912–1.173)	0.599
CRP (C-reactive protein)	1.003 (0.995–1.011)	0.490	1.002 (0.991–1.014)	0.696
Procalcitonin	1.036 (1.004–1.068)	**0.025**	1.033 (0.999–1.068)	0.057
Leukocytes	1.029 (0.924–1.146)	0.608	1.066 (0.909–1.249)	0.433

Table 3.12 continued

Variable	Univariable OR (95 % CI)	p value	Multivariable adjusted OR (95 % CI)	p value
Embolism	1.586 (0.621–4.048)	0.335	1.311 (0.368–4.673)	0.677
Intravenous drug user	0.497 (0.063–3.937)	0.508	1.202 (0.087–16.600)	0.891
Prosthetic valve endocarditis	2.157 (0.810–5.742)	0.124	0.849 (0.024–29.994)	0.928
Native valve endocarditis	0.464 (0.174–1.234)	0.124	0.840 (0.025–27.790)	0.922

Table 3.13

Detected microorganisms as predictors of hospital mortality by univariable and multivariable logistic regression analyses

Variable	Univariable OR (95 % CI)	p value	Multivariable adjusted OR (95 % CI)	p value
E. faecalis	2.325 (0.527–10.246)	0.265	1.310 (0.356–4.824)	0.685
Streptococcus spp.	1.788 (0.422–7.587)	0.430	1.073 (0.295–3.910)	0.915
S. aureus	4.408 (1.406–13.821)	**0.011**	3.332 (1.268–8.751)	**0.015**
Other microorganism	1.302 (0.243–6.962)	0.758	0.678 (0.149–3.089)	0.615

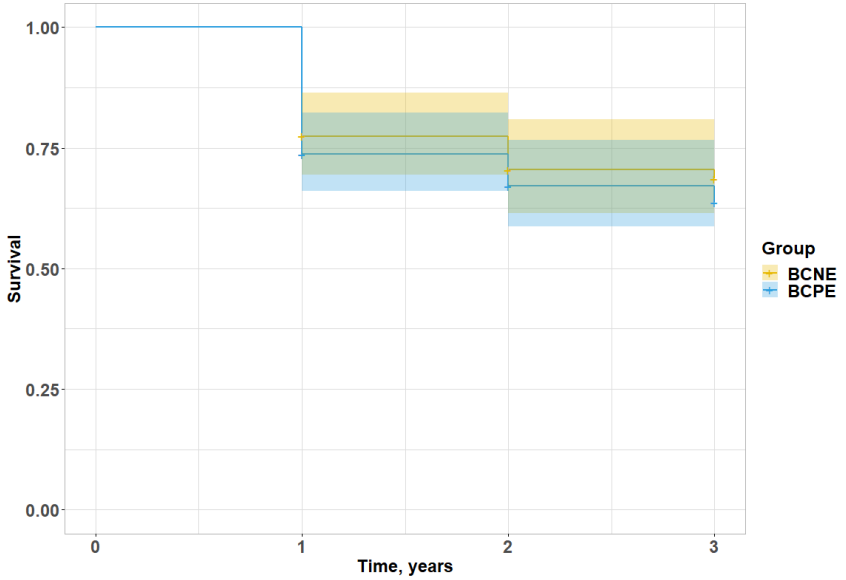

Figure 3.2 **Three-year follow-up survival in surgically treated BCPE and BCNE patients**

Summary of research results

1. There are no statistically significant differences between BCPE and BCNE groups regarding in-hospital mortality, hospital and ICU length of stay, as well as three-year mortality.
2. Although BCPE patients have higher in-hospital and three-year mortality than BCNE patients, BCPE is not independently associated with higher mortality in multivariate analysis.
3. The BCPE group had higher procalcitonin levels; however, elevated procalcitonin levels did not reveal an independent association with mortality in multivariate analysis.

4. The most common microorganism in the BCPE group was *S. aureus*. It was associated with independently higher in-hospital mortality (RR coefficient 3.332 and 4.408 in univariate and multivariate analysis, respectively) compared to other causative microorganisms.

3.3 Comparison of outcome between blood culture positive and negative IE patients undergoing cardiac surgery

In this study, 253 patients who underwent surgery for IE during the period from January 1, 2016 to September 1, 2020 were analysed. The indications for surgery were set based on the 2015 guidelines of the European Society of Cardiology for the management of IE (Table 1.1). In contrast to the previous study, which compared outcomes in BCNE, this study examined the impact of various common IE causative agents on outcomes. Of the 253 patient histories, the IE-causing microorganism was *S. aureus* in 44 cases, *Streptococcus spp.* in 35 cases, *E. faecalis* in 33 cases, and coagulase-negative staphylococci (CoNS) in 32 cases. Overall, 79.9 % of patients had native valve IE, and 20.3 % had prosthetic valve IE. The majority of native valve endocarditis cases were observed in *Streptococcus spp.* cases, while in prosthetic valve IE cases, coagulase-negative staphylococci (CoNS) were the most common causative agents (Table 3.14). Consequently, more perivalvular infection spread was observed in the CoNS group. In the *Streptococcus spp.* group, significantly more severe aortic regurgitation cases and fistula formation between heart chambers were observed. The association of several other echocardiographic parameters with specific microorganism groups was also examined, but no statistically significant differences were found (Table 3.15).

Table 3.14

Characteristics of patients with various IE causative microorganisms

Variable	***S. aureus*, N = 44**	***Streptococcus spp.*, N = 35**	***E. faecalis*, N = 33**	**CoNS, N = 32**	**p value**
Age, years	58.5 (45.0–66.0)	50.0 (37.0–65.5)	65.0 (56.0–73.0)	56.0 (48.2–69.2)	**0.037**
Males, %	77.3	85.7	87.9	68.8	0.195
BMI, mean, (kg/m^2)	24.7 (21.1–27.1)	24.7 (22.1–27.6)	24.4 (23.5–29.3)	26.6 (24.7–30.9)	**0.031**
EuroSCORE II risk, %	4.7 (2.2–7.0)	2.9 (2.0–4.9)	3.1 (2.0–8.0)	4.4 (2.1–10.9)	0.237
Native valve IE, %	82.5	94.1	78.1	62.5	**0.015**
Left heart side IE, %	80.5	97.1	96.9	90.6	0.059

Table 3.15

Echocardiographic parameters and valvular dysfunction in patients with various IE causative microorganisms

Variable	***S. aureus***	***Streptococcus spp***	***E. faecalis***	**CoNS**	**p value**
TOE performed, %	68.4	75.0	79.3	85.7	0.414
Size of vegetation, mm	16.0 (12.5–20.0)	14.0 (10.0–17.0)	15.0 (13.0–22.5)	15.0 (10.0–20.0)	0.250
Perivalvular complications, %	15.8	27.3	3.4	32.3	**0.024**
Fistula between cardiac chambers, %	2.7	15.2	0.0	3.2	**0.049**
Severe (grade 3–4) aortic regurgitation, %	20.5	57.1	27.3	28.1	**0.004**
Severe (grade 3–4) mitral regurgitation, %	20.5	28.6	33.3	18.8	0.455
Severe (grade 3–4) tricuspid regurgitation, %	15.9	2.9	9.1	6.2	0.235
Predominantly aortic stenosis	6.8	8.6	12.1	21.9	0.249

Table 3.15 continued

Variable	*S. aureus*	*Streptococcus spp*	*E. faecalis*	CoNS	p value
Predominantly mitral stenosis	2.3	0.0	9.1	12.5	0.074
EF, %	59.0 (52.0–60.0)	56.5 (54.2–60.0)	58.0 (50.0–65.0)	60.0 (55.0–65.0)	0.541
EDD, mm	53.0 (50.0–58.0)	56.5 (52.0–59.8)	53.0 (50.0–59.5)	53.0 (48.0–58.0)	0.272
ESD, mm	35.0 (31.0–38.0)	37.0 (33.0–38.8)	34.5 (30.5–37.2)	35.0 (31.0–39.0)	0.275
IVS, mm	11.0 (9.5–13.0)	10.0 (9.0–12.0)	10.0 (9.0–11.5)	11.0 (9.0–14.0)	0.496
TAPSE, mm	22.0 (18.5–25.5)	21.0 (18.0–24.0)	21.0 (17.5–23.5)	20.0 (18.5–24.2)	0.748
LAVI, ml/m^2	45.0 (31.0–59.0)	43.0 (37.0–51.0)	38.0 (31.8–55.2)	47.0 (36.2–57.0)	0.817
Ascending aortic diameter, mm	34.0 (32.2–35.8)	37.0 (35.0–44.0)	35.0 (33.2–37.5)	35.0 (31.0–37.0)	0.245

Embolic complications were observed in all microorganism groups, most frequently in the *S. aureus* group, and subsequently in the *E. faecalis*, CoNS, and *Streptococcus spp.* groups. They were more often found in the CNS, spleen, and kidneys. Notably, *S. aureus* IE was associated with a statistically significantly higher rate of CNS embolism ($p = 0.005$), observed in 25 % of all patients. Among microorganism groups, there were no differences in the length of hospitalization and time spent in the intensive care unit (Table 3.16). However, statistically significant differences were found in both one-year and three-year mortality rates among microorganism groups, where the *S. aureus* group had higher mortality, and the *E. faecalis* group had lower mortality (Figures 3.3 and 3.4).

Table 3.16

Clinical outcomes and complications in patients with various IE causative microorganisms

Variable	*S. aureus*	*Streptococcus spp*	*E. faecalis*	CoNS	p value
In-hospital stay, days	28.0 (19.0–42.0)	23.0 (15.5–31.0)	26.0 (17.0–37.0)	27.5 (22.0–35.0)	0.310
ICU stay, days	3.0 (1.0–9.8)	2.0 (1.0–5.0)	2.0 (1.0–4.0)	2.0 (1.0–4.0)	0.230
Embolic complications, %	42.5	8.8	34.4	15.6	**0.003**
CNS embolism, %	25.0	0.0	18.8	9.4	**0.005**
Spleen embolism, %	12.5	8.8	15.6	9.4	0.809
Kidney embolism, %	7.5	0.0	12.5	6.2	0.203

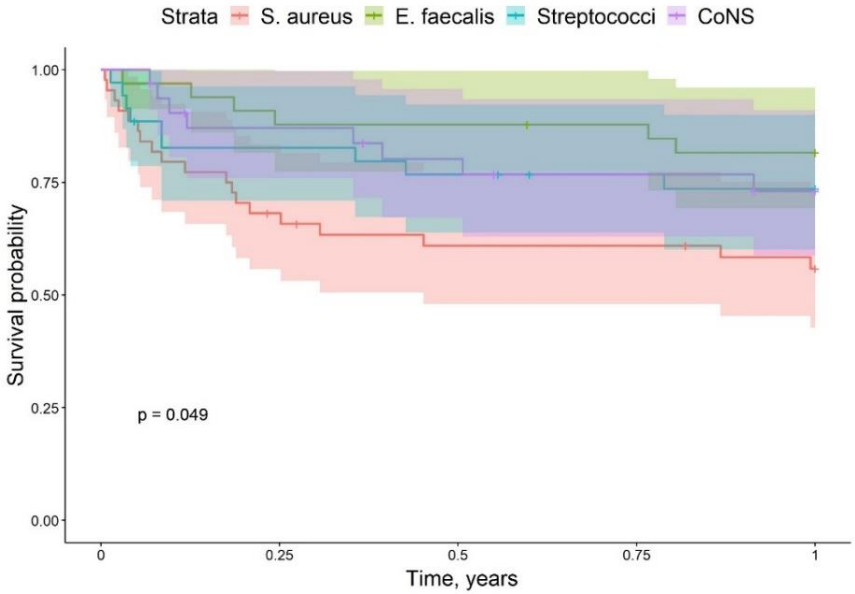

Figure 3.3 **Kaplan-Meier 1-year survival curves when comparing IE groups presented with different causative microorganisms. P-value represents the overall differences**

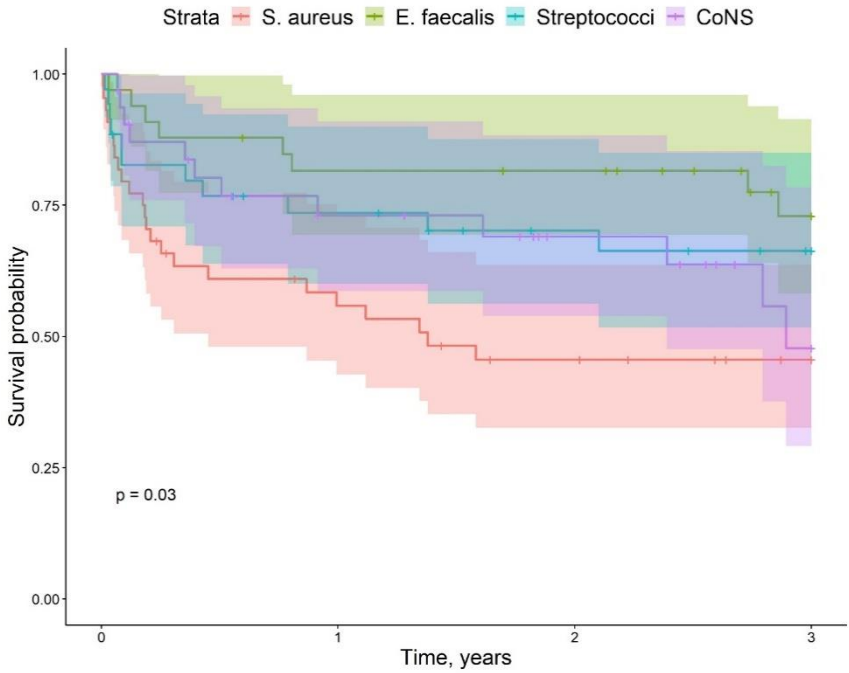

Figure 3.4 **Kaplan-Meier 3-year survival curves when comparing IE groups presented with different causative microorganisms. P-value represents the overall differences**

Summary of research results

1. Embolism was frequently observed in patients with *S. aureus* and *E. faecalis* IE.
2. Unlike *E. faecalis*, *S. aureus* is associated with a worse long-term prognosis for patients undergoing cardiac surgery for IE.
3. Perivalvular complications are most commonly associated with the presence of coagulase-negative staphylococci and prosthetic valve IE.

3.4 Application of 16S rRNA Next-generation sequencing in excised valve samples from IE patients

The study involved 46 patients. Among these patients, blood cultures were positive in 28 cases (60.1 %), whereas valve cultures were positive in only four cases (8.7 %). Blood culture-negative IE (BCNE) was diagnosed in 18 patients (39.1 %). Next-generation sequencing (NGS) method, in which the relative abundance of bacterial nucleic acids was > 90 % (thus conclusively indicating the causative pathogen), was found in 29 patients (63.0 %). All microorganisms identified in BCNE patients through blood or valve cultures were also detected using the 16S rRNA NGS method. The relative DNA abundance of microorganisms ranged from 22.6 % to 100 %. The 16S rRNA NGS technology proved particularly useful in cases where blood culture was negative, revealing the causative pathogen in 12 cases (66.7 %). An unexpected finding was the high incidence of *Bartonella spp.* in BCNE cases, detected in 11 out of 18 cases. In *Bartonella spp.* cases, there was a particularly high relative DNA abundance, with eight cases being 100 % and two cases being 95.4 % and 99.6 %, conclusively indicating the pathogenic microorganism. In cases of other microorganisms, a relative DNA abundance of 100 % was found in only 10.9 % of cases, whereas in *Bartonella spp.* cases, it was 72.7 %. Aetiology was not identified in six cases (8.7 %) because these patients had negative blood and valve culture results. However, 16S rRNA NGS analysis showed multiple microorganism DNA admixtures, with the relative DNA abundance of one microorganism ranging from 31.5 % to 71.7 %, making it impossible to determine the causative microorganism. In cases of IE caused by *Bartonella spp.*, patients had a lower left ventricular ejection fraction, significantly lower white blood cell and platelet counts, higher glucose, B-type natriuretic peptide (BNP), and creatinine levels (Table 3.17). Additionally, it is noteworthy that patients with *Bartonella spp.* endocarditis had a significantly higher history of

alcoholism. The outcomes such as hospital and ICU length of stay, as well as in-hospital mortality, did not statistically differ among *Bartonella spp*. patients.

Table 3.17

Comparison of non-*Bartonella spp.*- and *Bartonella spp.*-caused IE patients' characteristics, laboratory indices, and clinical outcomes

Variable	Non-*Bartonella spp.* IE, N = 35	*Bartonella spp.* IE, N = 11	p value
Age, years, mean (SD)	55.6 (14.8)	54.6 (9.8)	0.8217
Sex, male, N (%)	26 (74.3)	10 (9.9)	pY=0.4551
EuroSCORE II risk score, %, median (IQR)	6.5 (3.6–14.3)	6.3 (3.6–12.6)	pMW=0.9442
IE mortality risk score, %, median (IQR)	30.2 (13.6–41.1)	30.2 (21.5–34.9)	pMW=0.7988
Charlson Comorbidity index, points, median (IQR)	2.5 (1–5)	4 (3–5)	pMW=0.1868
Diabetes mellitus, N (%)	8 (22.9)	2 (18.2)	pY=0.9274
Alcoholism history, N (%)	5 (14.3)	7 (63.6)	pY=0.0124
Intravenous drug usage history, N (%)	3 (8.6)	0 (0.0)	pY=0.7609
Length of vegetation, mm, median (IQR)	12.0 (0.0–18.0)	13.0 (12.0–22.0)	pMW=0.1644
Embolism, N (%)	8 (22.9)	3 (27.3)	pY = 0.9158
Cerebral embolism, N (%)	7 (20.0)	2 (18.2)	pY=0.7618
Spleen embolism, N (%)	1 (2.9)	1 (9.1)	pY=0.9706
Kidney embolism, N (%)	2 (5.7)	0 (0.0)	pY=0.9706
EF of the left ventricle, %, mean (SD)	56.4 (7.8)	50.6 (9.4)	**0.0481**
Right ventricle systolic pressure, mm/Hg, median (IQR)	37.5 (30.0–56.3)	46.5 (43.8–56.3)	pMW=0.0627
Leukocytes, count 109 mL, median (IQR)	7.6 (5.8–9.7)	5.2 (4.2–6.0)	pMW=0.0038
Platelets, count 109 mL, median (IQR)	241.0 (186.0–325.0)	174.0 (82.0–218.0)	pMW=0.0185
Red blood cells, count 109 mL, median (IQR)	3.7 (3.2–4.4)	3.6 (3.1–4.1)	pMW=0.1987

Table 3.17 continued

Variable	Non-*Bartonella spp.* IE, N = 35	*Bartonella spp.* IE, N = 11	p value
Haemoglobin, g/L, mean (SD)	110.4 (22.2)	99.1 (14.1)	0.1205
CRP level before surgery, mg/dL, median (IQR)	32.8 (7.5–55.1)	33.5 (6.6–57.0)	pMW=0.7837
CRP level 2nd day after surgery, mg/dL, median (IQR)	185.8 (133.9–228.8)	157.8 (94.2–220.6)	pMW=0.2979
CRP level 4th day after surgery, mg/dL, median (IQR)	110.0 (72.7–159.3)	118.7 (104.8–215.1)	pMW=0.4110
CRP level 6th day after surgery, mg/dL, median (IQR)	57.6 (31.0–100.5)	71.9 (46.3–101.3)	pMW=0.4572
Procalcitonin, ng/mL, median (IQR)	0.12 (0.08–0.28)	0.22 (0.07–0.38)	pMW=0.4911
BNP, ng/mL, median (IQR)	429.8 (136.4–960.6)	1523.0 (692.9–3708.0)	pMW=0.0002
Glucose, mkmol/L, median (IQR)	5.4 (4.8–6.7)	4.7 (4.3–5.6)	pMW=0.0375
Creatinine, mmol/L, median (IQR)	77.0 (64.0–89.0)	104.0 (78.0–283.0)	pMW=0.0071
Blood loss, first day, mL, median (IQR)	372.5 (250.0–630.0)	420.0 (301.3–930.0)	pMW=0.3463
Resternotomy due to acute bleeding, N (%)	6 (17.1)	3 (27.3)	pY=0.7618
Mechanical lung ventilation, hours, median (IQR)	11.5 (8.0–72.0)	14.0 (9.0–25.0)	pMW=0.7533
Usage of vasopressors, N (%)	23 (65.7)	8 (72.7)	pY=0.9489
Usage of beta-agonists, N (%)	13 (37.1)	7 (63.6)	pY=0.2311
Days in-hospital, median (IQR)	31.0 (25.0–48.0)	37.0 (19.0–44.0)	pMW=0.5544
Days in the ICU, median (IQR)	3.0 (1.8–9.0)	3.0 (2.0–8.0)	pMW=0.9106
In-hospital death, N (%)	4 (11.4)	0 (0.0)	pY=0.5755

* Abbreviations: IE – IE, SD – standard deviation, IQR – interquartile range, EF – ejection fraction, CRP – C-reactive protein, BNP – Beta-type natriuretic peptide, ICU – intensive care unit, p – p-values from unpaired t-test,
pMW – p-values from Mann-Whitney U-test, pY – p-values from Chi2 test with Yates correction.

Summary of research results

1. The 16S rRNA NGS is a useful technology for diagnosing the causative agent of diseases, especially in cases of blood culture-negative IE.
2. Among the causative agents of blood culture-negative IE, *Bartonella spp.* constitutes the majority.
3. Patients with *Bartonella spp.* IE more frequently have a history of alcoholism, higher levels of creatinine and B-type natriuretic peptide, lower glucose levels, as well as lower white blood cell and platelet counts.

3.5 Comparison of morphological findings and neutrophil leukocyte activation in IE caused by *Bartonella spp.* and *non-Bartonella spp.*

In total, valve samples were obtained from 10 patients with *Bartonella spp.* IE, randomly selected 12 patients with non-*Bartonella spp.* IE, and 23 patients in the control group. The *non-Bartonella spp.* group included patients whose IE was caused by *S. aureus* in one patient, *E. faecalis* in two patients, coagulase-negative staphylococci in three patients, and *Streptococcus spp.* in six patients. The control group consisted of cardiac surgery patients without a diagnosis of IE, who underwent surgery for severe aortic or mitral valve insufficiency. Sixteen aortic and seven mitral valve patients of similar age and corresponding valve were selected.

3.5.1 Histopathological and histochemical findings

Histopathologically, *Bartonella spp.* patient valve material showed fibroblastic proliferation and predominance of mononuclear cells (Figure 3.5 B–C). Fibrotic changes and sometimes calcification were also observed. In vegetation samples obtained from patients with *Bartonella spp.* induced IE, sponge-like, eosinophilic masses were more frequently found. These

masses consisted of a fibrin network, with a small number of immune cells. A small amount of microorganisms was observed in them. Similar eosinophilic vegetation masses were found in samples obtained from patients with non-*Bartonella spp.* induced IE; however, the sponge-like appearance of the vegetation masses was less pronounced than in *Bartonella spp.* cases. In non-*Bartonella spp.* IE, visually, there was a higher number of immune cells and microorganisms in the vegetation mass.

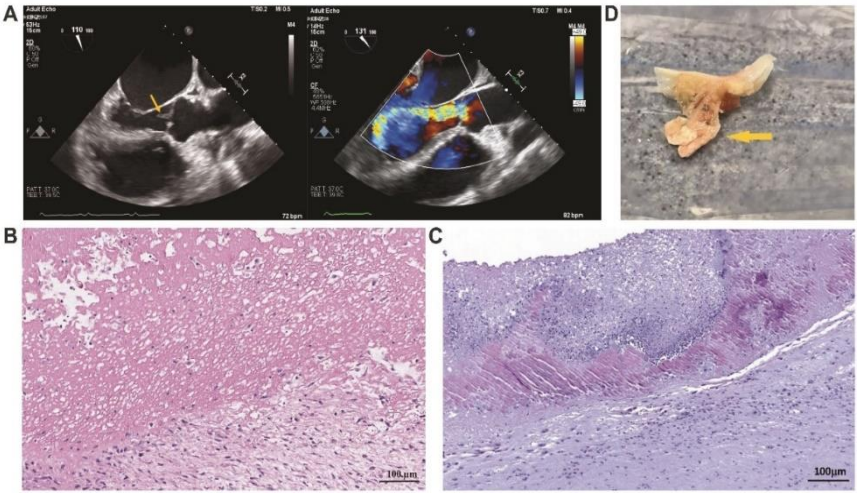

Figure 3.5 **Echocardiographic, macroscopic, and histopathological findings**

Bartonella spp. induced aortic valve endocarditis transoesophageal echocardiography (A). On the left side of the image, indicated by the arrow, vegetation attached to the aortic valve leaflet is shown, while on the right side of the visible image, using colour Doppler imaging, pronounced aortic valve insufficiency is visualised. In the lower images, histopathological examinations of valve vegetation in areas where it attaches to the valve leaflet in *Bartonella spp.* induced IE cases are shown. In the vegetation area, an amorphous material with a sponge-like appearance is observed, along with immune cells. Fibroblasts and mononuclear cells are also visible (B). In non-*Bartonella spp.* induced IE cases, more neutrophils, macrophages, bacteria, and fibrin fibres are observed, as well (C). H&E staining. Scale: 100 µm. Macroscopic section of the aortic valve leaflet with a vegetation appearance. The arrow indicates vegetation attached to the aortic valve leaflet (D).

Thereafter, histochemistry was applied to visualise most of the cardiac valve connective tissue components. To simultaneously label collagen reticular fibres and enhance bacterial visualization, a reticulin silver impregnation kit was used. In *Bartonella spp.* IE cases, reticular fibres in the valve leaflets formed a network and surrounded the amorphous vegetation mass. In non-*Bartonella spp.* IE cases, a slightly more parallel arrangement of reticular fibres was observed. It was found that this arrangement has statistically significant differences compared to that confirmed in control sample valve leaflets but not in vegetations. Using silver impregnation, black-coloured bacterial clusters were observed.

3.5.2 Immunohistochemical evaluation of neutrophil leukocyte activation markers in cardiac valve leaflets and vegetations

To study the expression of various neutrophil leukocyte granule, cytosolic enzyme, and histone in damaged heart valve tissues and vegetations, both *Bartonella spp.* and non-*Bartonella spp.* cases of IE used immunohistochemical methods. Neutrophil azurophilic granule components, including bactericidal enzymes such as neutrophil elastase (NE) and myeloperoxidase (MPO), as well as cytosolic protein calprotectin and histone H3, were evaluated both intracellularly and extracellularly. Valve leaflets and vegetations were examined separately and compared to the control group.

There was a significant difference in MPO expression between *Bartonella spp.* and non-*Bartonella spp.* IE. Anti-MPO antibodies strongly stained target cells, whereas in non-*Bartonella spp.* IE cases, the staining was more diffuse and mostly localised extracellularly. Both *Bartonella spp.* and non-*Bartonella spp.* groups showed some cells within newly formed blood vessels. MPO expression was significantly more pronounced in vegetations in *Bartonella spp.* ($p = 0.0437$) and non-*Bartonella spp.* ($p = 0.0027$) cases. In all IE cases, MPO expression significantly differed from the control group, except

for intracellularly determined MPO expression in *Bartonella spp.* IE cases. Both intracellularly and extracellularly, MPO was significantly more common in non-*Bartonella spp.* IE cases compared to *Bartonella spp.* IE cases ($p = 0.0279$ and $p = 0.0020$, respectively) and the control group ($p < 0.0001$). Extracellularly, MPO expression was significantly more common in the non-*Bartonella spp.* IE group ($p = 0.0279$).

Anti-NE antibodies produced intense staining of target cells, supplemented with moderately strong and fairly diffuse extracellular staining in both valve leaflets and vegetations of *Bartonella spp.* and non-*Bartonella spp.* IE cases. This neutrophil activation marker also showed statistically significantly higher expression in vegetations than in valve leaflets in both non-*Bartonella spp.* IE cases ($p = 0.0022$) and *Bartonella spp.* cases ($p = 0.0060$). Furthermore, when comparing the expression in valve leaflets, it was significantly higher in non-*Bartonella spp.* cases ($p = 0.0041$). In both *Bartonella spp.* and non-*Bartonella spp.* groups, NE expression, whether intracellular or extracellular, was significantly higher than in the control group ($p = 0.0218$, $p < 0.0001$, $p = 0.0154$, and $p = 0.0007$).

To investigate the role of adhesion molecules in the formation of platelet-leukocyte conjugates in damaged heart valve tissues and vegetations, immunohistochemistry was used. P-selectin expression was noted in damaged valve leaflets but was more pronounced in vegetations. Although non-*Bartonella spp.* IE cases showed higher P-selectin expression compared to *Bartonella spp.* IE, the difference was not statistically significant.

Alluvial diagrams were also created (Figure 3.6.) to illustrate the distribution of associations between categorical dimensions, including immunohistochemically assessed neutrophil leukocyte activation markers and clinical parameters in *Bartonella spp.* IE, non-*Bartonella spp.* IE, and control groups.

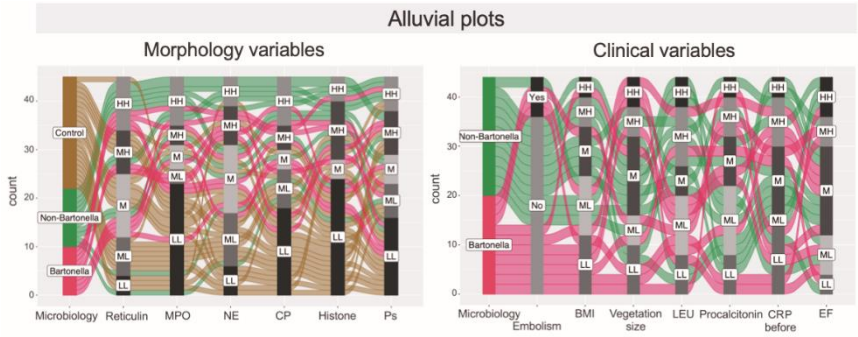

Figure 3.6 **Alluvial diagrams**

Alluvial diagrams represent patient groups as flows between nodes. Patient groups are depicted in rows, while immunohistochemically obtained neutrophil leukocyte activation markers are presented in columns. The number of each marker is stratified into five different levels. In the investigated groups, the width of each line and the resulting flow are proportional to the category's total. The left-side graph shows that in non-*Bartonella spp.* IE cases, all immunohistochemical manifestations indicating neutrophil leukocyte activation are mostly high and moderately high. On the right side, the graph indicates that the evaluated levels of clinical parameters in IE patient groups differ: CRP, leukocyte count, procalcitonin, and CRO reached the highest levels in patients with non-*Bartonella spp.* IE, while larger vegetations were observed in the *Bartonella spp.* patient group. Abbreviations: High, High (HH); Moderate, High (MH); Moderate (M); Moderate, Low (ML); Low, Low (LL); MPO – myeloperoxidase; NE – neutrophil elastase; CP – calprotectin; Histone – histone H3; Ps – P-selectin; BMI – body mass index; LEU – leukocyte count; CRP before – C-reactive protein level before surgery; EF – left ventricular ejection fraction.

Summary of research results

1. Neutrophil leukocyte extracellular traps are involved in the pathogenesis of IE but are not found in cardiac valve tissues with degenerative disease.
2. Differences in the morphology of vegetations on valve leaflets are observed between *Bartonella spp.* and non-*Bartonella spp.* IE patients.
3. More pronounced activation of neutrophil activation markers is observed in patients with non-*Bartonella spp.* induced IE.

4 Discussion

4.1 Risk factors associated with mortality in the IE patients requiring cardiac surgery

Despite advancing diagnostic capabilities and available guidelines for the treatment of IE, it remains a clinically challenging disease associated with a high risk of mortality (Habib et al., 2015b; Pettersson & Hussain, 2019). In our study, the in-hospital mortality for surgically treated IE patients was 11.2 %. In most cases, patient death was primarily associated with cardiopulmonary failure. Other causes of death included uncontrolled infection, intracerebral bleeding, and one patient's death intraoperatively due to massive bleeding. It is noteworthy that due to retrospective data, the long-term causes of death for most patients are unknown. Overall, our results are comparable to data reported by other centres, where in-hospital mortality ranges from 10 to 20 % (Abramczuk et al., 2015; Fernandes et al., 2017; Murdoch et al., 2009; Selton-Suty et al., 2019). In a large international multicentre study involving 58 hospitals and 2781 treated IE patients, the main risk factors associated with increased in-hospital mortality were patient age, pulmonary oedema, perivalvular complications, prosthetic valve IE, and the presence of staphylococcal IE (Murdoch et al., 2009). The study confirmed that in-hospital mortality is also dependent on the patient's preoperative condition during hospitalization and accompanying illnesses. Among IE patients, 26.45 % had chronic kidney disease, 11.56 % had diabetes mellitus, and 11.16 % had chronic obstructive pulmonary disease. Subclinical or clinically detected embolism was found in 26.86 % of patients. In left-sided IE cases, the central nervous system was most frequently affected. In this study, patients with both clinical and subclinical neurological changes were surgically treated based on the 2015 European Society of Cardiology guidelines on the prevention, diagnosis, and treatment of IE. For patients with intracranial bleeding and severe neurological damage, surgery was not performed, and these patients

received conservative treatment. In such cases, the operation was postponed for at least one month, revaluating its necessity. In general, IE patients often face a spectrum of complications, requiring careful monitoring after surgical treatment. In our study, one-year mortality for IE patients was 21.70 %, while three-year mortality was 28.70 %. These results align with reported data indicating that one-year mortality can reach 20–40 % (Cahill & Prendergast, 2016; Murdoch et al., 2009; Muthiah et al., 2020; Rohn et al., 2020). Several independent risk factors influencing IE patient survival after cardiac surgery were identified, such as *S. aureus* infection (RR 2.27, $p = 0.002$), the presence of perivalvular complications (RR 1.98, $p = 0.009$), and systemic vegetation embolism (RR 1.63, $p = 0.048$). Additionally, several laboratory parameters, such as elevated C-reactive protein levels (RR 1.2, $p = 0.050$), B-type natriuretic peptide (RR 1.28, $p = 0.020$), and procalcitonin (RR 1.2, $p < 0.001$), were associated with in-hospital mortality in univariate analysis. The International Collaboration on Endocarditis-Prospective Cohort Study reported several independent parameters affecting one-year mortality, including chronic haemodialysis (RR 2.278, $p < 0.001$), heart failure (RR 1.681, $p < 0.001$), diabetes mellitus (RR 1.461, $p = 0.0069$), and the use of biological valves (RR 1.298, $p = 0.0406$), among others (Murdoch et al., 2009). In our study, no significant difference was found in in-hospital and long-term mortality between IE patients receiving biological or mechanical valves. When identifying risk factors for IE, various factors must be taken into account, including patient age, count, local healthcare system, and treatment options (Heiro et al., 2007; Toyoda et al., 2017). The average length of hospitalization and stay in the intensive care unit calculated in this study for IE patients was 26.86 and 4.54 days, respectively. In some cases, patients received a full course of intravenous antibiotics in our hospital, while others were transferred to nearby hospitals to complete treatment closer to their place of residence. In cases where infection was well controlled after completing

the intravenous antibiotic course, patients were discharged home with recommendations to take antibiotics orally. Guidelines recommend that complicated IE patients, including those with comorbidities, be treated in a facility with an endocarditis team consisting of various specialty physicians (Habib et al., 2015b). The formation of an endocarditis team could improve diagnostic possibilities, reduce the development of complications, and increase short-term and long-term survival chances for patients.

4.2 Comparison of outcome between blood culture positive and negative IE patients undergoing cardiac surgery

In this retrospective study, data and clinical outcomes of upper gastrointestinal tract endocarditis (BCPE) and lower gastrointestinal tract endocarditis (BCNE) patients who underwent cardiac surgery were analysed. Out of 207 patients, 93 (44.9 %) were diagnosed with BCNE. In the literature, the frequency of BCNE is described as highly variable – ranging from 2.5 % to 70 %, indicating a high incidence of BCNE in this study (P Brouqui & Raoult, 2001). In a study conducted by Fournier and colleagues with 918 patients, the frequency of BCNE in prospective analysis was 30.8 %. Their proposed diagnostic strategy revealed the aetiology of endocarditis in 138 patients (78.0 %) out of 283 BCNE patients, with 135 identifying the microorganism causing endocarditis. Among them, intracellular bacteria such as *C. burnetii*, *Bartonella spp.*, and *T. whipplei* constituted 15.9 % of BCNE cases. Three patients (1.1 %) confirmed non-infectious causes. In BCNE cases, 56 out of 70 patients infected with gram-positive cocci had received antibiotics before blood culture, indicating early antibiotic use as a possible cause of BCNE (Fournier et al., 2017). Conventional bacterial culture methods were used in the study without additional diagnostic tests to identify causative agents, which could partially explain the high prevalence of BCNE and the low detection of intracellular

bacteria. Data from Lamas and colleagues suggest that BCNE often develops after previous antibiotic use (Lamas et al., 2016). Since many patients were transferred from different institutions to our hospital, we could not fully assess previous antibiotic use, which may have influenced our study results, resulting in lower inflammatory markers and a less severe course of illness in the BCNE group. Literature provides conflicting data on outcomes for BCNE patients. Our study results indicate that there is no statistically significant difference in in-hospital and three-year mortality between BCPE and BCNE patients, but there is a tendency towards worse outcomes in the BCPE group. Univariate analysis found higher in-hospital mortality in the BCPE group, but no statistically significant difference was found in multivariate analysis. This finding may be associated with a more severe initial patient condition and less controlled infection manifestations in the BCPE group. We observed lower levels of haemoglobin and haematocrit, higher procalcitonin levels, and other factors such as intravenous drug use and more comorbidities before surgery in the BCPE group. These results are consistent with studies by Phua and others, where culture-negative and culture-positive sepsis were investigated (Phua et al., 2013). Higher inflammatory markers, such as C- reactive protein and procalcitonin, are usually associated with a more severe preoperative condition. Recent studies recognise procalcitonin as the most extensively studied and suitable biomarker for antibiotic effectiveness (Gregoriano et al., 2020; Neeser et al., 2019). Studies suggest that higher initial C-reactive protein levels are independently associated with more frequent short-term adverse events, and higher procalcitonin levels are associated with increased in-hospital mortality (AlRawahi et al., 2019; Mohanan et al., 2018). Siciliano and colleagues conducted a comparative study with 221 patients analysing the impact of BCNE and BCPE, and the most frequently identified microorganism in our study was *S. aureus* (31.6 %), which was associated with in-hospital death compared to other microorganisms in both

univariate and multivariate analyses. This finding is consistent with results from other authors (A. Wang et al., 2018). *S. aureus* is a pathogen known for its aggressive clinical presentation and worse prognosis compared to other microorganisms (Han et al., 2017). The proportion of aggressive microorganisms such as *S. aureus* in the BCPE group may affect the outcome results and could be associated with a more severe preoperative condition.

Overall, there is very little published research on this topic, especially regarding the role of BCNE in cardiac surgery patients. Our cardiac surgery centre is the only one providing cardiac surgery in the country, representing all Latvian population patients undergoing cardiac surgery due to IE.

4.3 Comparison of outcome between blood culture positive and negative IE patients undergoing cardiac surgery

In this study, the aetiology, risk factors, and clinical outcomes of IE caused by various bacterial agents in surgically treated patients were analysed. Scientific literature increasingly provides evidence that the presence of *S. aureus* infection often correlates with worse clinical outcomes in cases of IE. In our study over a five-year period, *S. aureus* was the most frequently identified microorganism causing IE. These data align with other studies where *S. aureus* is the most common causative agent of endocarditis worldwide (Fernández Guerrero et al., 2009; Fowler et al., 2005; Murdoch et al., 2009). *S. aureus* is known for its aggressive nature and is often associated with a more severe course of the disease (Han et al., 2017).

Studies indicate that in left-sided IE, subclinical or clinically detectable cerebral embolism occurs in up to 30 % of cases (Snygg-Martin et al., 2008). Moreover, embolism to the central nervous system is most frequently observed in cases involving *S. aureus*. In our study, except for echocardiography, other imaging diagnostics were performed only based on clinical necessity. Systemic

imaging for detecting embolism was not conducted, and, therefore, embolic complications might be underreported. We found that patients who died in the hospital had significantly more embolic events compared to survivors. Studies suggest that the size of vegetations measured by echocardiography plays a crucial role in predicting embolic events. Patients with left-sided IE having vegetations larger than 10 mm are at a high risk of embolism. On the other hand, in studies involving right-sided IE, patients with vegetations larger than 25 mm were more likely to experience IE recurrences (Thuny et al., 2005; Ye et al., 2021). Measurement of vegetation size is crucial for assessing the risk of embolic events and their potential prevention (Habib, 2019).

Although in this study, in-hospital mortality for *S. aureus* group patients did not differ significantly from IE patients with other causative microorganisms, in the long term, it was significantly associated with a worse prognosis. This finding is consistent with results obtained by other authors, indicating that the presence of *S. aureus* is associated with poor outcomes and high mortality in IE cases (Cabezón et al., 2021). Comparatively, the course of *E. faecalis*-induced IE was less severe, although embolism was frequently observed. Overall, surgically treated patients had better outcomes.

Regarding echocardiographically detected damages, *Streptococcus spp.* were significantly more associated with severe aortic insufficiency and the formation of fistulas between cardiac chambers. Patients suspected of having paravalvular abscesses mostly underwent cardiac CT angiography to define anatomy and plan potential surgical correction. In patients with native valve IE, two cases of fistula were identified, one connecting the aorta with the right atrium and the other connecting the aorta with the left ventricle. The remaining fistula cases were associated with prosthetic valve IE. The relatively high mortality in the *Streptococcus spp.* group might be explained by the relatively high proportion of beta-haemolytic streptococcal infections observed in 10 (28.6 %) patients.

However, the small number of patients in this group is one of the study limitations, potentially impacting the results.

Overall, native valves (79.9 %) were more affected by infection compared to prosthetic valves (20.1 %). Coagulase-negative staphylococci (CoNS) were the most common causative agents of prosthetic valve endocarditis. These results are consistent with data published in other studies, indicating that CoNS constitute approximately 60 % of all prosthetic valve endocarditis cases (Noshak et al., 2020). Similarly, CoNS and prosthetic valve IE are associated with more frequently occurring perivalvular complications. Similar findings are also reported in other studies (Damlin et al., 2019; Trifunovic et al., 2018).

The duration of mechanical ventilation, the use of catecholamines, the need for haemodialysis, as well as higher SOFA scores were identified as risk factors, with higher values observed in the non-survivor IE patient group along with longer aortic occlusion and artificial circulation times. The results of this study need to be evaluated considering some limitations. Only patients undergoing cardiac surgery were analysed, and conservatively treated patients were not included. Most analysed patients had an active infection, but they were operated on at different time points based on the urgency of the procedure. Additionally, the unspecified and unanalysed type of surgery in this study could influence the obtained results. The majority of performed operations involved valve replacement and resolution of the infection process. As mentioned earlier, the relatively small number of patients in each microorganism group is another limitation of this study. If there were a more detailed distribution of Streptococcus spp., new characteristics of clinical outcomes could be revealed. However, it should be noted that other studies have investigated the course and prognosis of different streptococcal species and found no significant differences (Escrihuela-Vidal et al., 2021).

The cause of death associated with long-term mortality was not specified in this study. Also, the impact of comorbidities and therapy on IE patients was not analysed, which could provide new data and alternative interpretations.

4.4 Application of 16S rRNA Next-generation sequencing in excised valve samples from IE patients

In this study, the utility of 16S rRNA NGS technology was investigated by analysing excised valves in patients with BCNE. The effectiveness of the method was particularly observed in BCNE cases, where the causative microorganism was detected in 70.6 % of cases, leading to a need to change antibiotic therapy in 23.9 % of patients. This finding highlights the potential clinical significance of NGS for BCNE patients to receive more specific antibacterial therapy, especially in cases where routine microbiological methods fail to identify the pathogenic agent. Echocardiographically, BCNE patients exhibited larger vegetations, but systemic embolism was not more frequently observed. Similarly, the outcomes for BCNE and BCPE patients did not statistically differ. Scientific literature also lacks consensus on outcomes in BCNE and BCPE cases. Studies have compared BCNE with BCPE and sepsis with a known or unknown aetiology, but no significant differences in outcomes (including mortality) have been observed (Phua et al., 2013; Zamorano et al., 2001). However, a recent large study comparing outcomes from the EURO-ENDO registry found worse outcomes for BCNE patients. Long-term mortality in BCNE cases was higher than in BCPE patients (Kong et al., 2022).

Another finding in this study was the high prevalence of *Bartonella spp.* infection. *Bartonella spp.*, along with *C. burnetii*, are the most commonly identified microorganisms in the majority of BCNE cases (Godfrey et al., 2020). In our study, no patients with *Coxiella spp.* IE were found. There are also studies where *C. burnetii* is more frequently found compared to *Bartonella spp.*

(W. Wang et al., 2022). This could be explained by geographic location and local endemic status. Studies have emphasised that the number of *Bartonella spp.* IE cases has been increasing recently (Okaro et al., 2017). In one of the largest studies on *Bartonella spp.* endocarditis published, data from 101 patients in France from 1996 to 2001 were analysed. It was reported that *Bartonella spp.* as the causative microorganism of IE accounted for approximately 3 % of all endocarditis cases. Alcoholism and homelessness were mentioned as risk factors for the development of *Bartonella spp.* endocarditis. This finding aligns with our study data, where a history of alcoholism was observed significantly more frequently than in other microorganism-induced IE. The role of aminoglycoside antibiotics in the treatment of *Bartonella spp.* IE was emphasised. A treatment regimen including aminoglycosides was associated with significantly better outcomes (Raoult et al., 2003). *Bartonella spp.* are associated with various extracardiac damages, including kidney involvement.
In another study by Ehrlich et al., 44 % of patients with *Bartonella spp.* endocarditis showed signs of kidney damage, including glomerulonephritis and acute renal failure (Ehrlich et al., 2010). Pathogenetic mechanisms of kidney damage may vary in *Bartonella spp.* endocarditis cases (Raybould et al., 2016). In our study, patients with *Bartonella spp.* IE had significantly higher creatinine levels compared to other microorganisms, confirming the previously mentioned findings.

Several limitations of this study should be mentioned, potentially influencing the results. Firstly, this is a single-centre study and included a relatively small number of patients. Secondly, not all operated patients due to IE were included during the study, but rather randomly selected cases, thus the proportions of detected microorganisms may not reflect the real situation. As for the limitations of 16S rRNA NGS itself, its sensitivity and the ambiguous interpretation of results should be acknowledged. A significant drawback is that

this method can diagnose bacteria but cannot detect fungi and viruses. Unlike viruses, fungi, although rarely, can be causative agents of IE (Badiee et al., 2014). The relative quantity of nucleic acids of the detected microorganisms plays a crucial role in determining the pathogenic role using 16S rRNA NGS. Literature suggests that a microorganism can be considered the causative agent if the relative nucleic acid quantity for that microorganism is above 90 % (Flurin et al., 2022; Haddad et al., 2022; Santibáñez et al., 2021). Finally, *Bartonella spp.* with 16S rRNA NGS only allowed identification up to the genus, not species level. Therefore, it was not possible to determine the specific *Bartonella spp.* in the samples (Thornhill et al., 2018), potentially providing additional information.

In conclusion, the diagnosis of BCNE is complex and often requires a multidisciplinary approach, as well as new diagnostic possibilities. It is crucial for IE treatment to be tailored according to the pathogenic microorganism, the patient's clinical condition, and comorbidities. Clinicians should assess the most likely causes of BCNE, which could include prior antibiotic use, difficult-to-culture or intracellular microorganisms, as well as possible non-infectious causes of endocarditis. In our study, 16S rRNA NGS technology in excised valve samples was recognised as effective for diagnosing the causative microorganism of IE, especially in cases where blood culture is negative.

4.5 Comparison of morphological findings and neutrophil leukocyte activation in IE caused by *Bartonella spp.* and non-*Bartonella spp.*

The present study combines a diverse range of analytical methods and approaches to offer a deeper understanding of IE, particularly in the context of *Bartonella spp.*-caused cases, which is still relatively poorly understood. This study takes a multifaceted approach by analysing a range of factors related to IE, including patients' characteristics, laboratory indices, clinical outcomes, and histopathological assessments. It further delves into the immunohistochemical

expression of neutrophilic leukocyte activation markers and markers associated with NETosis, providing a comprehensive view of the disease. Analysis of ultrastructural changes in IE further contributes to the advancement of knowledge in this field. The fact that *Bartonella spp.* are facultative intracellular microorganisms, unlike the majority of common IE-causing agents, makes them unique in the context of IE. Therefore, there are currently not many studies on the pathogenesis of IE caused by these pathogens. Besides focusing on *Bartonella spp.*, the study also explored neutrophil leukocyte activation and the potential involvement of neutrophil extracellular traps (NETs) in the IE process. A comparison was made with classical IE triggers by immunohistochemically determining markers of neutrophil leukocyte activation associated with the NETosis process. Histopathological, histochemical, and ultrastructural findings were also investigated.

Considering that *Bartonella spp.* microorganisms are facultative intracellular, possible differences in NET involvement in IE pathogenesis were expected compared to other microorganisms. Therefore, various morphological approaches were used in our study to elucidate the involvement of neutrophil leukocyte extracellular traps in *Bartonella spp.* and non-*Bartonella spp.* microorganism cases.

Neutrophil leukocytes, along with platelets and other immune cells, play a direct role in the pathogenesis of IE by causing damage to the valve microenvironment, producing chemoattractants, and inducing an inflammatory reaction. In damaged valve tissues, neutrophil leukocytes actively release various enzymes, chemokines, cytokines, as well as NETs (S.-J. Kim & Jenne, 2016). Platelets in the damaged valve inhibit neutrophil apoptosis processes and promote defence mechanisms, such as stimulating the formation of free radicals, phagocytosis, and MPO release (S.-J. Kim & Jenne, 2016). Lipopolysaccharides, von Willebrand factor, and platelet factor 4, which react with neutrophil

receptors such as P-selectin, PSGL-1, GPIb-Integrin M2, initiate NET release mechanisms (Andrews et al., 2014; Carestia et al., 2016). Previous studies have shown that different signals from both bacteria and activated platelets can induce the NETosis process. This phenomenon was explored by authors Chiau-Jing Jung and Chiou Yueh Yeh in 2015 (Jung et al., 2015).

During NETosis, proteins such as NE, MPO, CP, histones, etc., are released, indirectly indicating the NETosis process and the presence of NETs. It has been demonstrated that after activation, NE is released from azurophilic granules and moves to the nucleus, where it partially divides laminin and histones, promoting nuclear chromatin decondensation. Later, MPO also participates in the process, further promoting the aforementioned process (Papayannopoulos et al., 2010). Recent studies indicate that activating isolated human neutrophils with NET-associated neutrophil proteases affects the integrity of NET-associated proteins, inducing NET formation (de Bont & Pruijn, 2023). Moreover, indications of the complex nature of the immune response to polymicrobial infections have been observed (Kao et al., 2023). Understanding the role of the nucleus in the NETosis process provides valuable insights into the behaviour of neutrophil leukocytes in immune reactions, especially in the context of inflammatory disorders. Understanding the role of the nucleus in the process of NETosis provides valuable insights into the behaviour of neutrophil leukocytes in immune reactions, particularly in the context of inflammatory disorders. At the ultrastructural level, chromatin decondensation of the nucleus is a crucial stage that occurs before its breakdown in the process of NET release. This is observed as swelling of neutrophil nuclei undergoing NETosis (Neubert et al., 2018). Several models of NETosis have been recognised so far (Delgado-Rizo et al., 2017). Previous transmission electron microscopy (TEM) studies have clearly shown nuclear dissolution in both "suicidal" and "vital" NET release. In "suicidal" NETosis, the nuclear envelope breaks down before

decondensed chromatin fills the cytoplasm, mixing with granule components, resulting in the lytic release of NETs (Manley et al., 2018). Earlier studies indicated the separation of internal and external nuclear membranes and the formation of vesicles containing nuclear DNA (Pilsczek et al., 2010). The initiation of NETosis may be associated with the inhibition of apoptosis, thereby enhancing the overall antimicrobial effect (Vorobjeva & Chernyak, 2020). In this study, the application of transmission electron microscopy to analyse the ultrastructural properties of heart valve leaflets and vegetations in IE, with a particular focus on the role of neutrophil leukocytes during NET formation, offered a new perspective on cellular interactions and processes. We confirmed irregularities in the perinuclear space, sometimes filled with fine filamentous material, as well as nuclear envelope ruptures with chromatin wedged between cytoplasmic organelles and components of the extracellular matrix. Meanwhile, the use of scanning electron microscopy to visualise bacterial adhesion, the formation of fibrin networks, and the appearance of platelets in vegetations in both *Bartonella spp.* and non-*Bartonella spp.* induced IE cases provided a visual representation of the disease process at the microscopic level. This approach was validated by studying the vegetation structure induced by various bacterial species to understand the pathophysiological mechanisms of vegetation formation (Hannachi et al., 2020; Onouchi et al., 2016).

In this study, we delved into the complex structure of altered heart valves associated with vegetation formation in IE. Various morphological approaches were used to carefully examine the extent of valve damage caused by the disease and to determine the involvement of neutrophil leukocytes in pathology. The histopathological examination of changes in heart valves and vegetation in IE provides insight into the distinctive features between cases induced by *Bartonella spp.* and those induced by non-*Bartonella spp.* The immunohistochemical analysis of neutrophil leukocyte markers, such as

MPO, NE, CP, and histone H3, highlights differences in expression patterns between *Bartonella spp.* induced and non-*Bartonella spp.* induced IE. This reveals potential differences in immune reactions and the NETosis process.

The most significant finding of this study is the identification of the expression of neutrophil leukocyte activation markers in IE valves, demonstrating pronounced involvement of neutrophil leukocytes and their extracellular traps in pathogenesis. Furthermore, significant differences in the expression of these markers were observed between *Bartonella spp.* and non-*Bartonella spp.* IE patients, which may be related to the fact that *Bartonella spp.* are facultative intracellular pathogens. Considering their rarity, there is also extremely limited research globally on the involvement of these pathogens in IE pathogenesis. Indeed, before the introduction of molecular methods such as PCR and NGS into research and clinical practice, the diagnosis of these pathogens was much rarer than it is today. Studies have emphasised an increase in *Bartonella spp.* infections, including IE, due to improved diagnostic possibilities (Okaro et al., 2017).

Conclusions

1. IE caused by various microorganisms is associated with different clinical outcomes, complications, and prognosis in patients undergoing cardiac surgery.
2. The prevalence of perivalvular infections is independently linked to increased in-hospital mortality.
3. There are no statistically significant differences between blood culture-positive and negative endocarditis groups in terms of in-hospital mortality, duration of hospitalization, time spent in the intensive care unit, and three-year mortality.
4. To diagnose the causative agent, especially in cases of blood culture-negative infectious endocarditis, 16S rRNA NGS is a useful technology.
5. The involvement of neutrophil leukocytes, their activation, and formation of extracellular traps is observed in the pathogenesis of infective endocarditis caused by all bacterial microorganisms.

Clinical recommendations

1. In cases of blood culture-negative infective endocarditis, 16S rRNA NGS in excised valve tissues is a recommended method for diagnosing the causative microorganism and refining treatment.
2. The establishment of an "Endocarditis Team" in Latvia for the diagnosis, treatment, and long-term monitoring of infectious endocarditis is necessary to improve treatment outcomes and patient prognosis.

List of publications and reports on the topic of the Thesis

Internationally citable publications:

1. **Meidrops, K.**, Zuravlova, A., Osipovs, J. D., Kalejs, M., Groma, V., Petrosina, E., Reinis, A., Strike, E., Dumpis, U., Erglis, A., & Stradins, P. (2021). Comparison of outcome between blood culture positive and negative IE patients undergoing cardiac surgery. Journal of Cardiothoracic Surgery, 16(1), 1–7. https://doi.org/10.1186/s13019-021-01532-9

2. **Meidrops, K**., Osipovs, J. D., Zuravlova, A., Groma, V., Kalejs, M., Petrosina, E., Leibuss, R., Strike, E., Dumpis, U., Erglis, A., & Stradins, P. (2022). Risk factors associated with mortality in the IE patients requiring cardiac surgery: a study based on Latvian population. The Journal of Cardiovascular Surgery, 63(4), 507–513. https://doi.org/10.23736/S0021-9509.22.12092-6

3. **Meidrops, K.**, Burkhardt, F. J., Osipovs, J. D., Petrosina, E., Groma, V., & Stradins, P. (2022). Etiology, Risk Factors and Clinical Outcomes in IE Patients Requiring Cardiac Surgery. Journal of Clinical Medicine, 11(7). https://doi.org/10.3390/jcm11071957

4. **Meidrops, K.**; Groma, V.; Goldins, N.R.; Apine, L.; Skuja, S.; Svirskis, S.; Gudra, D.; Fridmanis, D.; Stradins, P. (2024). Understanding Bartonella-Associated IE: Examining Heart Valve and Vegetation Appearance and the Role of Neutrophilic Leukocytes. Cells 2024, 13, 43. https://doi.org/10.3390/cells13010043

5. Goldins, N.R, **Meidrops, K.**, Apine, L., Petrosina, E., Stradins, P., Groma, V. (2023). Neutrophilic leukocytes and neutrophil extracellular traps in the native aortic valve endocarditis. Proceedings of the Latvian Academy of Sciences. Section B. Natural, Exact, and Applied Sciences., vol.77, no 1, 2023, pp.41–48. https://doi.org/10.2478/prolas-2023-0005

6. Brida, M., Balint, H.O., Bence, A., Panfile, E., Prokšelj, K., Kačar, P., Lebid, I.H., Šimkova, I., Bobocka, K., **Meidrops, K.**, Strenge, A., Perčin, L., Kapleriene, L., Gumbiene, L., Tomkiewicz-Pająk, L., Komar, M., Roos-Hesselink, J.W., Gatzoulis M.A., Diller G.P. IE in adults with congenital heart disease: Contemporary management and related outcomes in Central and South-Eastern European region. International Journal of Cardiology, 2023. ISSN 0167-5273. https://doi.org/10.1016/j.ijcard.2023.01.012.

Oral presentation at an international scientific conference:

1. **Meidrops K.** (2021). Incidence of blood culture negative IE in patients undergoing cardiac surgery. RSU Research Week 2021, Knowledge for Use in Practice. 24.–26.03.2021.

2. **Meidrops K.** (2021). IE: should we revise Duke criteria? RSU Research Week 2021, Knowledge for Use in Practice. 24.–26.03.2021.

3. **Meidrops K.** (2022). IE – a disease with many faces. Interventional Cardiology and Cardiac Surgery meeting Baltic Summer 2022. 17.06.2022.

4. **Meidrops K.** (2023). Causative microorganism association with outcome in cardiac surgery patients with IE. RSU Research Week 2023, Knowledge for Use in Practice. 29.–31.03.2023.

Poster presentation at an international scientific conference:

1. Kalējs M., Brecs I., **Meidrops K.**, Leibuss R., Klesmite A., Strike E., Stradins P. (2021). A Delayed Staged Treatment Strategy in a Double-valve Septic Endocarditis Patient with Influenza, Severe ARDS and an Aortic Aneurism. The International Society for Minimally Invasive Cardiothoracic Surgery meeting. 18.–20.06.2021.

2. **Meidrops K.**, Goldiņš N.R., Groma V., Kalējs M., Stradiņš P. Assessment of neutrophil leukocyte infiltration in aortic valves in patients with IE. RSU Research Week 2021, Knowledge for Use in Practice. 24.–26.03.2021.

Oral presentation at a local scientific conference:

1. **Meidrops K.** (2020). Infekciozs endokardīts. Klīniskais gadījums, patoģenēze un 5 gadu ķirurģiskās ārstēšanas pieredze Latvijā. Latvijas Hipertensijas un Aterosklerozes biedrība. 12.08.2020.

2. **Meidrops K.** (2021). Vai infekciozais endokardīts ir sirds slimība. Latvijas Ārstu Biedrības Pasaules sirds veselības diena. 25.09.2021.

3. **Meidrops K.** (2022)**.** Infekciozais endokardīts – kāpēc ar to saslimst un kā ārstēt? Latvijas Kardioloģijas biedrības sēde. 07.04.2022.

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4. AlRawahi, A. N., AlHinai, F. A., Doig, C. J., Ball, C. G., Dixon, E., Xiao, Z., & Kirkpatrick, A. W. (2019). The prognostic value of serum procalcitonin measurements in critically injured patients: a systematic review. *Critical Care (London, England)*, *23*(1), 390. https://doi.org/10.1186/s13054-019-2669-1

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