UNIVERSITY OF MEDICINE AND PHARMACY OF CRAIOVA
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THE ROLE OF METALLOPROTEINASES IN THE PROGRESS OF DILATED CARDIOMYOPATHY

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INTRODUCTION

Dilated cardiomyopathy (DCM) is mainly characterized by enlargement or dilation of the ventricular chamber with systolic dysfunction and increased thickness of the left ventricular wall, constituting the most common form of cardiomyopathy and largely a common cause of irreversible myocardial injury. It is induced by genetic or environmental factors and manifests itself clinically most often in the third or fourth decade of life, but also in young children. It has an estimated prevalence of 1/2500, and an incidence of 1 / 15,000-18,000 per year in adults and an estimated prevalence in children of 2/3 [20, 44].

DCM is characterized by remodelling and cardiac dysfunction, which is a major cause of heart failure [47]. From the physiopathological point of view, fibrogenesis is considered the pivotal factor that leads to maladaptation and heart failure [22]. The extracellular matrix (MEC) of the heart is a dynamic entity that undergoes constant fluctuation, and the integrity of its structure is maintained in balance by the function of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs). In DCM the levels of MMP and TIMP are altered, resulting in an imbalance between these two protein families.

In this study we aimed to investigate MMP and TIMP immunoexpression in DCM. The immunohistochemical characterization of the selected markers in relation to the histopathological characteristics, may lead to clarification of some aspects regarding the biological behaviour of the patients with DCM, in order to better stratify the patients to identify possible personalized therapies.

Keywords: dilated cardiomyopathy, histopathology, immunohistochemistry, matrix metalloproteinases

STAGE OF KNOWLEDGE

CHAPTER I. Epidemiology and etiology of dilated cardiomyopathy. The working group of the European Society of Cardiology defined DCM as a progressive and usually irreversible myocardial disorder, characterized by dilation of the left ventricle or both ventricles and global systolic contractile dysfunction, which is not explained by chronic loading conditions such as hypertension. and valvular disorders or coronary artery disease to cause a global systolic disorder [26]

Accurate data regarding the epidemiology of DCM are difficult to obtain, which makes the prevalence and incidence of the disease difficult to assess [28], because the diagnosis of the disease does not have strict clinical guidelines, the subclinical phase is asymptomatic and usually remains undiagnosed. In addition, DCM has a low frequency compared to the general population, with many reports establishing the diagnosis after autopsy [14]. In Europe, the incidence rate of DCM at autopsy is 4.5 cases per 100,000 people / year, and the clinical incidence is 6.95 per 100,000 people / year [28]. The disease is more common in men than women (3/1: men / women) and manifests clinically over a wide age range, but it occurs most commonly in the third and fourth decades of life [20 24].

Based on predominant organ involvement, the American Heart Association classifies DCM as a primitive cardiomyopathy when the disease only affects the myocardium and as a secondary cardiomyopathy when it is part of a disease that affects several organs [5]. The European Society of Cardiology classifies DCM into a genetic family form and a non-genetic family form [26]. Such differences in the classification of DCM have important clinical implications for the diagnosis, treatment and prognosis of the disease.

CHAPTER II. Extracellular matrix biomarkers with role in dilated cardiomyopathy.

To fully utilize the various therapeutic strategies, the diversity of mechanisms underlying DCM must be understood first, by studying the many biomarkers involved in disease progression. As a result, the discovery of genes and biomolecules that regulate cardiac remodelling will allow the development of new clinical therapies. Thus, the biomarkers involved in the onset and progression of DCM actually reflect the different pathobiological processes involved in the evolution of the disease, including: neurohormonal activation, oxidative stress, matrix remodelling, inflammation, etc. (Table 3).

Table 3. Biomarkers involved in the evolution on DCM [6, 13]

CATEGORIES OF BIOMARKERS	BIOMARKERS
Biomarkers of ECM Remodelling	MMP, TIMP, Collagen Peptides, Cardiotrophin-1
Neurohormonal biomarkers	Renin, Angiotensin, Aldosterone, Endothelin-1, Vasopressin
InflammationBiomarkers	C-reactive protein, natriuretic peptide type B, myeloperoxidase, galectin-3, chemerin, α-TNF, IL 1, IL 6, IL 18
Oxidative stress biomarkers	Nitric oxide, Oxygen free radicals
Other biomarkers	Tenascin-C, miRNA, TLR biomarkers

CHAPTER III. The role of matrix metalloproteinases and their inhibitors in the progression of fibrosis in dilated cardiomyopathy.

MMPs are a family of latent zinc and calcium enzymes, which when activated, are responsible for the clearance of ECM in many diseases [30]. Although ECM fibrosis is part of CDM pathology, its process of formation and progression is not fully understood. The progression of ventricular remodelling is mediated by MMP destruction of collagen and elastin, leading to hypertrophy and ventricular dilatation, the consequence of which could be severe congestive heart failure. MMPs involved in myocardial remodelling have been found to be collagenases (MMP-1 and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysin(MMP-3) type MMP (MMP-14) [21, 36, 37] (Table 4).

TABLE 4 Potentially relevant MMPs in DCM

Type	Subtype	Number	Substrate
Collagenase	Interstitial collagenase	MMP-1	Collagen I, II, III, VII and components of basal membranes
	Neutrophilic collagenase	MMP-8	Collagen I, II, III
	Collagenase3	MMP-13	Collagen I, II, III
Gelatinase	GelatinaseA	MMP-2	Gelatin, collagen I, II, III, VII and components of the basal membrane
	GelatinaseB	MMP-9	Gelatin, collagen I, II, III, VII and components of the basal membrane
Stromelysin	Stromelysin 1	MMP-3	Fibronectin, laminin, collagen III, IV, IX, and MMP activation
Membrane type MMP	MT1-MMP	MMP-14	Collagen I, II, III, fibronectin, laminin and proMMP-2 and proMMP-13 activation

Under normal conditions, the MMP / TIMP system is in a dynamic equilibrium, but under pathological conditions there is an over-expansion of MMP and decreased TIMP expression

[27]. Numerous MMPs and TIMPs have been shown to be associated with long-term prognosis in heart failure and DCM, but association with cardiac fibrosis has not been demonstrated [15]. TIMP binds to MMP and forms MMP-TIMP complexes that play an inhibitory control role on MMP. Several studies have highlighted the relationship between MMP and TIMP and that changes in the MMP / TIMP ratio correlate with hypertrophy and left ventricular dilation [48]. Loss of TIMP inhibitory control over MMP is correlated with progression of left ventricular remodelling through increased MMP activity, ECM proteolysis, and myocardial remodelling [34].

CHAPTER IV. Prognostic factors in dilated cardiomyopathy. To develop a more accurate prognosis of patients with DCM, a more precise stratification of the risk of death is required. Several studies on larger number of patients reported as the main predictors of DCM prognosis the morphological, clinical, hemodynamic and electrocardiographic characteristics, together with laboratory variables and the applied therapy [1, 11, 13, 25] (Table 5).

Table 5. Characteristics of the adverse prognosis in DCM

FEATURES	PARAMETERS
Clinical Features	Sex, Age, NYHA Class, History, Signs of Right Heart Failure
Ventriculographic characteristics	Decreased ratio of ventricular mass / volume, Overall anomaly of ventricular wall movement, Decreased ejection fraction of left ventricle, Large diastolic size of left ventricle, Expansion of right ventricle, Spherical geometry of left ventricle
Electrocardiographic features	Atrial fibrillation, Grade I / II AV block, Left branch block, Ventricular tachycardia
Hemodynamic characteristics	Cardiac index, Increased right atrial pressure, Low average blood pressure, Pulmonary capillary pressure> 20 mm Hg
Laboratory variables	Angiotensin II, Atrial natriuretic factor, Epinephrine (adrenaline), Norepinephrine (Noradrenaline)
Applied therapy	Angiotensin conversion enzymes, angiotensin II receptor blockers, β-blockers, heart transplant, etc.

OWN CONTRIBUTIONS

Motivation and purpose of the study. Identifying the relationships between the expression of the analyzed markers and the extent of the histopathologically evaluated changes may allow the elaboration of new criteria for stratifying the patients with this disease in order to apply effective therapies.

The specific objectives of the study include:

1. Extension of knowledge related to histopathological and immunohistochemical changes involved in DCM, in order to deepen the pathophysiology of the disease;
2. Identify the relationships between MMP expression and TIMP in DCM.
3. Establishing the usefulness of the immunohistochemical markers investigated in relation to the histopathological parameters of aggression of the DCM, in order to better stratify the patients to identify possible personalized therapies.

CHAPTER V. Material and methods. The study, a retrospective and prospective analytical type, included a number of 48 cases, which came from patients diagnosed with DCM that were hospitalized and died within the Cardiology Department of the Craiova County

Clinical Hospital. In addition, we added 6 cases of normal heart fragments collected during the autopsy of deceased patients without cardiac pathology.

• The histopathological study of the investigated cases aimed to identify the extent of changes in both myocytes and myocardial interstitium components. The extracted heart fragments were fixed in 10% buffered formalin, processed by the usual technique of inclusion in paraffin (the automatic BioOptica tissue processor), followed by 3-5 μm sectioning and staining with Hemalaum-Eosin (BioOptica kit), trichromic Masson (BioOptica kit), blue-alkali PAS (BioOptica kit) (BioOptica automatic coloring). The histopathological parameters were represented by:

- at the myocyteslevel: changes in myocytes volume, size and shape, as well as structural changes in the cytoplasm and nucleus;
- at the interstitial level: fibrosis, lipomatosis and inflammatory infiltrate.

For the semi-quantitative evaluation, we followed the assessment according to a 3-degree scale: grade 1 - focal ≤ 10%, grade 2 - zone 10-50%, grade 3 - diffuse ≥ 50%. We considered a low CHS for the value between 1-6 and high CHS for the cases with values between 8-12.

For the immunohistochemical processing, 46 cases were selected, represented by normal heart fragments (6 cases) and fragments from patients with the clinical diagnosis of DCM (40 cases) with low or high CHS. The immunohistochemical study was an enzymatic detection type using the LSAB-HRP (Labelled Streptavidin-Biotin2 System Horseradish Peroxidase, Dako, code K0675) technique. The antibodies used in this study are shown in Table 6 together with their clone and source, the dilution used, the disassembly mode and the tissue used for external control.

Table 6. Panel with antibodies used in immunohistochemical study

Antibody	Gene / Source	Dilution	Exposure	External control
MMP-1	EP1247y	1/50	Tris-EDTA buffer pH 9.0	Testicle
MMP-2	A-Gel VC2	1/50	Citrat, pH 6	Placenta
MMP-3	1B4	1/50	Citrat, pH 6	Prostate
MMP-8	Policlonal	1/50	Citrat, pH 6	Spleen
MMP-9	2C3	1/50	Citrat, pH 6	Lung
MMP-14	113 – 5B7	1/50	Citrat, pH 6	Breast carcinoma
TIMP-1	EPR18352	1/500	Tris-EDTA buffer pH 9.0	Liver
TIMP-2	3A4	1/500	Citrat, pH 6	Kidney

For the semiquantitative quantification of the analysed markers, we used a scoring system that evaluated the intensity of the reactions: score 3 (intense), score 2 (moderate), score 1 (weak). The scores obtained were then compared to the CHS values. Statistical analysis was performed using the Microsoft Excel software package (Microsoft Office 2007), using the test - "$\chi 2$" (square) to highlight the significant differences between the various groups.

CHAPTER VI. Results. The histopathological characteristics of the myocardium from the 48 patients who died from the diagnosis of DCM were varied, but quite non-specific.

Characteristics		Grade 1	Grade 2	Grade 3	Absent
Reduction of myocyte mass	No. of cases	**26**	**8**	**0**	**14**
	Percentage%	**54,2**	**16,3**	**0**	**28,5**
Atrophy	No. of cases	**29**	**8**	**2**	**9**
	Percentage%	**60,4**	**8,3**	**4,2**	**18,7**
Vacuolar degeneration	No. of cases	**19**	**11**	**3**	**15**

	Percentage%	**39,6**	**22,9**	**6,2**	**31.3**
Myocitolysis	No. of cases	**7**	**0**	**0**	**41**
	Percentage%	**14,6**	**0**	**0**	**85,4**
Mucin accumulation	No. of cases	**7**	**1**	**0**	**40**
	Percentage%	**14,5%**	**2,1**	**0**	**83,4**
Nuclear pleiomorphism	No. of cases	**22**	**5**	**2**	**19**
	Percentage%	**45,8**	**10,4**	**4,2**	**25**
Fibrosis	No. of cases	**11**	**27**	**10**	**0**
	Percentage%	**22,9**	**56,2**	**16,6**	**0**

The myocytes had a normal architectural disposition, but they showed changes in the volume of myocyte mass, size and shape, as well as structural changes in the cytoplasm and nucleus. The cytoplasm of myocytes showed numerous changes, such as: vacuolar degeneration, myocytolysis, reduction of myofibrils number, accumulation of lipofuscinic pigment or mucin. Myocyte nuclei also had modifications, both in shape and in tinctoriality. In addition, we analyzed several morphometric indicators in the normal heart and with DCM, in order to identify the indicators that can characterize the nuclear changes in the heart with DCM (nuclear area, nuclear perimeter, the ratio of diameters, nuclear roundness).
For the 48 cases of DCM taken in the study we calculated the composite histopathological score (CHS) by summing the values given to each analysed histopathological parameter (table 18).

Table 18. Distribution of casework according to the SHC

	Low SHC	**High SHC**
No. of cases	**39**	**9**
Percentage%	**81,2**	**18,8**

We analyzed a number of 46 cases immunohistochemically, represented by normal heart fragments (6 cases) and from patients with clinical diagnosis of DCM (40 cases) with low or high CHS. For all the cases taken in the study I followed the expression:
- collagenases: MMP-1, MMP-8
- stromelysins: MMP-3
- gelatinases: MMP-2, MMP9,
- membrane MMP -: MMP-14,
- tissue inhibitors of MMP: TIMP1 and TIMP2.

• Immunoreaction for MMP-1 was identified in all investigated cases, both in the normal heart and in those with selected DCM. We found that the increased intensity of the reaction for MMP-1 was present only in the stromal elements. Analysis of the intensity of MMP1 with respect to CHS indicated insignificant differences ($p = 0.081$, $\chi 2$ test). However, we found an association of the increased intensity of the markings with low CHS.
• Immunoreaction for MMP-8 was identified in all control cases as well as in all selected DCM cases. The intensity of MMP-8 immunoexpression was moderate in the normal heart compared to that with DCM in which the intensity of staining was moderate or increased. The analysis of MMP8 intensity in relation to the CHS level indicated significant differences ($p < 0.001$, $\chi 2$ test), the increased intensity of the markings being associated with low SHC.
• Immunocolouration for MMP-3 was identified in all cases of normal heart, as well as in all those with DCM. The pattern of MMP-3 expression was similar in the normal heart as compared to that of DCM, with immunophotography at the cytoplasmic level, only in myocardiocytes. Analysis of the intensity of MMP3 in relation to the CHS level indicated

insignificant differences ($p = 0.083$, $\chi 2$ test). However, we found an association of low and moderate intensity of low CHS markings.
• Immunoreaction for MMP-2 was identified in 31 of the cases with DCM (90%) analyzed and in all cases of normal heart. MMP-2 staining was present in myocardiocytes and stroma, with predominantly moderate intensity. The analysis of the MMP2 intensity in relation to the CHS level indicated significant differences ($p = 0.002$, $\chi 2$ test), the increased intensity of the markings being associated with low CHS.
• Immunoreaction for MMP-9 was identified in 37 of the selected DCM cases (92.5%) and in all cases of normal heart. The staining for MMP-9 was predominantly moderate in intensity, localized to both myocardiocytes and stromal cells. Analysis of MMP-9 intensity relative to the CHS level indicated insignificant differences ($p = 0.062$, $\chi 2$ test). However, we found an association of the high and moderate intensity of the markings with low CHS.
• Immunoreaction for MMP-14 was identified in 33 of the selected DCM cases (82.5%) and absent in normal heart cases. Staining for MMP-14 was present only in myocardiocytes, with predominantly moderate intensity. Analysis of MMP14 intensity relative to the CHS level indicated significant differences ($p < 0.001$, $\chi 2$ test), with low or moderate intensity of markings being associated with low SHC.
• Immunoreaction for TIMP-1 was identified in 34 of the selected DCM cases (85%) and in all cases of normal heart. TIMP-1 showed predominantly moderate intensity staining, located in the myocardiocytes and stromal cells. Analysis of TIMP1 intensity relative to CHS level indicated significant differences ($p < 0.001$, $\chi 2$ test), with moderate intensity of markings being associated with low CHS.
• Immunoreaction for TIMP-2 was identified in all selected DCM cases, as well as in all control cases. TIMP2 showed predominantly moderate staining in myocardiocytes and stroma. Analysis of TIMP-2 intensity in relation to CHS level indicated significant differences ($p < 0.001$, $\chi 2$ test), with moderate and high intensity of markings being associated with low CHS.

CHAPTER VII. Discussions.

Discussions on the histopathological study in dilated cardiomyopathy. Histopathological features of dilatative cardiomyopathy are nonspecific, the diagnosis being one of exclusion [7]. Therefore, the role of endomyocardial biopsy in the elaboration of the diagnosis of DCM is one of exclusion of secondary causes. Aspects described on endomyocardial biopsies from patients with DCM range from minimal differences in myocyte size to quite typical disease, with marked variations in myofibril size, myofibril loss, and interstitial fibrosis [7, 18]. In one study, the authors reported the association of interstitial fibrosis with hypertrophy of myofibrils, together with myocyte degeneration and atrophy in 60% of the cases of DCM investigated [2]. Myocardial atrophy is frequently mentioned among the histopathological features of DCM [18], being defined by the presence of elongated, thin myocytes with a narrow cytoplasmic border, their wavy appearance constituting an image indicating their overgrowth. Vacuolar degeneration has a low diagnostic value, but it may have important clinical implications depending on the content of the vacuoles, which electronically analysed can reveal their content and lead to the correct diagnosis [41]. One of the frequently mentioned changes in the myocardiocyte cytoplasm of DCM is the accumulation of lipofuscin pigment [9, 32, 35]. Nuclear pleiomorphism is commonly described in DCM, due to the presence of myocytes that have dysmetric and dysmorphic nuclei [3, 18, 29, 31]. The most typical change in DCM is the development of interstitial and perivascular fibrosis, of varying degrees [18, 33]. In DCM, fibrosis formation is a continuous process, in contrast to myocardial ischemia and hypertrophic cardiomyopathy, in which its formation stops after reaching a certain degree [4, 16, 42]. Myocardial fibrosis observed in dilatative cardiomyopathy is a reactive accumulation of

connective tissue in the interstitial space of the myocardium in order to fill the remaining space following the loss of myocytes [10].

Discussions on the immunohistochemical study on the expression of MMP and TIMP in dilative cardiomyopathy. The extracellular matrix (ECM) is responsible for the arrangement of cardiac cells and ensures the structural integrity of the myocardium. In normal myocardium ECM synthesis and degradation are balanced processes that are strictly controlled [45, 46]. In pathological processes there is an imbalance of the proteinase / antiproteinase systems, resulting in quantitative and qualitative changes of the ECM structure [48]. All four categories of proteinases are involved in proteolysis processes: serine, cysteine, aspartic proteinase and MMP. Because collagen is the major structural protein of ECM, collagenolytic MMPs play an essential role in cardiac remodelling.

Several studies have identified abnormalities of myocardial MMP expression and activity in DCM, as well as their association with progression of left ventricular remodelling [8, 12, 17, 39, 40, 49]. Studies on animal models have shown increased MMP production with the onset of left ventricular dilatation and may be an early event in DCM. Subsequently, DCM progression is characterized by a decrease in MMP activity due to increased TIMP production [22]. Of the 26 MMPs cloned and characterized in vertebrates, those involved in myocardial remodelling are MMP-1, MMP-3, MMP-8, MMP-13, MMP-2, MMP-9, MMP-12, MMP-28 and MMP membrane type (MT1-MMP / MMP-14) [40, 38, 23]. Usually, collagenases (MMP-1, MMP-8 and MMP-13) initiate the degradation process by cleaving all three chains of native α-collagen type I, II and III [19]. Gelatinases (MMP-2 and MMP-9) digest these products into smaller peptides which are then cleaved by nonspecific proteases [19]. In patients with end-stage DCM, myocardial tissue analysis from the left ventricle indicated decreased MMP-1 expression, increased MMP-3, MMP-9, TIMP-1 and TIMP-2 expression and no change in MMP-2 expression [43]. It is estimated that differences in expression between MMP and TIMP levels favour a persistent activation of myocardial MMP and probably contribute to the remodelling process of MEC from heart failure [39].

CHAPTER VIII. Conclusions.

The study included 48 cases of dilated cardiomyopathy and allowed the following observations:

Histopathological analysis revealed changes in both myocardiocytes and myocardial interstitium, the extent of which allowed the calculation of CHS:

• for myocardiocytes we observed a combination of hypertrophic, atrophic and normal myocardiocytes, frequently associated with cytoplasmic and nuclear changes.

• cytoplasmic changes were represented by hydropic degeneration (68.7%), myocytolysis (14.6%), cytoplasmic accumulation of lipofuscin (47.9%) and mucin (14.5%).

• the nuclear changes represented by the nuclear pleiomorphism were observed in 60.4% of the cases; the analysis of nuclear morphometric indicators such as nuclear area and perimeter did not reveal significant differences compared to the normal heart, as opposed to the nuclear roundness and the ratio of nuclear diameters that showed significant differences compared to the normal heart.

• at interstitial level we observed changes of fibrosis (100%), lipomatosis (25%) and rarely the presence of lymphocyte infiltrate (16.6%) and mast cell (10.4%).

• most of the cases corresponded to a low SHC, respectively 39 cases (81.2%), and only 10 cases (18.8%) presented with high CHS.

Immunohistochemical analysis aimed to analyse the intensity of expression of collagenases MMP-1 and MMP-8, stromelysinMMP-3, gelatinases MMP-2 and MMP9, MMP-membrane MMP-14, and tissue inhibitors have MMP, respectively TIMP1 and TIMP2.

• analysis of the intensity of the immunoexpression of MMP-1 and MMP-8 collagenases, in relation to CHS, showed no insignificant differences for MMP-1, with the association of the increased intensity of the markings associated in DCM with low CHS, as opposed to MMP-8 that showed significant differences, the increased intensity of the markings being associated with low CHS.
• identification of a signal with nuclear localization for MMP-1 opens a new field of research in cell biology.
• analysis of the intensity of MMP-3 stromelys in immunoexpression in relation to CHS showed insignificant differences, with an association of the low and moderate intensity of the markings with low CHS.
• analysis of the intensity of MMP-2 and MMP-9 gelatinase immuno-expression in relation to CHS, showed significant differences for both gelatinases, the increased intensity of markings being associated with low CHS for MMP-2, as opposed to MMP-9, which showed an association of high and moderate intensity of low CHS markings.
• analysis of the intensity of MMP-14 immunoexpression in relation to CHS, indicated significant differences, the low or moderate intensity of the markers being associated with low CHS.
• analysis of the intensity of TIMP-1 and TIMP-2 immunoexpression in relation to CHS, indicated significant differences, the moderate intensity of the markings being associated with low CHS.
• MMP and TIMP expression in the myocardium of patients with DCM is closely associated with myocardial remodelling and subsequent deterioration of cardiac performance.
• The MMP / TIMP ratio serves as a marker of fibrosis development in the extracellular matrix, with prognostic value in cardiovascular disease
• these correlations may be useful for a better stratification of the patients in order to evaluate the prognosis and to identify possible therapeutic targets.

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