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Research Article

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Association Between Histopathological Changes and Immunohistochemical Expression of CD3, CD20 & CD68 in Mitral Valvular Tissues in Rheumatic Heart Disease

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Abstract

Background: *Even though research on the pathophysiology of RHD has much advanced than before, little is known about the underlying mechanisms that lead to severe valve malfunction. The histopathological examination of diseased mitral valvular tissues, search for intralesional regulatory T cells, B cells and macrophages by immunohistochemical study and observing their association can contribute toward a better understanding of the immunopathogenesis of RHD, and help in developing a more precise targeted therapy.*

Objectives: *The aim of this study is to evaluate the association between histopathological changes in diseased mitral heart valves in RHD cases by Haematoxylin & eosin and Masson's trichrome staining and immunohistochemical expression of CD3, CD20 & CD68 in those tissues to identify the morphological changes and underlying immune mechanism responsible for the disease progression.*

Method: *A cross-sectional study was carried out in the collaboration of Department of Cardiac surgery and Department of Pathology, Bangabandhu Sheikh Mujib Medical University, Dhaka, from July 2022 to June 2024. A total of 55 patients aged between 18 and 65 years with rheumatic mitral valvular disease were included in this study. The excised mitral valves were histopathologically analyzed and immunohistochemical study was done.*

Result: *In gross morphological study, fibrous stenotic valve was 44%, elastic insufficient valve 40% and calcific stenotic mitral valve was 16%. In microscopic examination, pattern of inflammation was focal 25% and diffuse 75% cases. Fibrosis was mild in 24%, moderate in 40% and severe in 36%. Calcification was present as mild in 2%, moderate in 5% and severe in 9% (focal in 7% and diffuse in 9%). In immunohistochemical examination, for calcification, CD3 infiltration- occasional 44.5% and several groups 55.5% which was not statistically significant (P- value 0.581ns). CD20 infiltration- absent 22.2%, occasional 33.3%, several groups 33.3% and many groups 11.2% which was not statistically significant (P- value 0. 3945ns).CD68 infiltration- absent 11.1%, focal 55.6% and multifocal 33.3% which was statistically significant (P- value 0. 0036s). In case of fibrosis, CD3 infiltration- occasional 61.5% (mild), 45.5% (moderate), 10% (severe) and several groups 38.5%(mild), 54.5% (moderate), 90% (severe) which was statistically significant (P- value 0. 0056s). CD20 infiltration- absent 7.7% (mild), 22.8% (moderate), 5% (severe); occasional 61.5% (mild), 54.5% (moderate),5% (severe); several groups 15.4% (mild), 18.2% (moderate), 25% (se-*

vere) and many groups 15.4% (mild), 4.5% (moderate),65% (severe) which was statistically significant (P- value 0. 00015s). CD68 infiltration- absent 61.5% (mild), 27.3% (moderate), 5% (severe); focal 23.1% (mild), 68.2% (moderate),90% (severe) and multifocal 15.4% (mild), 4.5% (moderate), 5% (severe) which was statistically significant (P- value 0. 0027s).

Conclusion: *The significant association between immunohistochemical examination and histopathological changes observed in this study can provide new insight to disease presentation and hence can contribute to the development of more specific targeted therapy to halt disease progression.*

Abbreviation

CD: Cluster of Differentiation
IHC: Immunohistochemistry
RHVD: Rheumatic Heart Valve Disease
SPSS: Statistical Package for the Social Science
Th: T helper cells

Introduction

Rheumatic heart disease, which mainly affects school-age children and adolescents in low- and middle-income countries, is the most common cardiac ailment among those under 25. It is most likely caused by group A streptococcus transmission among students. It is not entirely clear why streptococcal infections are more common than Acute Rheumatic Fever (ARF). According to some research, immune related genes such human leukocyte antigen, toll like receptor and cytokines may be involved in genetic disposition [1].

The initial attack may happen in middle or later life. Most patients with rheumatic fever have antibodies to one or more streptococcal enzymes, such as streptolysin O and DNase B in their sera, even when pharyngeal cultures for streptococci are negative by the time the illness starts. Carditis and arthritis are the most prevalent clinical symptoms; minors are especially likely to have arthritis [2].

It is well recognized that chronic inflammation and a high level of fibrosis cause the valve apparatus to anatomically distort, resulting in valve malfunction. In contrast to other degenerative heart valve diseases, this one exhibits a distinct natural history and clinical manifestations. It is currently unknown how self-tolerance mechanisms fail in RHVD and how humoral and cellular inflammatory responses are related, despite the fact that more than 50 years have elapsed since the groundbreaking research on the disease's etiology [3].

Acute and recurring inflammation are the hallmark pathological features of rheumatic mitral valvular disease. Although leaflet thickening, calcification and retraction, periannular calcification with annular motion limitation, leaflet fusion, chordal thickening, shortening & fusion and papillary inflammation are all part of the rheumatic process, it is unclear which specific immunologic and inflammatory mechanisms cause valvulitis [4]. There have been reports of elevated IL-2 production in patients with acute rheumatic fever (ARF) and active RHD. Furthermore, these individuals have high levels of CD4+ and CD25+ cells, which may indicate that activated T CD4+ cells expanded in peripheral blood during the disease's active phase. It should be noted, nevertheless, that these cells might also be the CD4+CD25+ regulatory T cells, as described more recently. These results were validated by other authors, who also found that RF/RHD patients had higher plasma levels of TNF-α. The Aschoff nodule is regarded as a pathognomonic indication of ARF in cardiac lesions. It is a granulomatous lesion that is primarily found in the cardiac interstitium's perivascular areas, endocardium or subendocardium. Different cells (Anitschkow cells, multinucleated cells, few lymphocytes, macrophages, plasma cells, and polymorphonuclear leukocytes) are involved in the various stages of the Aschoff nodule. Aschoff nodule progression has been linked to the production of IL-1, TNF-α and IL-2 in the valvular lesions of ARF patients. In stages 1 and 2, monocytes/macrophages secreted IL-1 and TNF-α, whereas in stage 3, T cells produced IL-2. Additionally observed are CD8+ T cells, B cells and macrophages. It's possible that the Th2-type cytokine IL-5 stimulated these cells. Once activated, they may produce chemokines and a number of proinflammatory cytokines, including IL-6, IL-8, TNF, and IL-1, which may contribute to local inflammation [5].

We can learn more about the underlying structural alterations that cause valve dysfunction by examining diseased mitral valves both grossly and microscopically using Masson's trichrome staining and H&E. Furthermore, the identification of intralesional regulatory T cells, B cells and macrophages through immunohistochemical expression of CD3, CD20 and CD68 can undoubtedly aid in the development of new treatments targeted at reducing heart damage in RHD patients as well as a deeper comprehension of the immunopathogenesis of the disease.

So, my aim is to evaluate the association between histopathological changes in diseased mitral heart valves in RHD cases by Haematoxylin & eosin and Masson's trichrome staining and immunohistochemical expression of CD3, CD20 & CD68 in those tissues in this study, by which we can identify the morphological changes and underlying immune mechanism responsible for the disease progression.

Methods

This was a cross-sectional, descriptive and observational study. A total of 55 patients with RVHD aged between 18 to 65 years were included in this study. Any Cancer patients, covid & dengue positive patients and patients with cirrhosis of liver & ILD were excluded from the study. The study was conducted at the Department of Cardiac Surgery, Bangabandhu Sheikh Mujib Medical University, Dhaka, and Department of Pathology, Bangabandhu Sheikh Mujib Medical University, Dhaka, Bangladesh, from July 2022 to June 2024.

The valves were collected from the Department of Cardiac surgery immediately after MVR surgery and fixed with 10% neutral buffered formalin for 24 hours. The specimens were subsequent-

ly processed and examined at the Department of Pathology, Bangabandhu Sheikh Mujib Medical University, Dhaka for histopathological examination.

After meticulous gross examination, 3 blocks were submitted: 1 block for H&E, 1 for Masson's trichrome and 1 for immunohistochemical examination.

Scoring System for Immunohistochemical Study:
CD3:
- Absent: 0-10%
- Occasional: 10-30%
- Several groups: 30-60%
- Many groups: >60%
- (Ref: ISHLT-WF scoring guideline for immunopathology, 2013)

CD20:
- Absent: 0-10%
- Occasional: 10-30%
- Several groups: 30-60%
- Many groups: >60%
- (Ref: ISHLT-WF scoring guideline for immunopathology, 2013)

CD68:
- Absent: 0-10%
- Focal positivity: >10%- <50%
- Multifocal/diffuse positivity: >50%
- (Ref: ISHLT-WF scoring guideline for immunopathology, 2013)

Result

Distribution of Patients by Gross Histopathological Changes

Out of the 55 patients, casp thickness shows fibrous stenotic valve in 44%, elastic insufficient valve in 40% and calcific stenotic type mitral valve in 16% patients. 7% valves had focal and 9% had diffuse calcification. Only 4% valves had vegetations. Elasticity was reduced in 82% valves and were retained in 18% valves.

Table 1: Distribution of Patients by Gross Histopathological Changes (n=55)

Gross Morphological Changes	Frequency	Percentage
Casp Thickness		
Fibrous stenotic type	24	44
Elastic insufficient type	22	40
Calcific stenotic type	9	16
Degree of Calcification		
No calcification	46	84
Focal (vegetative)	4	7
Marked (diffuse)		
Presence of vegetation	5	9
Elasticity	2	4
Normal	10	18
Reduced	45	82

n= Total number of subjects f= frequency %= percentage

Distribution of Patients by Microscopic Histopathological Findings

Out of 55 patients, pattern of inflammation was focal in 25% patients and diffuse in 75% patients. Intensity of fibrosis was mild in 24%, moderate in 40% and severe in 36% patients. Calcification was present in 16% and absent in 84% patients.

Table 2: Distribution of Patients by Microscopic Histopathological Findings (n=55)

Microscopic changes	Frequency	Percentage
Pattern of inflammation		
Focal	14	25
Diffuse	41	75
Intensity of fibrosis		
Mild	13	24
Moderate	22	40
Severe	20	36
Calcification		
Absent	46	84
Present	9	16

n= Total number of subjects f= frequency %= percentage

Distribution of the Patients by CD3, CD20 & CD68 Expression

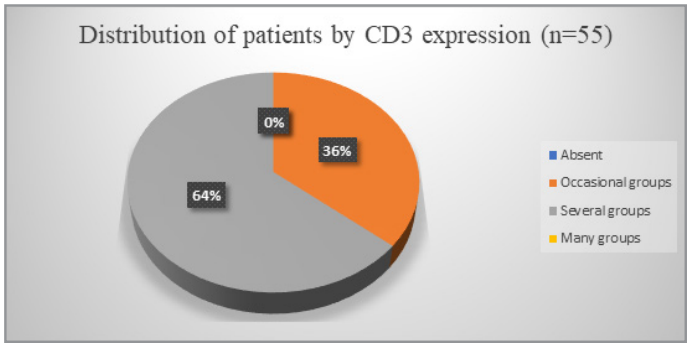

Figure 1: Distribution of the Patients by CD3 Expression (n=55)

Out of 55 patients, 20 (36%) valves showed occasional groups of CD3 positive cells are observed and in 35 (64%) of the valves of the patients there were several groups of CD3 positive cells.

Distribution of the Patients by CD20 Expression (n=55)

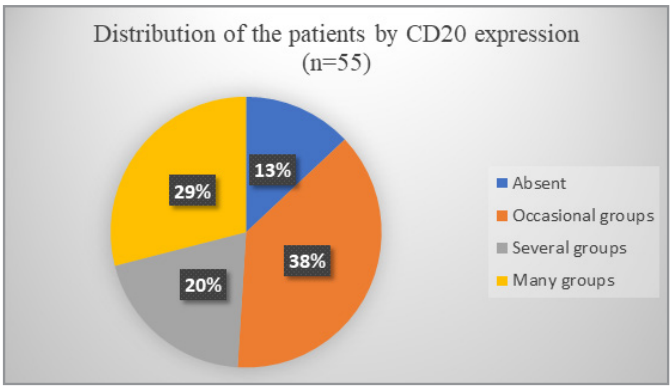

Figure 2: Distribution of the Patients by CD20 Expression (n=55)

Out of 55 patients, 7 (13%) of the valves were CD20 negative. 21 (38%) valves showed occasional CD20 positive groups of cells, 11 (20%) showed several groups of CD20 positive cells and 16 (29%) valves showed many groups of CD20 Immunopositive cells.

Distribution of the Patients by CD68 Expression (n=55)

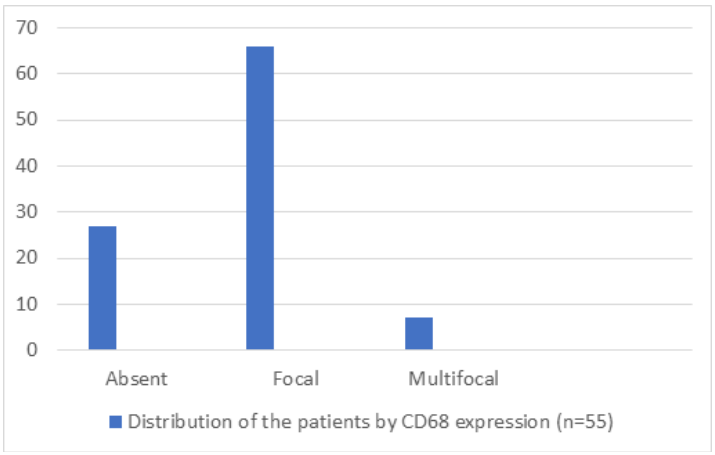

Figure 3: Distribution of the Patients by CD68 Expression (n=55)

Out of the 55 patients, 15 (27%) valves were CD68 negative, 36 (66%) showed focal CD68 positive cells and 4 (7%) showed multifocal CD68 immunopositive cells.

Association Between Presence of Calcification and CD3, CD20 and CD68 Among the Patients

Out of 9 calcified cases, CD3 infiltration was occasional in 44.5% and several groups in 55.5% which is not statistically significant (P- value 0.581ns). The CD20 infiltration was absent in 22.2%, occasional in 33.3%, several groups in 33.3% and many groups in 11.2% which is not statistically significant (P- value 0. 3945ns). The CD68 infiltration was absent in 11.1%, focal in 55.6% and multifocal in 33.3% which is statistically significant (P- value 0. 0036s).

Table 3: Association Between Presence of Calcification and CD3, CD20 and CD68 Among the Patients (n=55)

Variables	Calcification			
	Present	Absent	Total	
CD3				
Occasional	4 (44.5)	16 (34.8)	20 (36.36)	p value
Several groups	5 (55.5)	30 (65.2)	35 (63.63)	0.581ns
CD20				
Absent	2 (22.2)	5 (10.9)	7 (12.72)	p value
Occasional	3 (33.3)	18 (39.1)	21 (38.18)	0.3945ns
Several groups	3 (33.3)	8 (17.4)	11 (20.0)	
Many groups	1 (11.2)	15 (32.6)	16 (29.09)	
CD68				
Absent	1 (11.1)	14 (30.4)	15 (27.27)	p value
Focal	5 (55.6)	31 (67.4)	36 (65.45)	0.0036s
Multifocal	3 (33.3)	1 (2.2)	4 (7.27)	

n= Total number of subjects; ns= not significant; s= significant; A chi- square test of independence was performed to examine the relation between calcification and CD3, CD20 and CD68 immunopositivity; P value <0.05 was considered as significant.

Association Between Presence of Fibrosis and CD3, CD20 and CD68 Among the Patients

In case of fibrosis, CD3 infiltration was occasional 61.5% (mild), 45.5% (moderate), 10% (severe) and several groups 38.5%(mild), 54.5% (moderate), 90% (severe) which is statistically significant (P- value 0. 0056s). CD20 infiltration was absent 7.7% (mild), 22.8% (moderate), 5% (severe); occasional 61.5% (mild), 54.5% (moderate),5% (severe); several groups 15.4% (mild), 18.2% (moderate), 25% (severe) and many groups 15.4% (mild), 4.5% (moderate),65% (severe) which is statistically significant (P- value 0. 00015s). CD68 infiltration was absent 61.5% (mild), 27.3% (moderate), 5% (severe); focal 23.1% (mild), 68.2% (moderate),90% (severe) and multifocal 15.4% (mild), 4.5% (moderate), 5% (severe) which is statistically significant (P- value 0. 0027s).

Table 4: Association Between Presence of Fibrosis and CD3, CD20 and CD68 Among the Patients (n=55)

Variables	Fibrosis				
	Mild	Moderate	Severe	Total	
CD3					
Occasional	8 (61.5)	10 (45.5)	2 (10.0)	20 (36.36)	p value
Several groups	5 (38.5)	12 (54.5)	18 (90.0)	35 (63.63)	0.0056s
CD20					
Absent	1 (7.7)	5 (22.8)	1 (5.0)	7 (12.72)	p value
Occasional	8 (61.5)	12 (54.5)	1 (5.0)	21 (38.18)	0.00015s
Several groups	2 (15.4)	4 (18.2)	5 (25.0)	11 (20.0)	
Many groups	2 (15.4)	1 (4.5)	13 (65.0)	16 (29.09)	
CD68					
Absent	8 (61.5)	6 (27.3)	1 (5.0)	15 (27.27)	p value
Focal	3 (23.1)	15 (68.2)	18 (90.0)	36 (65.45)	0.0027s
Multifocal	2 (15.4)	1 (4.5)	1 (5.0)	4 (7.27)	

n= Total number of subjects s= significant ns= not significant A chi- square test of independence was performed to examine the relation between calcification and CD3, CD20 and CD68 immunopositivity. P value <0.05 was considered as significant.

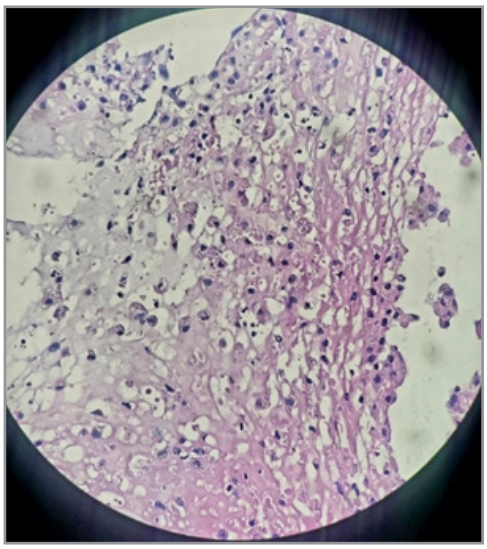

Figure 4: Photograph showing vegetation in valve casp (Case no:24, H&E, 40X)

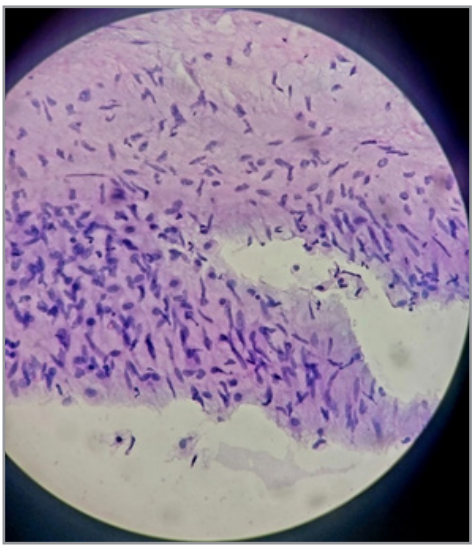

Figure 5: Photograph showing Anitschkow cell or caterpillar cells (Arrow, Case no: 9, H&E, 40X)

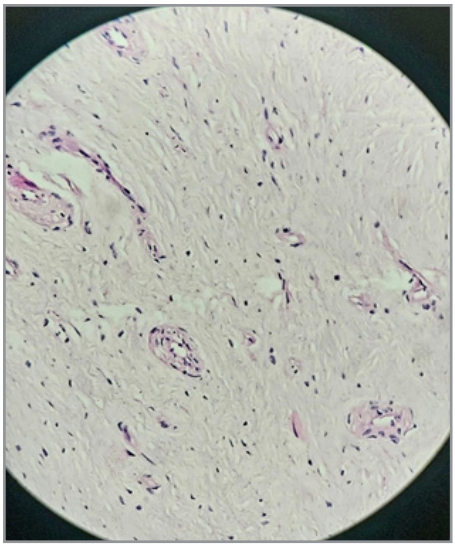

Figure 6: Photograph showing moderate infiltration of chronic inflammatory cells in the valve casp (Case no:10, H&E, 40X)

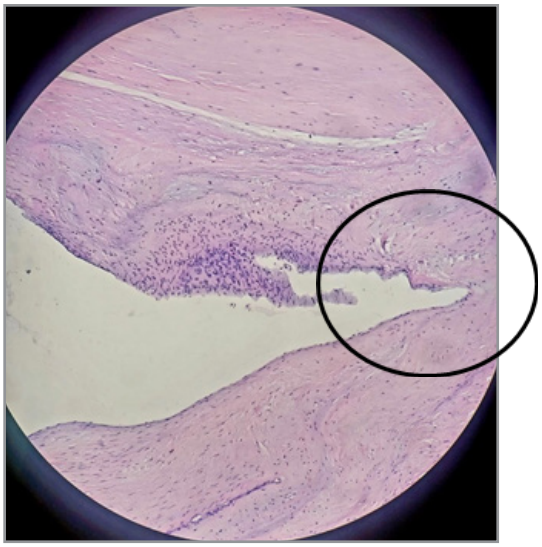

Figure 7: Photograph showing dense infiltration of chronic inflammatory cells in the valve casp (Case no:8, H&E, 40X)

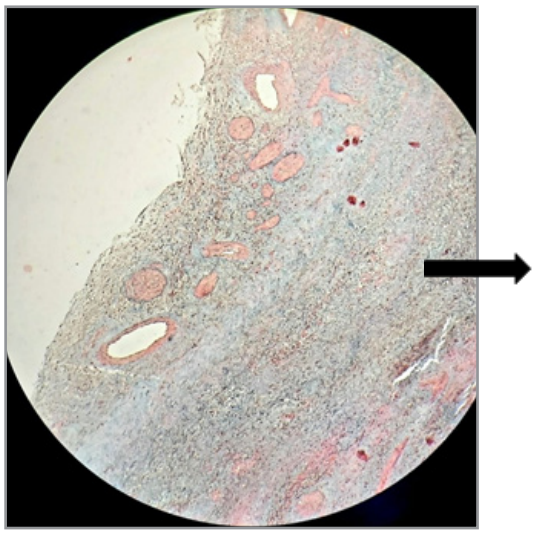

Figure 8: Photograph showing dense fibrosis in the valve casp (Case no:9, MT stain, 40X)

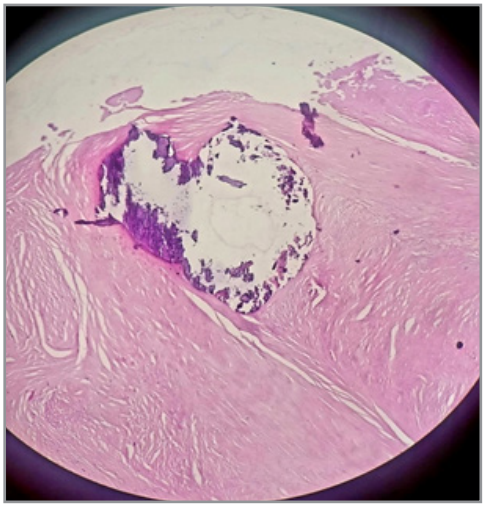

Figure 9: Photograph showing diffuse calcification in the valve casp (Case no: 12, H&E, 40X)

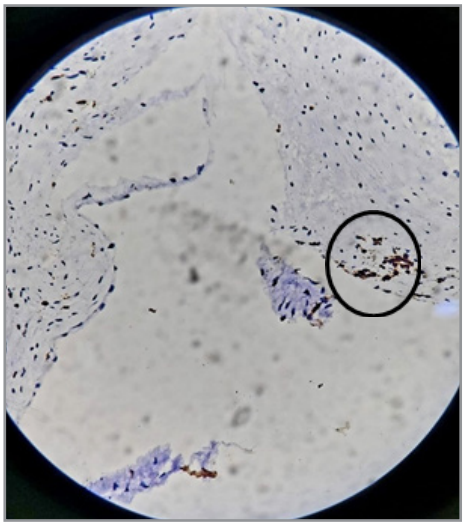

Figure 10: Photograph showing positive expression of CD3 immunohistochemistry in several groups of cells (Case no:6, 40X)

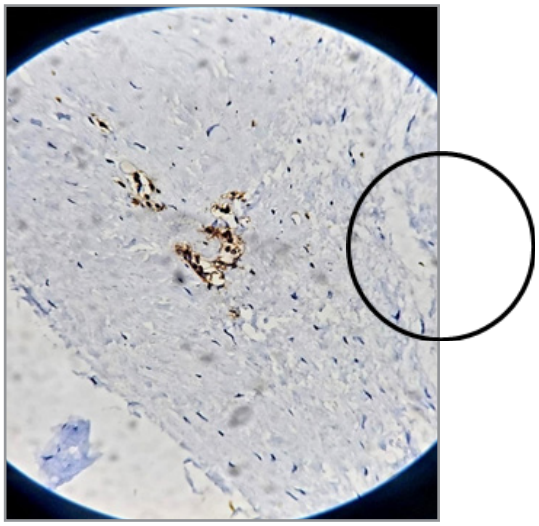

Figure 11: Photograph showing positive expression of CD3 immunohistochemistry in occasional groups of cells (Case no: 15, 40X)

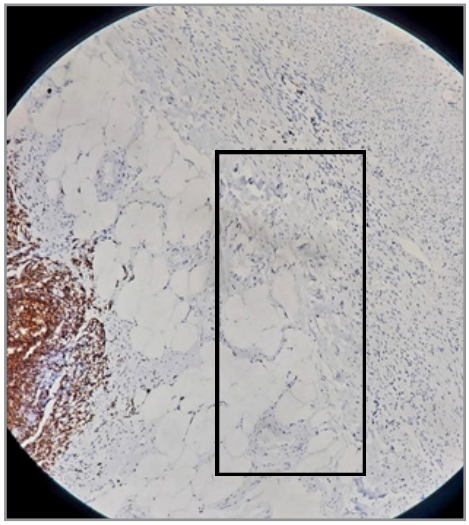

Figure 12: Photograph showing positive expression of CD20 immunohistochemistry in many groups of cells (Case no:24, 40X)

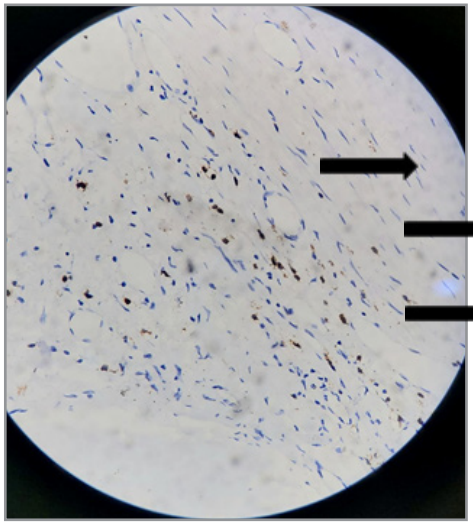

Figure 13: Photograph showing positive expression of CD20 immunohistochemistry in occasional groups of cells (Case no:2, 40X)

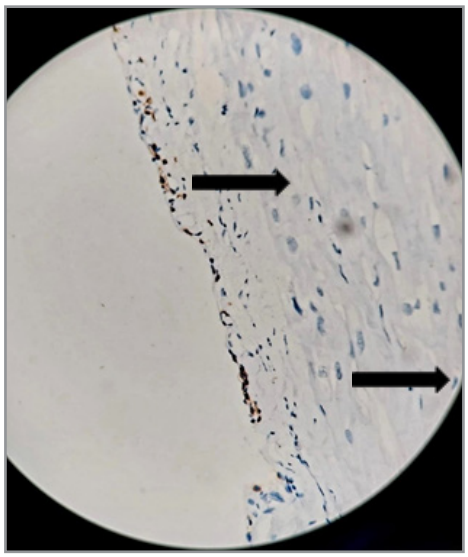

Figure 14: Photograph showing positive expression of CD68 immunohistochemistry in focal groups of cells (Case no:2, 40X)

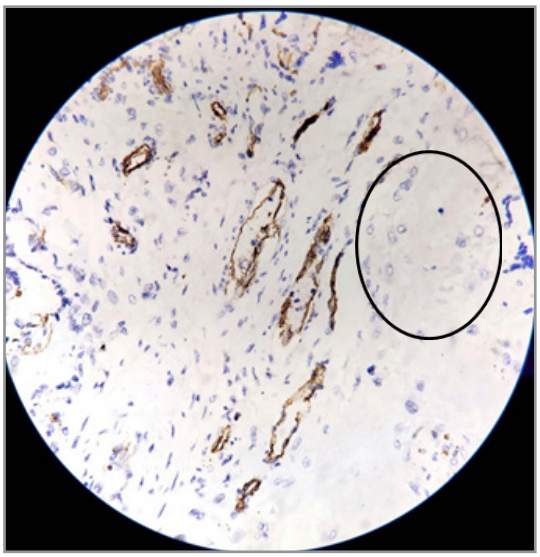

Figure 15: Photograph showing positive expression of CD68 immunohistochemistry in multifocal groups of cells (Case no:34, 40X)

Discussion

In the current study, out of the 55 patients, 44% patients had fibrous stenotic type mitral valve, whereas 40% had elastic insufficient and 16% had calcific stenotic type mitral valve. According to the study conducted by Nazarian et al., (1978), 26% of the valves were fibrous stenotic type and 40% were calcific stenotic type. In this study, most valves are fibrous stenotic type.

In the present study, pattern of inflammation was focal in 25% patients and diffuse in 75% patients. In the study by Rashed et al., (2006), perivascular lymphocytic aggregates tightly surrounding small & medium sized vessels was present in 16% valves and diffuse interstitial infiltrate of lymphocytes in 28%.8 Mutagaywa et al., (2022) observed 81.5% leucocytic inflammatory cell infiltrate. These findings align with this study.

Intensity of fibrosis was mild in 24%, moderate in 40% and severe in 36% patients in our study. Gomez et al., (2020) found moderate to marked fibrosis in 100% of their cases, in our study which is 76%.9 In the study conducted in Tanzania by Mutagaywa et al., (2022), the proportion of H&E stained tissue showed 72.2% fibrosis. Mutagaywa et al., (2022) used von kossa stain to observe fibrosis & calcification. In the current study, Masson's trichrome stain was used instead of Von Kossa stain and the findings are quite similar.

Calcification was absent in 84% valves and present in 16%. Nazarian et al., (1978) observed no calcification in 65% of the valves and present in 35%. Gomez et al., (2020) also found no calcification in 65% and calcification in 35% of valves. In Mutagaywa et al., (2022) study, 55.6% calcification was observed. These studies align with our study [6-9].

In this study, out of 9 calcified cases, CD3 infiltration was occasional in 44.5% cases and several groups in 55.5%. The relation between calcification and these variables was statistically insignificant (P- value 0.581ns). In case of CD20 in calcified cases, 22.2% showed no infiltration, 33.3% showed occasional groups, 33.3% showed several groups and 11.2% case showed many groups. The relation between calcification and these variables was statistically insignificant (P- value 0. 3945ns). In case of CD68 in calcified cases, 11.1% showed no infiltration, 55.6% showed focal and 33.3% showed diffuse cellular infiltration. The relation between calcification and these variables was statistically significant (P- value 0. 0036s). In the Mutagaywa et al., (2022) study, out of 30 calcified cases, CD3 infiltration was occasional 12.5% (mild calcification); several groups 37.5% (mild calcification) & 30% (severe calcification); and many groups 41.7% (mild calcification) & 70% (severe calcification). CD20 infiltration was occasional 37.5% (mild calcification) & 40% (moderate calcification); several groups 12.5% (mild calcification); and many groups 29.2% (mild calcification) & 60% (severe calcification). CD68 infiltration was occasional 8.3% (mild calcification); several groups 12.5% (mild calcification); and many groups 79.2% (mild calcification) & 90% (severe calcification). There was a statistically significant association between the extent of calcification and the degree of cellular infiltrations as marked by CD3, CD20 and CD68 staining. According to Rashed et al., (2006)'s theory, there might be negative CD3 and CD20 in calcified valves as it is a chronic process, but CD68 should be significant.6,8.

In our study, out of 13 cases with mild fibrosis, CD3 infiltration was occasional in 61.5% and several groups in 38.5%. Out of 22 cases of moderate fibrosis, 45.5% showed occasional and 54.5% showed several groups of CD3 infiltration. Among 20 cases with severe fibrosis, 10.0% showed occasional and 90.0% showed several CD3 group of cells. The relation between fibrosis and these variables was statistically significant (P- value 0. 0056s). In case of CD20, 61.5% showed occasional, 15.4% showed several groups and 15.4% showed many groups of CD20 cells among the mild fibrosis cases. In moderate fibrosis, 54.5% showed occasional, 18.2% showed several groups and 4.5% showed many groups of CD20 cells. In severe fibrosis cases, 5% showed occasional, 25% showed several groups and 65% showed many groups of CD20 cells. The relation between fibrosis and these variables was statistically significant (P- value 0. 00015s). In case of CD68, in mild fibrosis cases, 23.2% showed focal and 15.4% showed multifocal groups of cells. In case of moderate fibrosis, 68.2% showed focal and 4.5% showed multifocal groups of cells. In case of severe fibrosis, 90% showed focal and 5% showed multifocal CD68 positive groups of cells. The relation between fibrosis and these variables was significant (P- value 0. 0027s). No such study showing the association between fibrosis and CD3, CD20 and CD68 immunostain was conducted before.

Conclusion

There is significant association between immunohistochemical examination and histopathological changes observed in this study can provide new insight to disease presentation and hence can contribute to the development of more specific targeted therapy to halt disease progression.

Declarations

Acknowledgments

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Author contributions

Billah M prepared the research protocol and was responsible for data collection, arranging the data analysis and preparing the manuscript. Saba S was responsible for conducting the histopathological analysis, Alauddin M and Rahman M were responsible for conceptualizing the study and assisted in protocol development, manuscript preparation and proofreading.

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Conflicts of Interest

None.

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