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Original Paper

NEUTROPHILIC LEUKOCYTES AND NEUTROPHIL EXTRACELLULAR TRAPS IN NATIVE AORTIC VALVE ENDOCARDITIS

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Infective endocarditis is a disease that affects the endocardium and often alters heart valves, notably the aortic valve. Bacteraemia and valvular endothelial damage play an essential role in the pathogenesis of infective endocarditis. The pertinent literature suggests that neutrophil extracellular traps are important contributors to the development of the disease. However, features of the valvular damage and contribution of neutrophils to the alteration of cardiac tissue are not explored sufficiently. The purpose of this study was to investigate the occurrence and distribution of neutrophilic leukocytes and neutrophil extracellular traps in native aortic valves affected by infective endocarditis, using histopathology and immunohistochemistry assays. In addition, the presence of vegetations on the heart valve was determined. Infiltration of neutrophils into the valvular leaflet was significantly more severe at the free margin (mean 5.89 ± 3.00, p *< 0.001) and the middle portion (mean 4.58 ± 3.64, p = 0.032) when compared to the base portion (2.05 ± 1.90). No significant differences in neutrophilic leukocyte infiltrating inflammatory lesions were found between cusp layers. The presence of myeloperoxidase and citrullinated histone expression characteristic of neutrophil extracellular traps was demonstrated by the use of immunohistochemistry in IE-affected valvular leaflets and vegetations. Collectively, the study results suggest that the free cusp margin and its middle portion of the aortic valve are exposed to enforced blood flow; endothelial damage and vegetation formation are likely to occur along with the presence of infective endocarditis-related bacteraemia.*

Keywords: *infective endocarditis, NET, vegetation, immunohistochemistry.*

INTRODUCTION

Infective endocarditis (IE) is an infectious disease affecting the endocardium — the inner layer of the heart, which lines the chambers and cardiac valves. In recent years, the annual incidence of IE in the populations of the US and Europe increased from 9.3 to 15 (Chirillo, 2021) and from 3 to 12 per 100 000 people (Chambers *et al*., 2020; Liesenborghs *et al*., 2020; Rajani *et al*., 2020), respectively. The mortality rate of IE is up to 30%, despite early diagnostics and therapies available (Habib *et al*., 2019; Liesenborgh *et al*., 2020; Luehr *et al*., 2020). Clinical presentation of IE is greatly variable. In up to 30% of cases, IE manifests with septic embolism (Trifunovic *et al*., 2018; Luehr *et al*., 2020), whereas about 25% of left-sided IE develop cerebrovascular complications such as haemorrhagic and ischaemic stroke, and encephalopathy (Sotero *et al*., 2019; Salem *et al*., 2021). *Staphylococcus aureus*, *Enterococcus* spp., and *Streptococcus spp.* are common causative microorganisms of IE (Habib, 2006). However, the IE causative factors may

vary geographically (Holland *et al*., 2016). Furthermore, culture-negative IE may develop in up to 70% of cases (Fournier *et al*., 2017).

Application of antibiotic therapy before obtaining blood cultures, the existence of rare, fastidious microorganisms, the presence of low bacteriaemia during a blood draw, and the development of an inappropriate medium for a microorganism are common causes of culture-negative IE (Kupferwasser *et al*., 2001). Among heart valves, the aortic valve is one of the most commonly affected by IE. During a lifetime, the leaflets are exposed to different stresses, physical, and biochemical forces, but they maintain their function. Morphologically, the aortic valve leaflets are organised into three layers: *fibrosa layer* composed of stiff fibrillar collagen fibres and located on the aortic side of the valve, the middle *spongiosa layer* composed of glycosaminoglycans (GAG), proteoglycans (PG), and valvular interstitial cells (VIC), and the *ventricularis layer* rich in radially organised elastic fibres located on the ventricular side (Schoen, 2018; Buijtendijk *et al*., 2020). VIC presents with various phenotypes and contributes to the synthesis of PG, GAG, elastin, collagen, and other extracellular matrix (ECM) molecules (Rutkovskiy *et al*., 2017). A leaflet from both sides is covered with valve endothelial cells (VEC). The microstructure of leaflets is developed to endure bending stress, fluid shear, and axial stress (Sacks *et al*., 2009; O'Donnell *et al*., 2020). Several risk factors facilitate the remodelling process of normal, healthy valve leaflets. The presence of elevated blood pressure, chronic systemic inflammation, adiposity, as well as a congenital bicuspid aortic valve are major risk factors of IE (Habib *et al*., 2019; Luehr *et al*., 2020; Rajani *et al*., 2020; Salem *et al*., 2021). A damaged valve surface becomes adhesive to blood platelets and fibrin, neutrophils with their extracellular traps (NETs), and circulating microorganisms (Liesenborghs *et al*., 2020). Initially, a sterile aggregate composed of blood platelets forms vegetation that later on is colonised by microorganisms. Neutrophils and blood platelets are considered to be responsible for altering the valvular microenvironment, production of chemoattractants, and induction of inflammation (Kim *et al*., 2016). In lesioned heart valves, neutrophilic leukocytes are actively involved in tissue remodelling by the release of various enzymes, chemokines, cytokines, and the formation of NET.

Gradually, neutrophils build up a NET that contains extracellularly released chromatin packed by citrullinated nuclear histones, various defensines, and enzymes, such as myeloperoxidase (MPO) and neutrophil elastase. This web-like structure was initially recognised as a bacteria-, fungi-, and even virus-killing and trapping structure (Kim *et al*., 2016). A body of evidence suggests the contribution of NETs to the development of autoimmune diseases and acute respiratory distress immunothrombosis, characteristic of COVID-19 infectious disease (Brinkmann, 2018; Middleton, 2020). Furthermore, NETs are implicated in the formation of vegetations, they facilitate the encapsulation of bacteria in it, recruit blood platelets and immune system cells, and, thus promote the growth of vegetation (Jung *et al*., 2015; Hsu *et al*., 2019; Liesenborghs *et al*., 2020). Vegetation can form a structure with a size around 15 mm and more and easily detach from a valve leaflet and end up as an embolus in any tissue, but most often in the brain, spleen, or kidney (Trifunovic *et al*., 2018; Houard *et al*., 2020; Luehr *et al*., 2020; Kim *et al*., 2021). In IE patients, aggregates of blood platelets mimic the prothrombotic state and further contribute to the injury of valvular endothelium (Kim *et al*., 2016). Innate immune system cells easily adhere and invade the valvular leaflets. At the site of valvular injury, blood platelets inhibit neutrophil apoptosis and enhance neutrophil defence mechanisms, such as an increase in reactive oxygen species (ROS), phagocytosis, and MPO release against pathogens (Kim *et al*., 2016). Blood platelets activated by lipopolysaccharides, von Willebrand factor, fibrinogen, and platelet factor 4 interact with neutrophilic leukocytes through P-selectin, PSGL-1, and GPIbα — integrin $\alpha M_{\beta}2$ and mediate NET releasing mechanisms (Andrews *et al*., 2014; Carestia *et al*., 2016; Kim *et al*., 2016).

This study aimed to explore the occurrence and distribution of neutrophilic leukocytes and NETs in native aortic valves affected by IE, using histopathology and immunohistochemistry assays. In addition, the presence of vegetations on the heart valve were analysed.

MATERIAL AND METHODS

Aortic valve tissues obtained from nineteen patients diagnosed with IE and treated surgically at Pauls Stradiņš Clinical University Hospital, Rīga, Latvia, between January 2019 and September 2020, were used in this study. Patients were eligible for the study if they were aged 18 years or older and underwent cardiac surgery due to IE. A diagnosis of IE was based on modified Duke criteria.

The clinical and laboratory data obtained from the IE subjects included patient characteristics, laboratory indices, results of microbiological investigation, and vegetation parameters assessed by the use of echocardiography.

The aortic valve cusps with macroscopically observed vegetation were further processed for microscopical examination. The formalin-fixed, paraffin-embedded leaflet tissues were sectioned perpendicularly from the free edge of the leaflet through the vegetation structure towards the base or hinge of the leaflet. The sections were mounted on Histo-Bond+ adhesion slides (Paul Marienfeld, Germany) to further assess the expression of tissue antigens. In addition, parallel sections were mounted and routinely stained with haematoxylin and eosin (H&E) to obtain a panoramic view of the valve leaflet.

For immunohistochemical reactions, the sections were incubated overnight at 4 °C with the following primary antibodies: rabbit recombinant monoclonal anti-MPO (Abcam, Cambridge, UK, ab208670, dilution 1:1000) and rabbit polyclonal anti-histone H3 (Abcam, Cambridge, UK, ab5103, dilution 1:50).

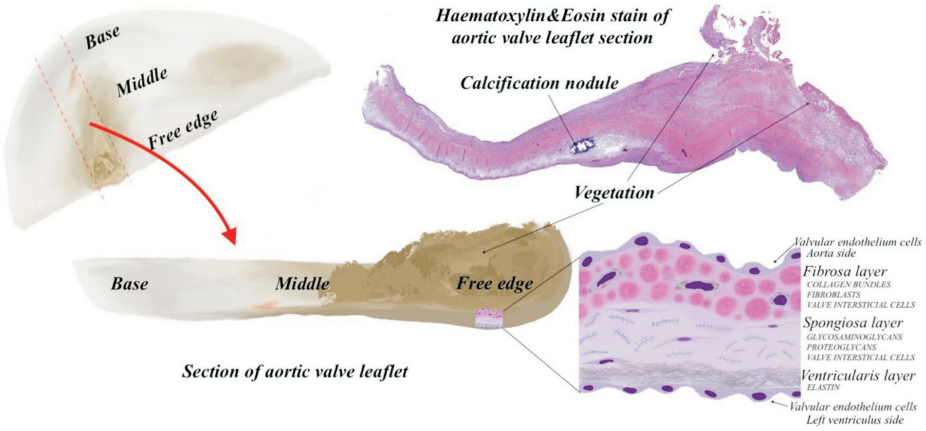

Fig. 1. Scheme of infective endocarditis-affected native aortic valve that highlights the valvular portions (base, middle, free edge) and the histological layers (*fibrosa, spongiosa, ventricularis*) assessed in the study.

The amplification of primary antibody and visualisation of reaction products were performed using the HiDef Detection HRP Polymer system (CellMarque, Rocklin, CA, USA). The antigen sites were visualised with a 3,30 diaminobenzidine (DAB) tetrahydrochloride kit (DAB+Chromogen and DAB+Substrate buffer, Cell Marque, Rocklin, CA, USA) applied for 5 min. The cell nuclei were further counterstained with Mayer's hematoxylin. Finally, the sections were washed, dehydrated, cleared, mounted in Roti® Histokitt (Carl Roth, Karlsruhe, Germany), and coverslipped.

For better microscopical assessment of the structural features of lesioned leaflets, the tissue was stratified into three equal portions or regions. The regions were distinguished as follows: i) free edge, ii) middle part, and iii) the base or hinge (Fig. 1). In addition, infiltration of neutrophils was separately assessed in the aortic leaflet layers — *fibrosa*, *spongiosa,* and *ventricularis*.

Neutrophilic leukocytes were estimated semi-quantitively in 10 randomly selected visual fields of each sample (magnification 400×) and graded as follows: "0" – no neutrophils, "1" – 1–9 neutrophils, "2" – 10-49 neutrophils, and "3" – more than 50 neutrophils found.

For both antibodies, the assessment of immunostaining was performed semi-quantitatively for the aortic valve tissue and vegetation in 10 randomly selected visual fields of each sample (magnification 400×) representing the regions of interest. Vegetation was recognised as a region attached to the valvular endothelium above the *fibrosa* and down from the *ventricularis layer* that microscopically displayed a fibrin-thrombocyte mass structure. The expression of antigens was graded as follows: 0 – negative; 1 – positive; 2 – strongly positive, 3 – very strongly positive expression.

The statistical analyses were performed using RStudio Version 2021.09.1. For quantitative variables, descriptive statistics used were mean and SD or median and interquartile range, and for categorical variables total count and relative count were applied. One-way ANOVA with or without Welch's correction and post hoc comparison by the Games-Howell test were performed for comparison between the three groups. A two paired sample t-test was used for comparison between 2 groups. A *p*-value of less than 0.05 was considered statistically significant.

RESULTS

Patients' characteristics. Nineteen native valve IE patients (12 males, 7 females) were included in this study. Among 19 aortic valves, six valves presented with a bicuspid aortic valve morphology (Sievers classification I type). In three patients, the size of vegetation exceeded 20 mm when measured using echocardiography. Five patients had embolic complications in the spleen, kidney, or brain already at the time of diagnosis. Characteristics of the IE patientsare summarised in Table 1. Blood cultures were negative in 12 (63%) patients, whereas *Viridans* group streptococci were

Table 1. Patient characteristics, n (%) or median (IQR)

Sex	
Female	7 (36.8%)
Male	12 (63.2%)
Age, years	52 (42.0–64.5)
Height, cm	173 (168.5–177.0)
Weight, kg	77 (69.0– 92.8)
Body mass index, kg/m^2	24.91 (22.5–30.7)
Aortic valve type	
Tricuspid	13 (68.4%)
Bicuspid	6 (31.5%)
Vegetation size in echo, mm	
5–9 mm	7 (36.9%)
10–14 mm	5 (26.3%)
15–19 mm	4 (21.1%)
>20 mm	3 (15.7%)
Chronic heart failure, NYHA class	
No data	1 (5.2%)
I	2 (10.7%)
II	8 (42.1%)
III	7 (36.8%)
IV	1 (5.2%)
Septic embolism, n = 5 (% of all embolism)	
CNS	3 (60%)
Spleen	4 (80%)
Kidney	3 (60%)

IQR, interquartile range; NYHA, New York Heart Association; CNS, central nervous system

Table 2. IE patient laboratory indices, median (IQR)

Erythrocytes, median	$3.80 \cdot 10^{12}$ /l (3.59–4.22)
Leukocyte count, median	$8.20 \cdot 10^{9}$ /l (6.80–9.15)
Neutrophils, median	$5.60 \cdot 10^{9}$ /l (4.40–6.93)
Thrombocytes, median	$259 \cdot 10^{9}$ /l (233.50–299.50)
C-reactive protein, median	23.41 mg/l (14.91–75.56)
Blood glucose, median	5.30 mmol/l (4.80–6.03)
BNP, median	307.85 pg/ml (159.85–1078.32)
Blood culture results, n = 19	
Blood culture negative IE	12 (63.6%)
Streptococcus gallolyticus	1 (5.2 %)
Streptococcus salivarius	1 (5.2 %)
Streptococcus viridans	1 (5.2 %)
Staphylococcus aureus	1 (5.2 %)
Staphylococcus hominis	1 (5.2 %)
Micrococcus luteus	1 (5.2 %)
Neisseria elongata	1 (5.2 %)

IQR, interquartile range; BNP, B-type natriuretic peptide

commonly diagnosed in IE patients with positive blood microbiology. Laboratory indices and blood microbiology of IE patients are shown in Table 2.

Histopathology and immunohistochemical analysis of the occurrence and distribution of neutrophilic leukocytes and neutrophil extracellular traps in IE-affected native aortic valves. Microscopically, neoangiogenesis was observed in the aortic valve leaflets of 16 (84%) and calcification in 13 (68%) patients. Inflammation and infiltration of neutrophils into the valvular leaflet was significantly more severe at the free edge and middle portion — mean 5.89 ± 3.00 ($p < 0.001$) and 4.58 ± 3.64 ($p = 0.032$), when compared to the base (hinge) portion (mean 2.05 ± 1.90) (Fig. 2).

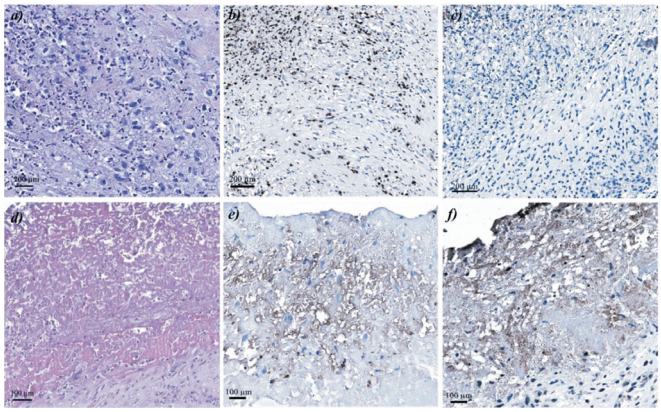

Fig. 2. Histopathology of the aortic valve, myeloperoxidase, and histone H3 immunohistochemistry. Representative images demonstrating infective endocarditis-affected valvular tissue (a) and vegetation (d), H&E staining; MPO-positive valvular tissue (b), and vegetation (e) antigens recognised by the presence of brown reaction products; histone H3-positive neutrophil extracellular traps localised within valvular tissue (c) and vegetation (f). Scale bars: a, b, c – 200 μm; d, e, f – 100 μm.

A statistically significant difference in neutrophilic leukocyte infiltrating inflammatory lesions was lacking for valvular structures stratified into three histological layers. However, mean neutrophil infiltration in the *fibrosa layer* of the leaflet (mean 5.26 ± 2.33) was higher when compared to the *spongiosa* (mean 3.63 ± 2.54) and *ventricularis* (mean 3.63 ± 2.85) *layer* (Fig. 3).

Immunohistochemically, the median values of the expression of neutrophil biomarkers (MPO and histone H3) suggestive of NETs in the valve leaflet tissue was 0.7 (IQR 0.67–1.67), and 0.20 (IQR 0.00–0.20), respectively (Fig. 4).

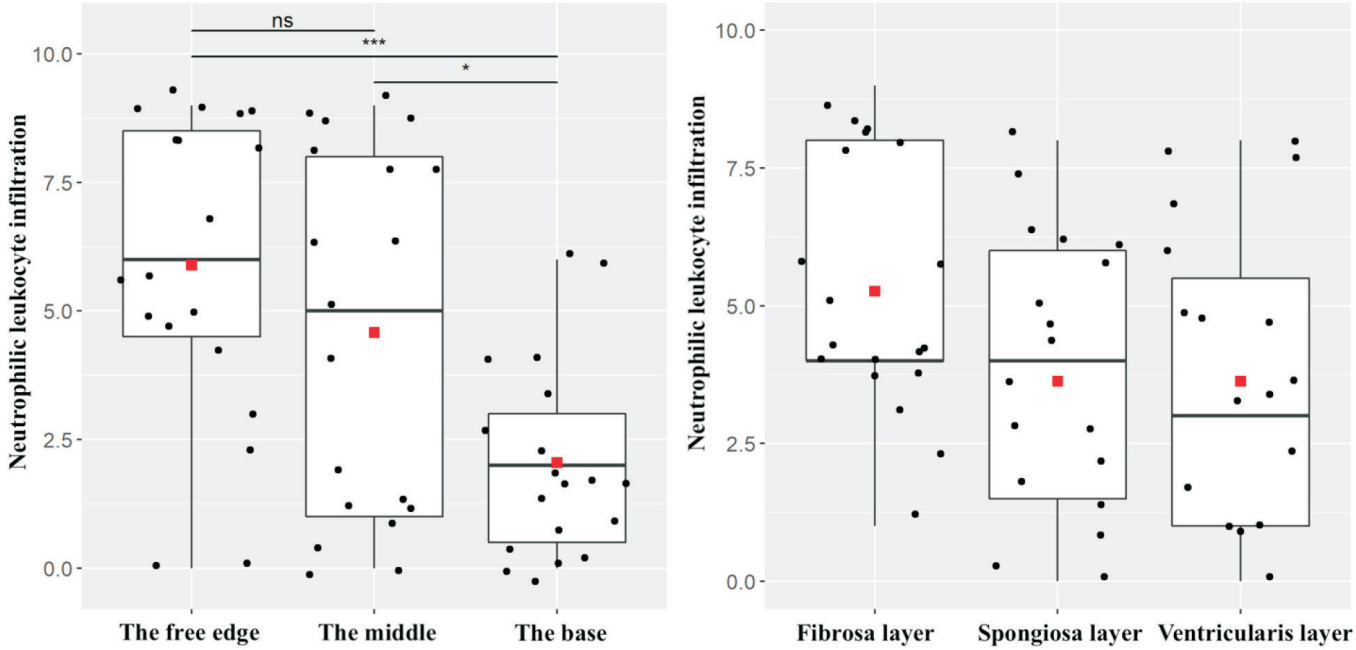

Fig. 3. Assessment of neutrophilic leukocyte infiltration distinguished in the valvular leaflet portions (a) and histological layers (b).

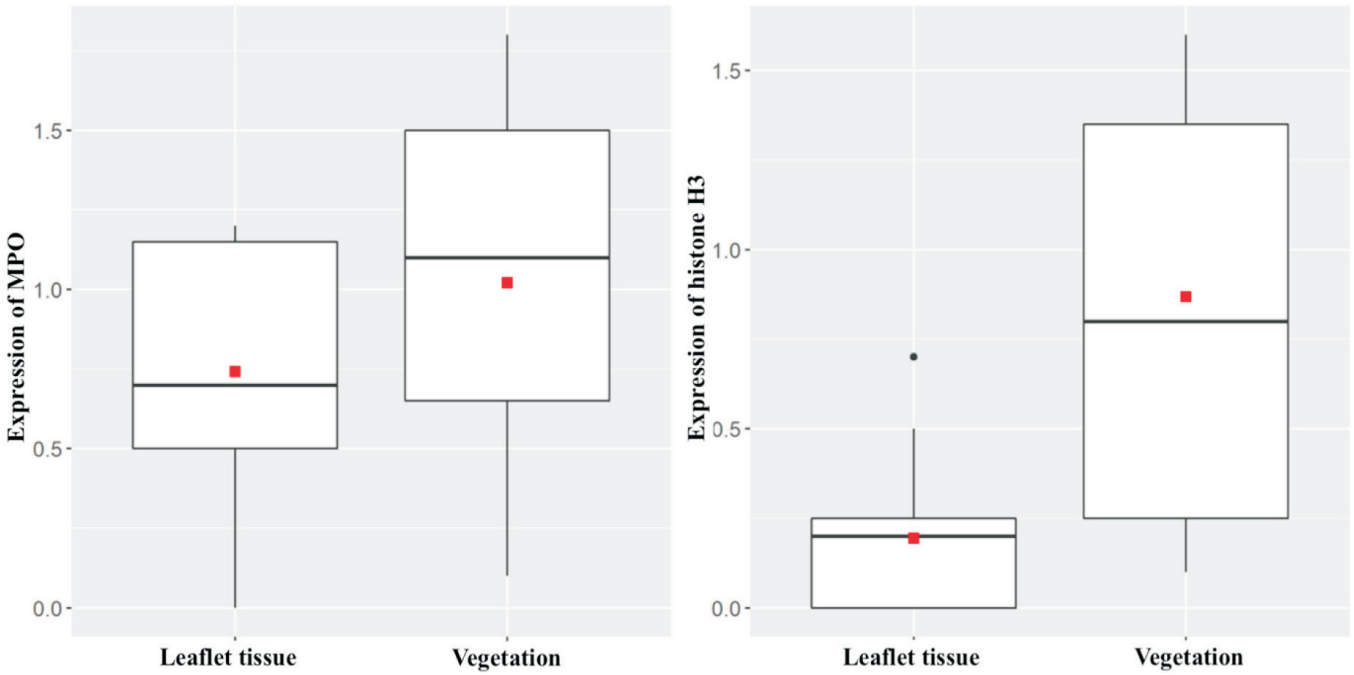

Fig. 4. Assessment of immunohistochemical expresssion of myeloperoxidase (a) and histone H3 (b) within the valvular leaflet and vegetation.

Structural and immunohistochemical analysis of the occurrence and distribution of neutrophilic leukocytes and neutrophil extracellular traps in the valvular vegetation. Microscopically, the valvular vegetation demonstrated a friable structure and were composed of fibrin and blood platelets intermixed with necrotic cellular debris, inflammatory cells, and bacterial colonies.

The presence of neutrophilic leukocytes in the valvular vegetation was confirmed in 17 (89%) samples. Notably, the median values reflecting the expression of immunostaining for MPO were significantly higher in the valvular vegetation compared to the leaflet: (median 1.10, IQR 0.65–1.48) and (median 0.70, IQR 0.5–1.18, $p = 0.012$).

Furthermore, the expression of histone H3 differed significantly between the valvular leaflet and vegetation — (median 0.20, IQR 0–0.20) and (median 0.8, IQR 0.25–1.13, $p < 0.001$), respectively (Fig. 4).

DISCUSSION

In the present study, the occurrence and distribution of neutrophilic leukocytes along with the formation of NETs in IE-affected native aortic valves were explored. In addition, the contribution of neutrophilic leukocytes to the structural features of the valvular vegetation was assessed by the use of morphology assays.

The pertinent literature suggests that bacteriaemia and endothelial damage play a key role in the pathogenesis of IE (Holland *et al*., 2016). A pre-existing heart condition, such as a bicuspid aortic valve, is associated with a higher risk of development of IE (Becerra-Munoz *et al*., 2016). Furthermore, other studies suggest that both abnormal conditions like bicuspid aortic valve endocarditis and mitral valve prolapse are associated with odontogenic *Viridans* group *Streptococcus* IE (Zegri-Reiriz *et al*., 2018). These findings are in line with our study, where the presence of a bicuspid aortic valve was confirmed in 31.6% of patients, and the most common IE causative microorganisms detected were *Viridans* streptococci. In our study, the presence of blood culture-negative IE was confirmed 63.6% of patients, which agrees with the literature data, which shows that it can occur in up to 70% of all IE cases (Fournier *et al*., 2017). Several interpretations have been used to explain the large percentage of blood culture-negative IE — the use of antibiotic therapy before obtaining blood culture, low bacteriaemia at the time of blood draw, rare species and intracellular bacteria, as well as an inappropriate type of media for the microorganisms (Brouqui *et al*., 2001; Kupferwasser *et al*., 2001). The results of our study support previously reported data and suggest that at least one fourth of IE patients present with an embolic event (Salem *et al*., 2021). Furthermore, the recognition of the primary origin of emboli, either mitral or aortic, remains a major task for further studies, since a higher risk of this complication is reported in mitral valve IE, compared to aortic (Yang *et al*., 2019).

The structural components of the extracellular matrix of the valvular leaflet are modified in the case of cardiovascular disease (Schoen, 2018; Tayem *et al*., 2022) and ageing (Gumpangseth *et al*., 2020). The aforementioned changes, including the cusp tissue calcification, alter the valve endothelial invariance, promote the thickening of a leaflet, and affect blood throughflow. Higher shear stress of the blood flow enhances platelet adhesion to the valvular surfaces (Hu *et al*., 2021).

In addition, maximum velocity of blood needs to be present at the free edges of the aortic valve leaflet (Butcher *et al*., 2007). During the leaflet invasion, neutrophilic leukocytes further promote cusp tissue inflammation and enzymatic remodulation. In this study, infiltration of the valvular cusp with neutrophilic leukocytes colonising the free edge of the leaflet was demonstrated, thus supporting the literature. In contrast, no significant differences in neutrophilic leukocyte infiltration of lesioned valvular leaflets were found between cusp histological layers.

It is believed that neutrophilic leukocytes and blood platelets modify the structure of the aortic valve, and promote IE vegetation growth and the formation of NETs (Jung *et al*., 2015). Not only risk factors and the structural alterations of the leaflet and inflammation, but also IE vegetation associated with protease activity caused by neutrophils and other immune cells affect the sustainability of leaflets (Al-Salih *et al*., 2012). In patients with cardiovascular disease, high levels of MPO negatively affect endothelial function and contribute to sustained tissue inflammation and proteolytic degradation (Etwebi *et al*., 2018).

In this study, the presence of MPO secreted by activated neutrophilic leukocytes was demonstrated by immunohistochemistry assay in IE-affected valvular leaflets and vegetation. Previous studies have demonstrated the similarity of IE vegetation on affected heart valves with biofilm infections (Lerche *et al*., 2021). In cardiac vegetation, the microorganisms often form aggregates, and the gradient-sensing mechanism in bacterial activity exists. However, it currently remains unclear what governs the dynamics of bacterial aggregations (Brumley *et al*., 2019). The results of our study, which confirm the presence of histone H3 expression, suggestive of NET formation, provide some insight into the understanding of the complex pathobiology of IE.

When the results are viewed in light of limitations, the authors are ready to admit a relatively small sample size. A second limitation to mention was the absence of healthy valve controls, which might create an interesting sample group to compare.

Collectively, the study highlights the necessity for further clinical and morphological exploration of the contribution of neutrophilic leukocytes and NETs in native aortic valve endocarditis.

CONCLUSIONS

Tissue damage diversity was demonstrated in native aortic valve endocarditis; the free edge and the middle portion of the valvular leaflet appeared to be more vulnerable and more severely subjected to neutrophilic leukocytes infiltrating inflammatory lesions. The formation of vegetation and damage of the endothelial layer occurred along with the presence of IE-related bacteraemia. An increase of enzymatic activity of neutrophilic leukocytes and release of citrullinated histones H3 characteristic of NETs was common in cardiac vegetation.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICS

The study was approved by the Central Medical Ethics committee of Latvia (Decision No. 5070) and conducted according to the Declaration of Helsinki.

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NEITROFILIE LEIKOCĪTI UN NEITROFILO LEIKOCĪTU ĀRPUSŠŪNU LAMATAS PACIENTIEM AR AORTAS VĀRSTUĻA INFEKCIOZO ENDOKARDĪTU

Infekciozais endokardīts ir slimība, kas skar endokardu, pamatā sirds vārstuļus. Aortas vārstulis ir viens no visbiežāk skartajiem vārstuļiem. Infekciozā endokardīta patoģenēzē būtiska loma ir bakterēmijai un vārstuļu endotēlija bojājumam, taču pētījumi liecina, ka būtiska nozīme var būt arī vārstuļu lapiņas neitrofilo leikocītu infiltrācijai un to ārpussūnu lamatām (*neutrophil extracellular traps*). Šī pētījuma mērķis bija analizēt neitrofilo leikocītu un neitrofilo leikocītu ārpussūnu lamatu izplatību gaismas mikroskopijā ar rutīnas krāsošanas un imūnhistoķīmijas metodēm aortas vārstuļa audos un veģetācijās pacientiem ar aortas vārstuļa infekciozo endokardītu. Statistiski nozīmīgas atšķirības novērotas, salīdzinot vārstuļa daļas, respektīvi, lielākā neitrofilo leikocītu infiltrācija tika novērota lapiņas brīvajā malā (vidējais 5.89 ± 3.00, $p < 0.001$) un vidusdaļā (vidējais 4.58 ± 3.64, $p = 0.032$), salīdzinot ar bazālo jeb aortas sienai tuvāko daļu (2.05 ± 1.90). Netika novērotas statistiski nozīmīgas atšķirības neitrofilo leikocītu infiltrācijā, sadalot vārstuli pa morfoloģiskajiem slāņiem – *fibrosa, spongiosa, ventricularis*. Izmantojot imūnhistoķīmijas metodes, tika vizualizēti neitrofilo leikocītu ārpussūnu lamatu struktūrelementi — mieloperoksidāze un citrulinēti histoni H3. Tika novērota abu antivielu pozitīva ekspresija infekciozā endokardīta skartās aortālās vārstules lapiņā un lielākoties veģetāciju struktūrā. Šis pētījums apstiprina ideju, ka vārstuļa lapiņas brīvā mala un vidējā daļa ir pakļauta pastiprinātai asins plūsmai; endotēlija bojājums un veģetāciju veidošānās visticamāk notiek kopā ar infekciozu endokardītu saistītu bakterēmiju.