Role of matrix metalloproteinases in mitral valve regurgitation: Association between the of MMP-1, MMP-9, TIMP-1, and TIMP-2 expression, degree of mitral valve insufficiency, and pathologic etiology

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Abstract

Background: The pathogenesis of mitral valve insufficiency is not yet fully understood. Several studies stressed the role of matrix metalloproteinases (MMPs) in the emergence of valvular pathologies. The primary objective of the present study is to analyze the role of selected MMPs and their inhibitors in mitral valve insufficiency.

Patients and Methods: Eighty patients (33 female/47 male, mean age 67 years) underwent cardiopulmonary bypass surgery for mitral valve reconstruction between 2007 and 2015. All patients suffered from mitral insufficiency (MI) Stages iii and iv. When tissue resection was acquired specimens were taken immediately frozen and used for histological examination. Expression of MMP-1, MMP-9, tissue inhibitor of metalloproteinase (TIMP)-1, and TIMP-2 was examined immunohistochemically and distribution was analyzed in regard to preoperative clinical, echocardiographic, and histopathological findings.

Results: A clear correlation between the MMP expression and the MI degree of severity could be shown. The expression of MMPs proved to be high in relation to mild insufficiencies and relatively weak in the case of severe ones. Additionally, the etiology of the MI was considered in the analysis and a significant difference in the expression of MMPs between the mitral valves with endocarditis and the ones featuring a degenerative disease could be shown. Within the group of valves with degenerative diseases, no significant difference could be established between the subgroups (myxoid and sclerosed valves).

Conclusion: The increased expression of MMPs and their inhibitors in mild insufficiencies could prove that the molecular changes in the valve precede the...
1 | INTRODUCTION

Mitral valve regurgitation is one of the most common heart valve diseases in Europe whose prevalence is expected to increase as a result of demographic development in society and an increase in the proportion of older persons. It results from the leakage of the mitral valve allowing some blood to flow back from the left ventricle into the left atrium during systole. In the past few years, there has been a strong interest in studying mitral valve regurgitation. Out of the various etiologies of mitral valve disease, degenerative mitral valve regurgitation accounts for about 60%-70% of the mitral valve insufficiency cases in Western countries. This is followed by ischemic mitral valve regurgitation accounting for approximately 20% of the cases. Additionally, 2%-5% of the cases result either from endocarditis or are rheumatic in nature. The remaining etiologies are rather rare. Mitral valve regurgitation is classified into chronic and acute forms. The chronic form is common while the acute form is rare which results from rapidly progressive destructive processes.

Currently, mitral regurgitation is diagnosed through echocardiography which provides important data on the extent of mitral valve regurgitation with a high degree of accuracy. Despite that, echocardiography still has a major disadvantage of subjectivity making the therapy decision challenging. To overcome this disadvantage, matrix metalloproteinase (MMPs) can be used instead. MMPs are already in use for the diagnosis and treatment of several diseases such as cancers and inflammatory diseases which brought interest to their importance in the field of cardiovascular diseases.

The MMPs are extracellular proteins but can be found inside the cells as well. There are 24 known MMPs which are divided into six groups based on their structure and function. These proteins are synthesized as inactive zymogens that are activated to perform a wide range of functions such as remodeling processes and tumor invasion as well as their role in growth factors and their receptors, molecular cell adhesion, and proteolytic process of cytokines. The MMP-2 and MMP-9 belong to the group of gelatinases and are involved in digesting gelatin and collagen. The activity of MMPs is inhibited by endogenous specific inhibitors called tissue inhibitors of metalloproteinases (TIMPs). Four types of TIMPs are present in vertebrates namely TIMP-1, TIMP-2, TIMP-3, and TIMP-4.

MMP-1, TIMP-1, and TIMP-2 are present in all heart valves. MMP-2 is only present in the aortic and pulmonary valves. It is suggested MMP-3 and MMP-9 do not express in heart valves. But numerous studies have shown a link between MMPs and TIMPs to cardiovascular diseases and their pathological processes. Investigating further the differential expression of MMPs and their inhibitors in the mitral valve under both health and disease can, therefore, contribute to improved understanding of mitral valve regurgitation opening new avenues for better diagnosis and treatments.

In the current study, we analyzed the expression of MMPs in mitral valve regurgitation to establish a relationship between MMPs and the disease. We selected two MMPs (MMP-1 and MMP-9) and two inhibitors (TIMP-1 and TIMP-2) for this purpose and examined their expression through immunohistochemistry in the valvular tissue of patients suffering from mitral valve regurgitation. We compared these findings with the clinicopathological data available.

2 | METHODS

2.1 | Ethical approval

This study was approved by the ethics committee of the Philipps University of Marburg in September 2016 (Az.: 70/16).

2.2 | Subjects

We examined the clinical reports of 80 patients (33 women and 47 men) who suffered from severe mitral valve regurgitation and were surgically operated on in the period from 2007 to 2015. The age of onset of patients ranged from 32 to 89 years with a median age of 67 years. A tissue sample of the affected mitral valve was collected from each patient during surgery. The sample was fixed in a 10% formalin solution, embedded in paraffin, and archived at the Institute for Pathology at the Philipps University of Marburg.

Several patients were symptomatic at the time of diagnosis. Exertional dyspnea was the most common symptom in 29 patients followed by unexplained fever and fatigue in 12 patients and septic shock in 5 patients. A secondary diagnosis of at least one more disease was made in 71 patients along with mitral valve regurgitation. Nineteen of these patients had one, 21 had two, and 31 had three or more secondary diseases.
patients had no other relevant comorbidities. The most common secondary diseases were cardiac arrhythmias, coronary heart disease, arterial hypertension, renal insufficiency, and type 2 diabetes mellitus. Additionally, aortic disease, peripheral arterial occlusive disease, chronic obstructive pulmonary disease, tricuspid regurgitation, and apoplectic insult were also identified (Table 1).

### 2.3 | Echocardiography

Echocardiography was performed in patients to confirm mitral valve regurgitation. Out of the 80 patients, 8 had acute mitral insufficiency (MI)-Grade 1, 27 had MI-Grade 2, and 45 had MI-Grade 3. Forty-eight patients had normal ejection fraction (EF) (>55%), nine had slightly impaired EF (46%–55%), and eight had moderately impaired EF (36%–45%). For the remaining 15 patients, information was lacking on exact measurements of EF.

### 2.4 | Tissue preparation for staining

Archived tissue samples were taken for staining. Sections of approximately 2 µm thickness were prepared using a sled microtome (SM2000 R; Leica) and were mounted on glass slides (Thermo Fisher Scientific). The slides were placed in the incubator at 60°C and were dewaxed for at least 60 min.

### 2.5 | Hematoxylin and eosin staining

The tissue samples were stained with hematoxylin and eosin in a continuous linear stainer (Tissue Stainer COT 20) according to the standard protocol. Xylene, alcohol (100%, 96%, and 70%), hemalum, eosin, alcohol (96% and 100%), and xylene were used to stain the slides in the given order. The tissue sections were subsequently assessed to evaluate the quality of the sample and the etiology of the disease.

### 2.6 | Immunohistochemical staining

The tissue slides were dewaxed by immersing in xylene, followed by ethanol (100%, 96%, and 70%) for 5 min each time. The slides were then immersed in the unmasking solution, were placed in a steam cooker for 45 min, and were rinsed with distilled water. The endogenous enzymes were blocked with a peroxidase-inhibiting solution and were rinsed with wash buffer. Primary antibodies were applied to the slides for 45 min (MMP-1: MAB3307; Millipore; MMP-9: NCL-MMP9-439; Leica Biosystems; TIMP-1: M7293, Fa. Dako; TIMP-2: NCI-TIMP2-487; Leica Biosystems). The slides were rinsed with wash buffer followed by the application of secondary antibodies. The excess of secondary antibodies was removed with wash buffer and the slides were immersed in Dako Real Envision polymer conjugate for 20 min followed by rewashing. The slides were then immersed in chromogen for 12 min and were rinsed with wash buffer and distilled water. They were counterstained with hematoxylin solution for 5 min. The tissue sections were then dehydrated with ethanol (70%, 96%, and 100%) to ensure complete dehydration. The slides were finally immersed in xylene. A few drops of Entellan were poured on the tissue sections were covered with a coverslip (Figure 1).

Afterward, the slides were examined under a light microscope (Olympus) with ×4, ×10, and ×40 magnification. Positive control tissues were also used. The stained tissues were classified semiquantitatively based on the percentage of stained cells. The following scale was used: 0 = negative (no visible color), 1 = positive in very few cells, 2 = positive in a moderate proportion of cells, and 3 = strongly positive for a larger proportion of cells. To make an appropriate

| Comorbidities           | Frequency | Percentage |
|-------------------------|-----------|------------|
| Arterial hypertension   | 41        | 51.2%      |
| Atrial fibrillation     | 39        | 48.8%      |
| CIHD                    | 20        | 25%        |
| Chronic renal insufficiency | 14    | 17.5%      |
| Diabetes type 2         | 13        | 16.25%     |

Abbreviation: CIHD, chronic ischemic heart disease.
evaluation, the percentages of stained cells were determined by two individuals independently and were jointly reevaluated (Figure 2).

2.7 | Imaging

The images were processed and recorded with a photomicroscope (Leica) using Leica Im50 Software. Data of blinded specimens were evaluated by two independent experienced pathologists.

2.8 | Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics version 24 software. p < .05 was considered significant for all statistical tests. For continuous variables, descriptive statistics (mean, median, minimum, and maximum) were used. For categorical variables, frequencies were evaluated. The relationship between the expression of MMPs and their inhibitors, the degree of mitral valve regurgitation, and the etiology were assessed by χ² test. Since the independent variable (MMP score) is an ordinal categorical variable in the present case, the relationship between linear and linear was used to evaluate the Mantel–Haenszel χ² test.

3 | RESULTS

3.1 | Pathological findings

The examined mitral valve tissues (posterior leaflets) of patients were categorized into two groups, endocarditis and degenerative mitral valve regurgitation. The endocarditis form was present in 44% of patients while degenerative mitral valve regurgitation was present in the remaining 56% of patients. Degenerative mitral valve regurgitation was further subcategorized into myxoid (27% patients), sclerotic (18% patients), and mixed (myxoid and sclerotic) (11% patients) (Figure 3).

3.2 | Expression of MMPs

MMP-1 expression was observed in all mitral valve tissue samples, so its expression signal intensity was scored from 1 to 3 in immunohistochemical evaluation (Figure 4A). MMP-9, however, was not observed in all samples and therefore its signal was scored from 0 to 3 (Figure 4B).

3.3 | Expression of TIMPs

The expression of TIMP-1 was detected in 86% of tissue preparations and TIMP-2 was detected in 95% of tissues. Their expression signal was scored from 0 to 3 in immunohistochemical evaluation (Figure 4C.D).

3.4 | Correlation between the expression of MMPs and TIMPs

To identify the correlation between MMPs and TIMPs, gene expression signal intensity levels were divided into two groups (weak for signal intensity Scores 0 and 1 and strong for Scores 2 and 3). Consequently, a strong correlation between the expression of MMP-1 and MMP-9 was found in 64 tissue samples (80%). A similar correlation was also identified between the expression of TIMP-1 and MMP-1 in 62 tissue samples (77.5%). A less noticeable correlation was found between TIMP-1 and TIMP-2 (75%), MMP-9 and TIMP-2 (72.5%), MMP-9 and TIMP-1 (67.5%), and MMP-1 and TIMP-2 (65%).

3.5 | Relationship between the expression of MMPs and TIMPs with the severity of mitral valve regurgitation

In patients with mitral valve regurgitation Grade 1 (MI-Grade 1), a high MMP1 expression was observed by immunohistochemistry as 25% had an expression intensity score 2%, and 75% of patients had a score of 3. In patients with MI-Grade 2, the expression of MMP1 was lower compared to the patients with MI-Grade 1 as 11.1% of patients had an expression intensity score of 1, 51.8% had an intensity score of 2 and only 37% of the cases had a score of 3. In patients with MI-Grade 3, MMP-1 expression decreased slightly further as 26.6% patients had an expression intensity score of 1, 44.4% had an intensity score 2%, and 29% of patients had a score of 3. These changes in the MMP-1 indicate that the MMP-1 expression decreases with an increase in MI severity (Figure 5).

FIGURE 2  Example for grading of histological specimen featuring specific antibody for matrix metalloproteinase-9
The expression of MMP-9 was similar in patients with MI-Grade 1 and MI-Grade 2 as shown in where the intensity score of 2 and 3 was assigned jointly to approximately 85% of patients. The combined expression of Scores 2 and 3 was, however, reduced to 60% of patients in MI-Grade 3 (Figure 6). This decrease in MMP-9 expression with an increase in MI severity is, thus, similar to MMP-1 expression variation as explained above. A statistically significant linear-by-linear association was observed ($p = .019$) between the intensity of MMP-9 expression with increasing MI severity.

The expression of TIMP-1 decreased as the severity of mitral valve regurgitation increased. As Figure 7 shows, 88% of patients with Mi-grade 1% and 89% of patients with MI-Grade 2 had a combined TIMP-1 expression intensity score of 2 and 3. The percentage of patients with an expression intensity score of 3, however, reduced to 40% with the severity of MI-Grade 3. A statistically significant linear-by-linear association was found with a significance level of .0001.

The expression of TIMP-2 also decreased as the severity of mitral valve regurgitation increased. As Figure 8 shows, 62% of patients with MI-Grade 1 and 81% patients with MI-Grade 2 had a combined TIMP-1 expression intensity score of 2 and 3. These intensity scores of 2 and 3 were, however, observed in only 31% of patients with the severity of MI-Grade 3. The significance level of the linear-by-linear relationship was .002.
3.6 Relationship between the expression of MMP and TIMPs with etiology of mitral valve regurgitation

Patients with endocarditis had increased expression of MMP-1 compared to the patients with degenerative mitral valve regurgitation as 65.7% of the endocarditis samples had an intensity score of 3 compared to 13.3% of the degenerative form samples (Table 1). The difference was, however, less prominent for an intensity score of 2 where 31.4% of tissue samples were from patients with endocarditis and 55.5% from degenerative form. The difference in expression between the two etiologies was statistically significant ($p = .000$)

MMP-9 expression was not detected in 8.5% of the endocarditis tissue samples and 4.4% of degenerative tissue samples. But 79.9% of patients with endocarditis had a combined intensity score of 2 and 3 compared to 64.4% of the patients with degenerative form (Figure 9A–D) For TIMP-1, combined intensity score of 2 and 3 was observed in 88.6% of patients with endocarditis compared to 40% patients with degenerative. Thus, there was a statistically significant increase ($p = .0001$) in the expression of TIMP-1 in endocarditis tissue samples. TIMP-2 expression was not detected in 11.4% of the endocarditis tissue samples and 4.4% of degenerative tissue samples. 62.8% of patients with endocarditis had the combined expression intensity score of 2 and 3 compared to 42.1% of the patients with degenerative form.

3.7 Relationship between the expression of MMPs and TIMPs in degenerative mitral regurgitation

The degenerative mitral valve samples were divided into three subgroups based on their pathological findings, namely myxoid valve insufficiency, sclerosed mitral valve, and myxoid-sclerosed valve. No significant difference was identified in the expression of MMPs and TIMPs among these three subgroups.
3.8 Effect of secondary diagnoses on the expression of MMPs and TIMPs

No significant difference was observed in the expression of MMPs and TIMPs based on the secondary findings (arterial hypertension, atrial fibrillation, and coronary artery disease).

3.9 Relationship of the expression of MMPs and TIMPs with postoperative complications

No significant differences were found in the expression of MMPs and TIMPs among complication-free courses and cases with mild and severe complications after surgery using the $\chi^2$ test.

4 DISCUSSION

MMPs, also known as matrix metallopeptidases or matrixins, are calcium-dependent zinc-containing endopeptidases. The enzymes are capable of degrading all kinds of extracellular matrix proteins, but also can process a number of bioactive molecules. The MMPs play an important role in tissue remodeling associated with various physiological or pathological processes, such as morphogenesis, angiogenesis, tissue repair, cirrhosis, arthritis, and metastasis. MMP-1 is thought to be important in rheumatoid arthritis and osteoarthritis. Dysregulation of the balance between MMPs and TIMPs is also a characteristic of acute and chronic cardiovascular diseases. Especially, MMP-1 are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis and metastasis. Specifically, MMP-1 breaks down the interstitial collagens, Types I, II, and III. MMP9 may play an important role in angiogenesis and neovascularization. For example, MMP9 appears to be involved in the remodeling associated with malignant glioma neovascularization. It is also a key regulator of growth plate formation—both growth plate angiogenesis and the generation of hypertrophic chondrocytes. MMP9 coordinates epithelial wound repair. TIMP1 plays a central role in extracellular matrix composition, wound healing, and pregnancy. TIMP1 is an inhibitory molecule that regulates MMPs, transcription of the TIMP 1 gene is highly inducible in response to many cytokines and hormones. In addition to an inhibitory role against metalloproteinases, the TIMP-2 has a unique role among TIMP family members in its ability to directly suppress the proliferation of endothelial cells. As a result, the encoded protein may be critical to the maintenance of tissue homeostasis by suppressing the proliferation of quiescent tissues in response to angiogenic factors, and by inhibiting protease activity in tissues undergoing remodeling of the extracellular matrix.
4.1 | MMP-9 as a potential marker for mitral valve regurgitation

In our study, we found MMP-9 expression in the diseased mitral valve tissues. This means an interesting point. The absence of MMP-9 expression in healthy human mitral valve tissues has previously been reported.\(^{13}\) MMP-9 expression in diseased tissues was also reported in another study conducted on tricuspid valves of dogs where MMP-9 was only expressed in chronically diseased tricuspid valves compared to the healthy valves.\(^{17}\) The increased expression of MMP-9 was also demonstrated in other heart diseases, such as myocardial infarction, atherosclerosis, and arterial hypertension.\(^{18}\) Our finding is, therefore, in accordance with the previous studies and is of particular interest. The exclusive expression of MMP-9 in the diseased tissue indicates the possible role it plays in the pathological remodeling processes in mitral valve regurgitation. The expression of MMP-9 in the mitral valve can therefore act as a suitable marker for the detection of the disease.

4.2 | The role of MMP inhibitors in mitral valve regurgitation

We obtained a significant positive correlation between the expression of MMPs and their inhibitors in diseased tissues. This result contradicts the general assumption of TIMPs as the specific inhibitors of MMPs as an inverse relationship is rather expected. Since TIMPs are also involved in performing many other functions, their direct role in the pathological process of mitral valve destruction thus cannot be ruled out in this case. Increased expression of TIMP-1 and TIMP-2 has already been associated with cardiac fibrosis and dysfunction in a previous study.\(^{19}\)

TIMPs also play a role in the activation of MMPs in addition to their inhibition, which can also explain the positive correlation observed between the expressions of MMPs and TIMPs.\(^{20,21}\) The other possibility of a positive correlation could be that the increased expression of MMPs stimulates the synthesis of TIMPs thereby inhibiting MMPs to prevent further degradation of the mitral valve. This hypothesis, however, has not been researched yet.

4.3 | MMPs as indicators of mitral valve regurgitation severity

We found a significant negative correlation between the expression of MMPs and the severity of mitral valve regurgitation from MI-Grade 1 to MI-Grade 3. Previous studies on cardiovascular diseases have, however, shown both a negative and a positive correlation.\(^{22,23}\) Our findings might be explained by the possibility that MMPs and TIMPs are expressed during mitral valve regurgitation in a dynamic destruction process whereby the high expression of MMPs in the early stages of the disease accelerates the destruction of the valve. During the later phases, however, when the destruction of the valve is nearly complete, the synthesis of MMPs and their inhibitors might play a less important role resulting in a decrease in their expression. A lower expression of the MMPs and TIMPs in such a case could, therefore, be used as a possible indicator of high mitral valve regurgitation severity.

4.4 | Varied MMP expression with etiology of mitral valve regurgitation

Our findings show a significantly increased expression of MMPs and their inhibitors in endocarditis etiology of mitral valve regurgitation compared to the degenerative form. A similar relationship between MMPs and their etiology was reported in previous studies where the MMP-12 gene was upregulated in the heart valves with infective endocarditis.\(^{24}\) Endocarditis is caused by a certain pathogen that leads to the local inflammation of the endocardium stimulating an immune response. As a result, monocytes and other inflammatory cells migrate to the valve tissues from the bloodstream.\(^{25}\) Inflammatory cells such as macrophages and certain growth factors also stimulate MMP synthesis.\(^{26}\) The increased expression of MMPs in infective endocarditis mitral valve disease can, thus, be explained to have occurred from a stimulated immune response. On the other hand, there is no stimulation of the immune response in degenerative heart valve disease. The increased MMPs expression in infective endocarditis may also indicate that a stronger remodeling of the extracellular matrix occurs resulting in a prominent mitral valve degradation compared to the degenerative form.

4.5 | Limitations of the study and future directions

In our study, the first-degree mitral valve regurgitation was under-represented resulting in the uneven distribution of the degrees of severity in the investigated tissue samples. This happened because mild insufficiencies are usually not treated with surgeries and hence the tissues were not available in the tissue archive. Similarly, the echocardiography findings were taken from the patient’s files instead of the images; we were, therefore, unable to perform a reassessment. We, however, tried to reduce this limitation by considering semiquantitative parameters such as the size of the vena contracta in addition to qualitative parameters such as the morphology of the mitral valve to access the degree of severity. In the case of the signal observation, the intensity signals of MMP-9 expression in tissues were independently assessed by two evaluators to increase the objectivity of the assessment. Despite that, evaluation errors cannot be ruled out because of subjective perception.

In the comparison made between the endocarditis and degenerative mitral valves, there is a possibility that endocarditis occurs in an already defective valve apparatus that has been previously defected by degenerative mitral valve disease.\(^{27}\) In such a case differentiating tissues based on the two types is not fairly possible. The comparison of myxoid and sclerosed subgroups of degenerative mitral valve regurgitation also presented a challenge as the histological separation of the two subgroups proved to be difficult. For our observation on
the MMP expression in the postoperative course, we only considered the time immediately after the operation until the discharge of the patient. In the future, investigating the change in MMP expression for long-term postoperation will rather be helpful in identifying the exact influence of MMPs on the prognosis of the disease.

In summary, our findings of the expression of MMP-9 exclusively in the diseased tissues as well as the correlation found between MMPs and the severity of mitral valve regurgitation indicate the importance of MMPs in the diagnosis of the disease. A further study of the measurement of MMPs expression with polymerase chain reaction or some other quantitative methodology will be helpful in confirming the results. MMP-9 expression together with echocardiography can hence help in improved diagnosis and better selection of the treatment offered. Furthermore, the therapeutic utility of inhibiting MMP-9 expression in the early stages of mitral valve destruction to stop the disease progression can also be considered. It should be examined in further studies. Herein, the correlation of the MMP blood levels with MMP tissue expression should be addressed. Assessment of the MMP expression in the mitral valve could additionally be relevant to the surgical therapeutic decision. As shown in the present study, this decision should not be based solely on the degree of severity but should also consider the etiology of the MI. The suitability of MMP inhibitor therapy as an alternative should be examined further. Meanwhile, we know about the beneficial effect of Losartan. It was noted to have antiapoptotic and antifibrotic effects on neurohormonal antagonists by transforming growth factor (TGF)-β inhibition. This opposes mitral valve fibrosis after myocardial infarction that is associated with excessive endothelial-to-mesenchymal transition, driven by TGF-β overexpression. Its reduction in leaflet thickness and favorable results of therapy are evidenced with modulation of profibrotic changes of tethered mitral valve leaflets without eliminating adaptive growth. 28

ACKNOWLEDGEMENT
Open access funding enabled and organized by Projekt DEAL.

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How to cite this article:IRQSUSI M, Mansouri AL, Ramaswamy A, et al. Role of matrix metalloproteinases in mitral valve regurgitation: association between the of MMP-1, MMP-9, TIMP-1, and TIMP-2 expression, degree of mitral valve insufficiency, and pathologic etiology. J Card Surg. 2022;37:1613-1622. doi:10.1111/jocs.16449