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# MOLECULAR MECHANISMS WITHIN CHRONIC VIRUS-POSITIVE AND INFLAMMATORY DILATED CARDIOMYOPATHY

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# MOLEKULINIAI MECHANIZMAI, ĮTAKOJANTYS VIRUSINĖS IR UŽDEGIMINĖS KILMĖS LĖTINIŲ DILATACINIŲ KARDIOMIOPATIJŲ VYSTYMĄSI

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# 1. ABBREVIATION LIST

Activated partial thromboplastin time (aPTT)

Active memory T-lymphocytes ($CD45Ro^{+}$)

Adenosine triphosphate (ATP)

Adenovirus (ADV)

Adiponectin (APN)

American Heart Association (AHA)

Apoptosis protease-activating factor (APAF)

Aspartate aminotransferase (AST)

Atrial fibrillation (AF)

Bovine serum albumin (BSA)

Brain natriuretic protein (BNP)

Cardiac index (CI)

Cardiac resynchronization therapy (CRT)

Collagen I 1-α chain (COL1A1)

Collagen I C-terminal telopeptide (CITP)

Collagen type I (Col I)

Collagen type III (Col III)

Coronary artery disease (CAD)

Creatine kinase MB (CK-MB)

Cytomegalovirus (CMV)

Death-inducing signaling complex (DISC)

Dilated cardiomyopathy (DCM)

Endomyocardial biopsy (EMB)

Enterovirus (EV)

Enzyme linked immunosorbent assay (ELISA)

Epstein-Barr virus (EBV)

European Society of Cardiology (ESC)

Extracellular matrix (ECM)

Fas ligand (FasL)

Fas-associated death domain (FADD)

Fatty acid synthetase (Fas)

Haematoxylin and Eosin (H&E)

Heart failure (HF)

Heat shock protein-60 (HSP60)

Hemoglobin A1c (HbA1c)

Hepatitis C virus (HCV)

High sensitivity C-reactive protein (hsCRP)

High-density lipoprotein (HDL)

High-sensitivity troponin T (hsTnT)

Human herpesvirus type 6 (HHV-6)

Human immunodeficiency virus (HIV)

Human leukocyte antigen (HLA)

Hypertrophic cardiomyopathy (HCM)

Implantable cardiac defibrillator (ICD)

Inducible nitric oxide synthase (iNOS)

Inhibitors of apoptosis proteins (IAPs)

Interleukin-1 (IL-1)

Interleukin-6 (IL-6)

Left ventricle (LV)

Left ventricular ejection fraction (LVEF)

Left ventricular end-diastolic diameter (LVEDD)

Left ventricular end-diastolic diameter index (LVEDDI)

Low-density lipoprotein (LDL)

Macrophages ($CD68^{+}$)

Matrix metalloproteinase (MMP)

Matrix metalloproteinase-1 (MMP1)

Matrix metalloproteinase-13 (MMP13)

Matrix metalloproteinase-2 (MMP2)

Matrix metalloproteinase-9 (MMP9)

Messenger RNA (mRNA)

Mitochondrial outer membrane permeability (MoMP)

Natural killer cells (NK)

New York Heart Association (NYHA)

Parvovirus B19 (PVB19)

Platelet cells (PT)

Polymerase chain reaction (PCR)

Procollagen I C-terminal propeptide (PICP)

Procollagen I N-terminal propeptide (PINP)

Procollagen III C-terminal propeptide (PIIICP)

Procollagen III N-terminal propeptide (PIIINP)

Pro-interleukin-1 beta (IL-1β)

Prothrombin time (PT)

Pulmonary artery (PA)

Pulmonary artery pressure (PAP)

Pulmonary capillary wedge pressure (PCWP)

Pulmonary hypertension (PH)

Pulmonary vascular resistance (PVR)

Reactive oxygen species (ROS)

Receptor-interacting protein (RIP)

Red blood cells (RBC)

Regions of interest (ROI)

Relative light units (RLU)

Restrictive cardiomyopathy (RCM)

Right atrial pressure (RAP)

Right ventricular cardiomyopathy (RVCM)

Tissue inhibitor of metalloproteinase-1 (TIMP1)

T-lymphocytes ($CD3^+$)

TNF receptor-associated death domain (TRADD)

Transforming growth factor β1 (TGF-β1)

Tumor necrosis factor (TNF)

Tumor necrosis factor alpha (TNF-α)

Unclassified cardiomyopathy (UCM)

Varicella-zoster virus (VZV)

White blood cells (WBC)

# 2. INTRODUCTION

## *2.1. Background*

Cardiomyopathies constitute a group of myocardial disorders in which the structural and functional abnormality of the heart muscle is the dominant feature (in the absence of coronary artery disease, hypertension, valvular disease and congenital heart disease sufficient to cause the observed myocardial abnormality). Cardiomyopathies are classified into five morphological and functional phenotypes with distinct hemodynamic properties that can be caused by genetic and non-genetic mechanisms: dilated (DCM), hypertrophic (HCM), restrictive (RCM), arrhythmogenic right ventricular (ARVC) and unclassified cardiomyopathies (UCM) [1]. Dilated cardiomyopathy, mainly characterized by left ventricular chamber enlargement and impaired myocardial contractility, is the most common form of cardiomyopathy in both adults and children and currently is the most frequent indication for heart transplantation [2-4]. The management of patients with non-ischemic DCM can be extremely challenging with a disease course, difficult to predict. DCM occurs more frequently in men than in women, and is most common between the ages of 20 and 60 years [5]. The history and clinical features are often nonspecific. Clinical manifestation of inflammatory cardiomyopathy varies, with a broad spectrum of symptoms ranging from asymptomatic courses over presentations with signs of myocardial infarction to devastating illness with cardiogenic shock. Symptoms usually develop gradually, and some patients are asymptomatic despite left ventricular dilatation for months or even years. The most striking symptoms of DCM are those of left ventricular systolic failure, but right-sided heart failure may also occur and is associated with a particularly poor prognosis [6, 7]. The diagnosis of inflammatory DCM cannot be established without endomyocardial biopsy. Practical and specific serological markers are not available during the acute phase of the disease.

The recent progress in study on etiology of DCM has shown that viral infection, genetic abnormalities and autoimmune mechanisms are the major causes [8-10]. Starting from the early 1950s at least three distinct mechanisms were identified to explain the development of DCM. Currently the most accepted potential disease mechanism was developed in 1990s. It encompasses a slow, chronic, and continuous

destruction of cardiac myocytes or impairment of myocyte function, which is considered as a direct, albeit delayed, consequence of the initial viral infection and/or a virus-initiated immunologic process. Although viral infection has been already identified as a main cause of DCM, the heart can be also the primary target for bacterial, protozoa or parasitic infection. Moreover, it could be involved in the "collateral damage" of infective organisms (by toxins, chemokines or cytokines) and cross-reactive antibodies. A causal involvement of myocardial inflammation and viral agents in the pathogenesis of DCM is supported by the findings of myocardial viral genome persistence in DCM patients [1, 11-13]. The spectrum of the infectious agents that could be involved in the inflammatory cardiomyopathy varies with the geographic region, the patient's age, application of different therapeutic procedures, and additional diseases. Infective agents show a remarkable organotropicity: viral infections, toxic and auto-reactive processes primarily affect the myocardium and the pericardium [14].

The heart relies on a complex network of cells to maintain appropriate function. The contracting cells in the heart (cardiomyocytes) exist in a three-dimensional network of endothelial cells, vascular smooth muscle, and an abundance of fibroblasts as well as transient populations of immune cells. The connections of cardiomyocytes to the extracellular matrix (ECM) transduce the force and coordinate the overall contraction of the heart. The development of interstitial and perivascular fibrosis is a hallmark of pathology in the heart. Focal fibrosis composed primarily of collagen types I and III, occurs in the early stages of cardiomyopathy, but over time fibrosis increases and directly compromises the function of cardiomyocytes. The presence of collagen-rich regions in the myocardium cause disruption of excitation–contraction coupling between cardiomyocyte and increased stiffness of the myocardium, which leads to decreased contractility in the heart [15]. The different types of cell death, such as apoptosis, necrosis/oncosis, autophagy and proteasome degradation are contributing to the loss of cardiac myocytes depending on the intensity and origin of heart damage [16-18]. Given the complexity of the coordinated efforts of many proteins existing in multimeric complexes, and also complexity of inter-cellular signaling, dysfunction of heart occurs when these interactions are disrupted [19]. Although the heart may functionally tolerate a variety of pathological insults, adaptive responses that aim to

maintain function eventually fail resulting in a wide range of functional deficits or cardiomyopathy.

Myocardial inflammation has been identified as an important causal factor responsible for the progression to DCM [20-25]. This includes autoimmune, viral or post-viral inflammation, mediated by the native and acquired immune response (cytotoxic T-lymphocytes, natural killer (NK) cells and macrophages) [26-28]. The damage of myocardium is carried out by a loco-regional effects of inflammatory mediators such as inducible nitric oxide synthase (iNOS) and cytokines released by the infiltrated lymphocytes, macrophages and/or endothelial cells. The further heart damage is caused by direct interaction of the antibodies against the β- and other plasma membrane receptors, myolemma, mitochondrial and microsomal membrane or intra-organelle proteins [29-32]. Finally, certain toxins (alcohol, anthracyclines, cocaine, etc.) might impair the membrane transport mechanisms or biochemical processes also induce the loss or dysfunction of the matrix proteins such as dystrophin, laminins, etc. Initial myocardial insults are difficult to identify, however, they very often lead to an autoimmune response and development of inflammatory DCM [24]. DCM is a consequence of persistent heart exposure to various stress signals, including pro-inflammatory, viral, oxidative, neuro-hormonal, and other micro- or macro-environmental factors subsequently leading to chronic heart failure (CHF) [33]. The understanding of CFH development has been changed from a simplistic disease to a multisystem disorder affecting immune, musculoskeletal, renal, and other systems.

The application of molecular-biological, histological and immunohistochemical diagnostic techniques has identified the sub-groups of specific diseases which may better respond to certain therapies such as immuno-modulation, immuno-suppression or antiviral based on the underlying pathomechanisms [14, 34-37]. These new insights of the disease are mandatory allowing developing novel etiology-directed treatment strategies. Although significant progress has been achieved in the treatment of heart failure, as a result of cardiomyopathy, it is still a matter of fact that there are many patients with refractory heart failure that do not respond to available treatment. No doubt, that the best way to treat inflammatory DCM is to identify exactly and then eliminate the causes initiating heart injury. Since this is often a hard task, it is of high importance to understand the molecular mechanisms behind myocarditis progressing

to inflammatory DCM. This breakthrough could facilitate the search of the better treatment for the injured myocardium. As a proof of it, recent studies have provided evidence of a positive clinical impact of immunosuppressive therapy in up to 90% of patients with negative cardiac PCR for the main cardiotropic viruses [14, 36, 38]. Unfortunately, so far there are no unanimous agreements about the benefit of specific treatment in addition to conventional anti-heart failure therapy.

### *2.2. Hypothesis*

Molecular mechanisms ongoing in chronic dilated cardiomyopathy can differ depending on the intensity and origin of toxic exposures. Digital evaluation of cardiac fibrosis is more accurate and can replace the pathologist evaluation.

### *2.3. Aims*

The aim of this study was to establish molecular mechanisms dominating in virus-positive and idiopathic inflammatory dilated cardiomyopathy, and to develop and validate a tool for cardiac fibrosis quantification.

### *2.4. Objectives*

1. To analyze virus-positive and virus-negative DCM biopsies and serums and investigate:
   1.1. Induction of inflammation, fibrosis and molecular mechanisms of intra-myocardial cell death.
   1.2. Molecular mechanisms regulating extracellular matrix and fibrosis.
2. To analyze biopsies and serums of inflammation-positive and inflammation-negative sub groups and to:
   2.1. Estimate the release of inflammatory cytokines and infiltration of inflammatory cells in myocardium.
   2.2. Investigate role of intrinsic apoptotic pathway in cardiomyocyte death.
   2.3. Explore role of extrinsic apoptotic pathway in cardiomyocyte death.
   2.4. Investigate molecular mechanisms regulating development of cardiac fibrosis and changes of extracellular matrix.
3. To evaluate the accuracy of digital image analysis (Genie and Colocalization) and the pathologist's visual scoring for the measurement of fibrosis in human myocardial biopsies.

### *2.5. The novelty and significance*

Despite intensive search for therapeutic interventions, DCM remains the major cause of heart failure in the patients of relatively young age eventually leading to heart transplantation. Limited availability of donor hearts results in long waiting of transplantation. Many patients with end-stage of heart failure perish before a donor

heart becomes available.

The common proof of presence of the virus in the DCM myocardium is PCR analysis, whereas the inflammatory infiltrates in EMB supports the inflammatory origin of the disease. However, the data about the molecular mechanisms playing a key role in the pathogenesis of development of various origins of DCM are still missing. Therefore, the main goal of this study was to investigate in more details the molecular mechanisms in virus-positive and inflammatory-positive myocardiums. The identification of the processes dominating in DCM development and also the ability to regulate and control them is one of the main aims for clinicians. Additionally, the search of biomarkers for early identification of myocardial failure is not of less importance. Only the timely identification of secreted biomarkers being coincident with processes in the myocardium will allow us to improve diagnosis and proper treatment of heart preventing further destructive processes of DCM.

One more important objective of this study was to develop a tool to quantify the cardiac fibrosis and also to evaluate the immunohistochemical stainings of the EMB. As a matter of fact, so far the interpretation of histomorphometric parameters in most clinical routine analysis and research studies is still primarily based on human visual scoring, which is not only hugely subjective and involving a substantial workload on a pathologist, but also has many limitations inherent to the traditional pathology. Consequently, since the evaluation of fibrosis was important for the interpretation of DCM molecular mechanisms, the digital algorithms for fibrosis estimation were validated against a criterion standard obtained by point counting used in interactive stereology.

### *2.6. Cooperation*

This study was conducted in cooperation with the following science institutions:

1. Vilnius University, Faculty of Medicine, Department of Pathology, Forensic Medicine and Pharmacology, Vilnius, Lithuania.
2. State Research Institute, Center for Innovative Medicine, Dept. of Stem Cell Biology, Vilnius, Lithuania.
3. National Center of Pathology, Affiliate of Vilnius University Hospital Santariskiu Klinikos, Vilnius, Lithuania.

4. Vilnius University Hospital Santariskiu Klinikos; Hematology, Oncology and Transfusion Medicine Center, Vilnius, Lithuania.
5. Vilnius University, Faculty of Medicine, Department of Physiology, Biochemistry, Microbiology and Laboratory Medicine, Vilnius, Lithuania.
6. Universitätsmedizin Mannheim, Department of Integrative Pathophysiology, Mannheim, Germany.
7. University of Heidelberg, Medical Faculty Mannheim, Mannheim, Germany.
8. University Hospital Gießen & Marburg, Department of Cardiology, Marburg, Germany.
9. University of Normandy, Path-Image/BioTiCla, Unicaen, Caen, France.
10. Hull York Medical School, Departament of Cardiology, Castle Hill Hospital, Cottingham United Kingdom.

# 3. LITERATURE REVIEW

## *3.1. Suggested mechanisms of DCM*

A historically based literature review of the relationship between acute viral myocarditis and the subsequent development of DCM reveals an evolution of understanding of the DCM and starts in the early 1950s. The first scenario considered that DCM was a direct consequence of viral myocarditis and divided into acute or sub-acute phases [39]. The injury was predominated by the myocyte necrosis caused by both acute viral infection and the intra-myocardial inflammatory cell infiltrate in response to the acute viral infection [40-42]. This mechanism was undisputedly supported with direct causal relation of Coxsackie B virus (CBV) myocarditis and subsequent sub-acute or chronic development of DCM. DCM state was considered as a direct consequence of myocyte necrosis and subsequent myocardial scarring that is directly attributable to the CBV infection.

The second potential mechanism developed in 1990s encompasses a slow, chronic and continuous destruction of cardiomyocytes and impairment of cardiomyocyte function through continuous heart-specific inflammation carried out by the innate immune response. Postulated specific mediators of such a chronic process include protracted active viral infection and/or a virus-initiated immunologic process [43, 44]. The inflammation may persist because of mistaken recognition of endogenous heart antigens as pathogenic entities. Although the development of DCM is temporally remote from the acute bout of myocarditis, it must be considered as a direct, albeit delayed consequence of the initial infection along with auto-immune processes playing a key role [45]. This mechanism is still considered as the basis for further DCM analysis.

The third and the most recent mechanism identifies a distinct and indirect mechanistic possibility of DCM developing long after complete resolution of the initial episode of myocarditis. The complete resolution of the process implies the absence of residual necrosis, scarring, or left ventricular (LV) dysfunction. In this case DCM develops as a result of an undefined process in which an episode of remote viral infection renders the healed myocardium more susceptible to the remote development

of idiopathic DCM. While the first two mechanisms are supported by the literature, the third mechanism remains controversial while hard evidence for the development of an idiopathic DCM as a late downstream complication following complete recovery from a bout of myocarditis is still not clear [46].

### *3.2. Classification of inflammatory heart disease*

The gold standard for diagnosis of myocarditis and inflammatory DCM is endomyocardial biopsy (EMB). It is now recognized that the Dallas criteria are not sensitive because they do not consider the presence of viral genome in the heart [47]. The use of the new tools of immunohistochemistry and viral polymerase chain reaction (PCR) has provided a better identification of the aetiology of inflammatory DCM and renewed interest in the mechanisms of the inflammatory process in the heart [14, 22, 48].

Myocarditis is defined as inflammation of the heart after immunohistochemical detection of focal or diffuse mononuclear infiltrates with >14 leukocytes per 1 $mm^2$ ($CD3^+$ T lymphocytes and/or $CD68^+$ macrophages) independent of the clinical phenotype or the presence of heart failure or ventricular dilatation [13, 49-51]. If there is hemodynamic compromise together with cardiac dilatation (LVEDD [left ventricular end-diastolic diameter] >55 mm and the ejection fraction is <50 %, histological myocarditis is categorized as inflammatory DCM [49]. The classification of inflammatory heart disease, myocarditis and peri-myocarditis is based on several consensus documents on the classification of cardiomyopathies [4, 51], the American Heart Association (AHA) guidelines on heart failure [52], the European Society of Cardiology **(**ESC) guidelines on the management of pericardial and peri-myocardial disease [53] and current knowledge on the diagnosis and treatment of cardiac inflammation from peer review publications [6, 8, 34, 35, 47, 54-61].

On the basis of results from biopsy sample analysis of immunohistochemistry (IHC) and PCR for virus detection it is possible to identify distinct subentities of myocarditis and inflammatory DCM with possible ethiopatogenetic treatment options (Fig. 1).

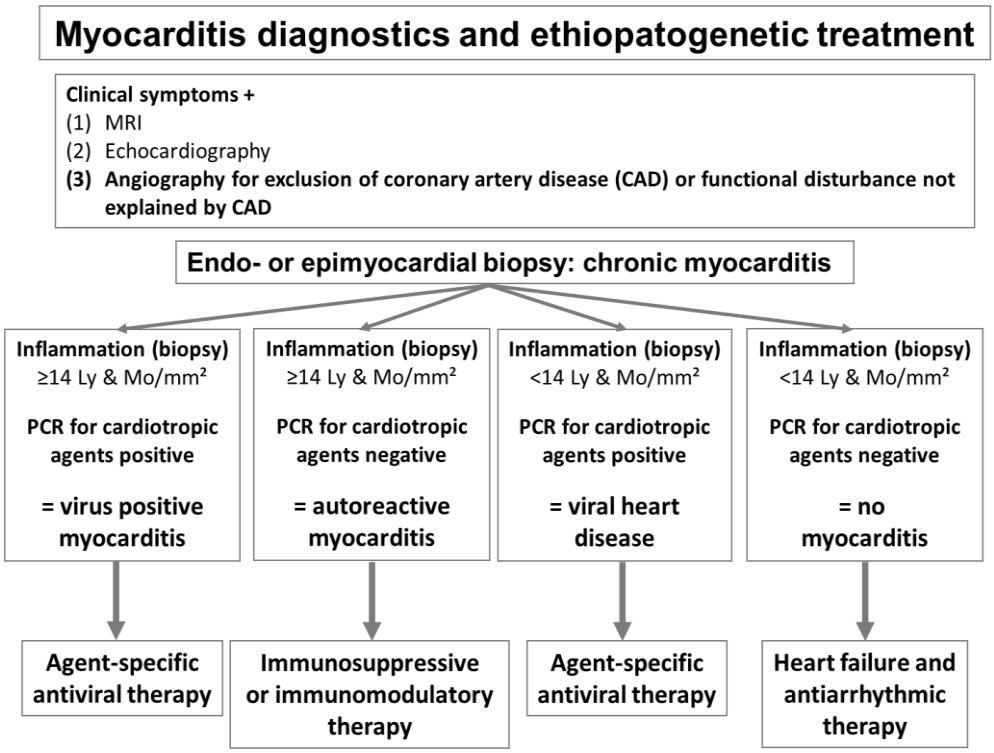


**Fig. 1. Myocarditis diagnostics and possible ethiopatogenetic treatment (adapted from [14]).**

### *3.3. The influence of virus genome on myocardium functioning*

Viral infection of the heart is recognized as an important cause of both acute and chronic heart failure [11, 12, 54, 62, 63]. It might be a main reason behind myocarditis with ensuing DCM [24]. A large variety of molecular mechanisms are suggested to initiate myocardial viral infection, yet most of them do not enjoy unanimous approbation [23]. On the other hand, some authors disagree with implication of viruses in development of DCM [64, 65]. The controversy might be explained by the variation of investigation models and different intensity of intramyocardial viral infection. The presence of viral genome in endomyocardial biopsy samples has also been reported in a subset of patients with idiopathic DCM even in the absence of classic histological myocarditis [12, 66]. Recent biopsy series in patients with DCM have revealed that long-term presence of cardiotropic viruses triggers heart failure: >70 % of patients with idiopathic DCM carry a cardiotropic virus in the heart [12, 61].

It is known, that coxackievirus, belonging to the enteroviruses, infect cardiomyocytes, B cells, $CD4^+$ T cells, macrophages and fibroblasts. In contrast to enteroviruses, all other viruses often detected in the human heart cannot infect myocytes, due to absence of the correspondent viral receptors. PB19 infects exclusively endothelial cells (this virus was found in endothelial cells of children and

adult patients with myocarditis). Herpesviruses including human herpesvirus type 6 (HHV6) and Epstein-Barr virus (EBV), which also do not infect cardiomyocytes, were detected in cardiac inflammatory cells (macrophages, T or B lymphocytes). Furthermore, it was found that cytomegalovirus (CMV) infects macrophages, fibroblasts and endothelial cells, whereas human immunodeficiency virus (HIV) affects $CD4^+$ T cells and macrophages [67].

Thus, numerous cardiotropic viruses do not damage the heart via cytolysis of cardiomyocytes but most likely via expression of cardiotoxic chemokines and cytokines from infected endothelial or immune cells, contributing to further attraction of potentially harmful immune cells into the heart. It is known that, for example, HHV6 may induce the expression of the proinflammatory cytokine IL-6 which is decisive for the invasion of T cells into infected organs [68]. Direct cytopathic effects and immune dysregulation induced by the viral myocarditis trigger cardiac dysfunction. Cardiotropic viruses are able to degrade cell-cell, cell-matrix, and intracellular elements. The proteases aim to facilitate the entry of the virus into cells, but also result in myocyte slippage, injury, and cardiac dysfunction [69]. The presence of viral genomes on endomyocardial biopsy is associated with subsequent worsening of heart function, the need for cardiac transplantation and sometimes even with a patient death [12, 50]. The intramyocardial virus can also be used to guide the treatment in acute and chronic inflammatory DCM [22].

### *3.4. Types of cardiotropic viruses*

The myocardium can be infected by a wide variety of viruses (Table 1).

If was established in 1950's that the main cardio-tropic viruses were coxackievirus A and B, but these were later replaced by enteroviruses (EV) and adenoviruses (ADV) [62]. With the development of molecular techniques (e.g. PCR) to examine endomyocardial tissue, many other viruses and viral co-infections have been recognized: case reports and series have associated DCM with approximately 20 viruses [12, 62, 66, 70]. At the turn-point in the year 1995, the prevalence of enterovirus decreased and soon this also happened with adenovirus. The recent day research revealed a new set of prevalent viruses: PVB19 followed by CMV, HHV-6, hepatitis C virus (HCV), EBV and only then the classic EV and ADV [10, 11, 47, 62,

64, 71-74]. Although the pathogenic role of enteroviruses in myocarditis and chronic DCM is well established, it remains unclear whether PB19 being currently the most commonly detected viral genome is incidental or pathogenic [12, 13, 50].

**Table 1 Cardiotropic viruses.**

| ***Viral agents of myocardium infection*** |
|---|
| **Adenovirus**; Arbovirus; **Coxsackievirus B**; **Cytomegalovirus**; Dengue virus; Echovirus; **Enterovirus**; **Epstein-Barr virus**; **Hepatitis C**; **Human Herpesvirus**; **Human immunodeficiency virus**; **Influenza virus;** Mumps; **Parvovirus B19**; Poliomyelitis; Rabies; Rubella; Rubeola; **Varicella**; Variola; Yellow fever. |

Most important viruses in DCM pathogenesis are in bold phase type.

Most humans are infected with **parvovirus B19** early in life without any major sequela. It was recently recognized that PB19 can cause myocarditis and either latent or active viral cardiomyopathy with high virus copy numbers in endomyocardial biopsies [75]. Mean numbers of viral copies detected in patients with inflammatory DCM were up to 50-fold higher when compared to hypertrophic cardiomyopathy [76]. In the recent PCR series PB19 has been observed in 30% to 67% of investigated endomyocardial biopsy samples of patients with DCM and myocarditis [11, 77, 78].

**Adenoviruses** account for 3–5 % of acute respiratory infections in children and less than 2 % of respiratory illnesses in adults. However, nearly 100 % of adults have serum antibody to multiple serotypes of this virus. The frequency of adenoviruses in PCR-positive DCM patients detected by nested PCR was from 1.6 % to 12.8 % [11, 76, 79]. The detection of ADV was associated with considerably reduced graft survival after cardiac transplantation in a pediatric population [80].

**Enteroviruses** (EV), in particular group B coxackievirus, have been detected in EMBs of myocarditis and DCM patients [81]. They have been linked to the transition from myocarditis to DCM and are considered to be important prognostic factors in DCM [82]. Despite the introduction of sensitive molecular biological methods for the detection of EV genomes, the incidence of EV infections in DCM was rather low, and thus, the hypothesized viral cause of DCM appeared to be confined to a small subset of DCM patients. The common rate of EV presence in the EBM is up to 9.4 % [11]. The evolvement of cardiac inflammation in EV infections is guided by viral cytotoxicity

and virus persistence.

In **herpesvirus** infections, the pathophysiology is rather determined by primary immune-mediated pathogenicity. Thus, herpesviruses including **Epstein-Barr virus** and **human herpesvirus type 6** infections rarely induce cardiac symptoms in immunocompetent patients. Prevalence for HHV6 genomes detected in patients with myocarditis or DCM ranged from 8 to 21.6 % and for EBV genomes from 0 to 8 % [11, 67].

The infections with **human cytomegalovirus** of unrecognized origin are common in childhood, and subsequently the majority of the adult population carry antibodies to CMV [83]. Primary infection after the age of 35 years, however, is uncommon and generalized infections usually occur in immunosuppressed patients only [84]. The frequency of this virus in PCR-positive DCM patients is from 0.8 % up to 3 % [11, 76].

The involvement of cardiac system in hepatitis is rare. There are contested data implicating **hepatitis virus C** infection as an etiologic factor in at least some cases of human viral cardiomyopathy [73]. In rare cases, fulminant myocarditis with congestive heart failure, hypotension, and death may occur.

Although clinically apparent myocarditis is rarely seen in **influenza**, the presence of preexisting cardiovascular disease greatly increases the risk of morbidity and mortality [85]. During epidemics, 5–10 % of infected patients may experience cardiac symptoms [86]. Postmortem findings in fatal cases include biventricular dilatation, with evidence of a mononuclear infiltrate, especially in perivascular areas. The frequency of this virus in PCR-positive DCM patients is up to 0.5 % [76].

Cardiac involvement occurs in up to 50 % of patients infected with **human immunodeficiency virus** (HIV). However, it leads to clinically apparent heart disease in only approximately 10 %. Congestive heart failure due to left ventricular dilatation and dysfunction is the most common finding in these patients [87].

### *3.5.Inflammatory infiltrates in DCM*

Although it is clear that viral genomes can be identified in a subset of patients with acute myocarditis and DCM, the impact of the presence of viral genomes on cardiac function and clinical outcome is still controversial. One clinical study showed the

association between viral persistence in the heart and progressive cardiac dysfunction [12]. In contrast, another clinical study reported that the presence of viral genomes per se could not be a predictor of cardiac death or heart transplantation in patients with clinically suspected myocarditis [50]. The latter clinical study further pointed out that the presence of inflammatory T cells and/or macrophages with enhanced expression of human leukocyte antigen (HLA) class II molecules in the heart can be a promising predictor of the clinical outcome even in the absence of viral genomes and Dallas criteria–positive findings [50].

The currently most accepted model is that viral infections trigger an inflammatory response leading to post-viral autoimmunity, chronic inflammation, cardiac injury and cardiomyopathy. The progression from acute injury to chronic DCM may be simplified into three phases (Fig. 2). Phase 1 is dominated by viral infection. Initial cardiomyocyte damage occurs in this phase by: direct viral damage, also by released perforins from cytolytic T cells and by many other ways. Acute cardiomyocyte injury leads to exposure of pathogens and intracellular sequestered antigens such as cardiac myosin or laminin and subsequent activation of the innate immune system. The innate immune response includes Toll-like receptor activation, release of cytokines, nitric oxide expression, and the recruitment of natural killer T cells [88]. The replication-deficient enterovirus may be capable of cleaving the host cytoskeletal proteins without activation of immune response [89]. Phase 2 is characterized by the onset of multiple autoimmune (innate and acquired) reactions, as innate immune system is activated to support phagocytosis of viral particles. In this phase the development of antibodies and antigen-specific T cells occurs. Antibodies to pathogens may cross-react with endogenous epitopes (cardiac myosin, laminin and β-adrenergic receptor) causing further damage to the heart. They key role is played by regulatory T helper cells, which mainly decide whether the process will be a self-limiting or go further to become auto-immune. Phase 3 is finalized by recovery or persistent cardiomyopathy. In most cases, the pathogen is cleared and the immune reaction is down-regulated with little or no lasting cardiac damage. However, in a minority of cases either the virus or the inflammatory reaction persists and contributes to further progression to cardiomyopathy with or without an infectious agent and cardiac inflammation [11, 14, 63, 88].

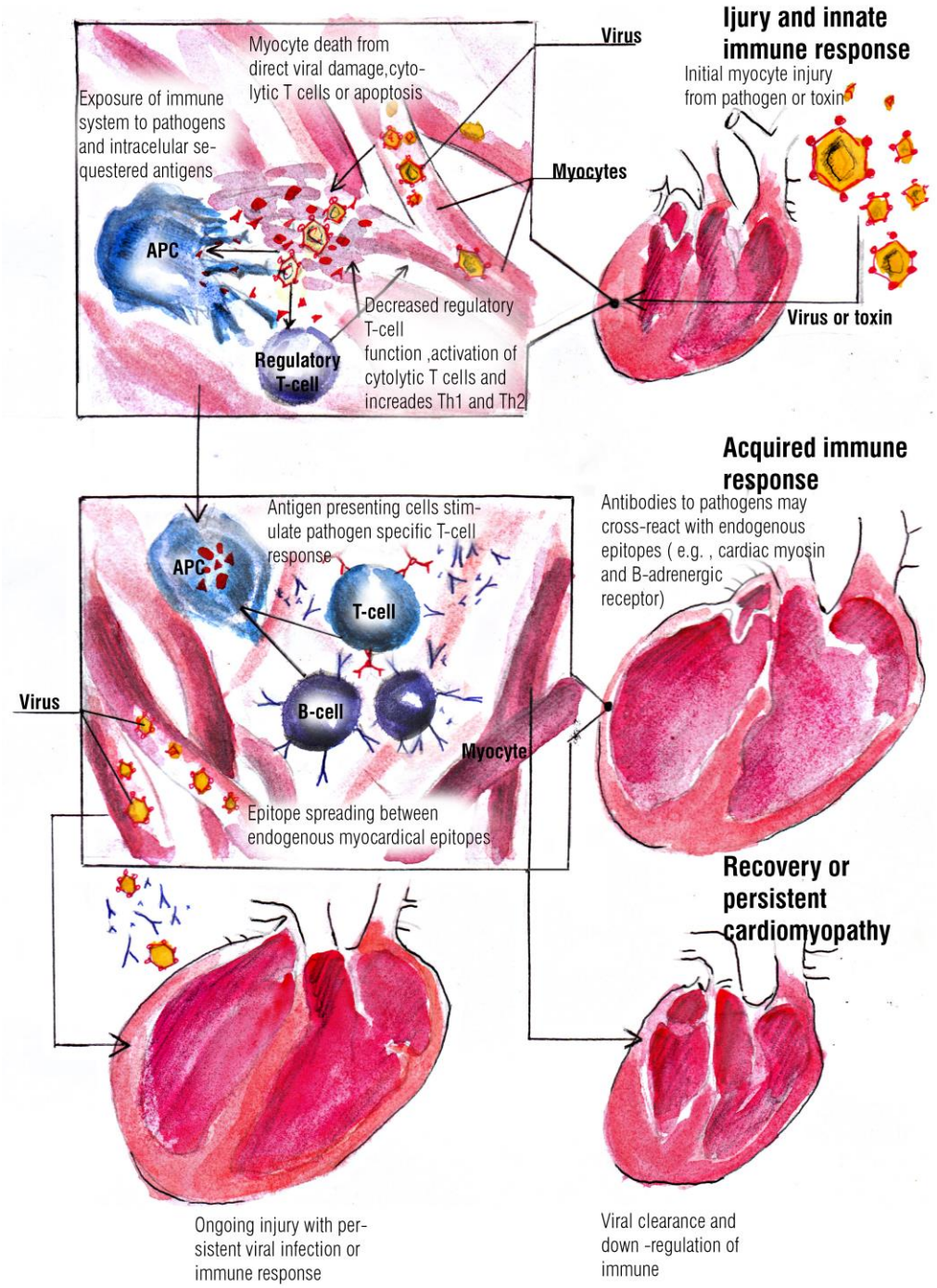


**Fig. 2. The suggested mechanism of DCM progression from acute injury may be simplified into a three-stage process. Acute injury leads to cardiac damage, exposure of intracellular antigens such as cardiac myosin, and activation of the innate immune system. Over weeks, specific immunity that is mediated by T lymphocytes and antibodies directed against pathogens and similar endogenous heart epitopes cause robust inflammation. In most patients, the pathogen is cleared and the immune reaction is downregulated with few sequelae. However, in other patients, the virus is not cleared and causes persistent myocyte damage, and heart-specific inflammation may persist because of mistaken recognition of endogenous heart antigens as pathogenic entities. Abbreviations: APC – antigen-presenting cell; Th1 – type 1 helper T cell; Th2 – type 2 helper T cell. Adapted from [90].**

The heart-specific inflammation may persist because of mistaken recognition of endogenous heart antigens as pathogenic entities. This phenomenon is explained through molecular mimicry, a chronic immune reaction, which may be stimulated by host antigens such as cardiac myosin or laminin (resembling pathogen proteins) long after the initial infection has been cleared [91]. Auto-antibodies can also cause powerful inflammatory responses leading to further damage of myocardium and progression of the heart dilation, thereby making viral myocarditis one of the main causes of DCM [92, 93]. Therefore, it is clinically important to identify biomarkers that may be predictive for early viral heart pathology.

### *3.6.Cytokines involved in progression of DCM*

In most cases, the initial myocardial injury activates the immune response rather than causes congestive heart failure [94]. Moreover, the low level of inflammation activates the immune system and fights tissue injuries, whereas inadequate level or duration of inflammation causes myocarditis and inflammatory DCM [95]. The accumulated inflammatory infiltrates in an infected myocardium may eliminate toxic agents and, on the other hand, insult properly functioning myocardium trough induction of apoptosis and other pathological changes [96-98].

Cytokines as regulators of the heart responses to toxic exposures, also contribute to either heart recovery or injury [99, 100]. Interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) are autocrine/paracrine compensatory agents and have been referred to as pro-inflammatory cytokines that are mainly initiated by activated immune system [101]. The high concentrations and/or long-term of stresses leading to uncontrolled expression of pro-inflammatory cytokines may be maladaptive and cause myocardial injury [101]. On the other hand, it was shown that pro-inflammatory cytokines are not constitutively expressed in the heart but are rapidly upregulated in response to cardiac stress and might function as an alarm system defending cardiomyocytes against low level of ischemic and other types of injuries [102, 103]. However, the possible molecular mechanisms dominating *in vivo* and progressing chronic inflammatory DCM are still under debate.

Transforming growth factor β1 (TGF-β1), a polypeptide member of the transforming growth factor beta superfamily of cytokines, is an important factor for

stimulation of fibroblast proliferation and synthesis of extra-cellular matrix molecules [104]. Inhibition of TGF-β1 induced epithelial-mesenchymal transition simultaneously reduces collagen synthesis in fibroblasts [105]. Recent studies also indicate that TGF-β1 is related to the dilated, ischemic and hypertrophic forms of cardiomyopathy through the activation of the TGF-β1-Smad pathway and stimulation of the collagen I gene promoter site [106]. Beside its involvement in collagen metabolism, TGF-β1 signaling participates in cell differentiation and even apoptosis [107, 108].

### *3.7.Involvement of hormones in regulation of DCM*

Adiponectin (APN) – a possible regulator of DCM development, is a protein hormone mainly produced by adipose tissue, though cardiomyocytes are also capable to produce APN by stimulating the APN receptors 1 and 2 [109]. APN mainly exhibits anti-apoptotic, anti-inflammatory and pro-angiogenic features regulating proper heart function [110-113]. On the other hand, APN can have pro-inflammatory, anti-angiogenic and anti-proliferating effects as well [114-117]. However, some studies advocate that high plasma adiponectin levels were associated with lower risk of myocardial infarction and coronary artery diseases, whereas other studies demonstrate correlation of high plasma adiponectin level with an increased risk of mortality in chronic heart failure patients [112, 118, 119]. So far, little is known about the relation between adiponectin and viral myocardial infections.

Brain natriuretic protein (BNP) is another hormone mainly produced by myocardium of the cardiac ventricular wall. The synthesis of BNP is up-regulated in the setting of volume expansion or pressure overload which leads to increased ventricular wall stretching often occurring in DCM [120]. The release of BNP results in improved myocardial relaxation and serves an important regulatory role in response to acute increase of ventricular volume by opposing the vasoconstriction, sodium retention and antidiuretic effects of the activated renin–angiotensin–aldosterone system [121]. BNP is widely used by clinicians as a diagnostic biomarker of severity of heart failure, left ventricular (LV) dysfunction as the circulating levels adversely increase in accordance with the degree of LV wall stretch [122, 123]. It also serves as an indicator of regional conditions and structural change in myocytes and also signals the risk of cardiovascular events and death [124]. BNP assessment is useful in

predicting the long-term risk of re-decompensation in non-ischemic DCM, even in low risk outpatients [125].

Galectin-3 is a member of a large family of β-galactoside-binding lectins with the size of 30 kDa. It is expressed and secreted mainly by macrophages at the phagocytic cups and phagosomes during the process of phagocytosis [126]. It can be localized in the cytoplasm and nuclei of the cells and can act both extra- and intra-cellularly [127, 128]. Galectin-3 interacts with various ligands located at the extracellular matrix, including laminin, collagen, synexin, and integrins [129, 130]. Extracellular galectin-3 mediates cell migration and cell-cell interactions, whereas intracellular galectin-3 regulates cell cycle and apoptosis [131]. Galectin-3 first came to attention in animal studies searching for potential mediators of decompensated heart failure. Galectin-3 is one of the most explored and promising heart failure biomarkers, a predictor of mortality, which appears to play an important role in the cardiac remodeling and is associated with development of myocardial fibrosis [132, 133].

Heat shock protein-60 (HSP60) is an intracellular protein (80-85 % of which is primarily located inside the mitochondria, with the rest found in the cytosol) constitutively expressed in the majority of cells [134, 135]. Its expression is up-regulated by a variety of stressors such as anoxia, oxidative stress, infection and inflammation [136]. HSP60 is known to have a protective role against stress-induced injury by maintaining cellular homeostasis and 3-dimensional structure of proteins [134, 135, 137]. In patients with heart failure, HSP60 has been reported to translocate to the myocardial cell surface before being released into the plasma [138, 139], also the increased levels of HSP60 in plasma membrane and serum have been paradoxically associated with an increase in myocardial apoptosis (caspase activation and DNA fragmentation) [140, 141]. Various studies have shown that serum HSP60 levels have been linked with endothelial dysfunction and higher risk of coronary heart disease [142, 143], pro-inflammatory status (increase TNF-α) [144], suggesting an important role in the activation of vascular cells and also the immune system. Serum HSP60 level is related to the severity of the disease and associated with a high risk of cardiac events in patients with advanced chronic heart failure [145].

Circulating troponin T is a very sensitive and specific biomarker of cardiomyocyte injury, and is used as the diagnostic and prognostic marker both in acute coronary

syndromes and heart failure (increased troponin T levels have been correlated with the severity and adverse outcomes) [146-149]. With the use of the standard assay for troponin T the elevations were detectable in only a small fraction of patients with heart failure, because the lower detection limit was only 0.01 ng/mL. With the recent development of the assay for a high-sensitivity troponin T (hsTnT) it became possible to measure concentrations about 10-fold lower than the lower detection limit of the previous standard assay [150]. In patients with heart failure, hs-TnT correlates with cardiac dysfunction (evaluated by echocardiography) and natriuretic peptides. The elevation of hs-TnT levels in heart failure may represent cardiac dysfunction due to minor and ongoing myocardial injury [151].

All previously mentioned data point out that the interaction of signaling pathways and molecules in regulation of viral and inflammatory DCM is very complicated. The understanding of these processes relies on complex cell-type, physiological and experimental condition-dependent processes, and, therefore not surprisingly, the investigation of molecular mechanisms of the heart damage and the finding of biomarkers identifying an early start of heart infection is of major importance.

### *3.8.Myocardial cell death mechanisms in DCM*

The different types of cell death, such as apoptosis, necrosis, oncosis, autophagy and proteasome degradation are contributing to the loss of cardiac myocytes depending on the intensity and origin of heart damage [16-18]. Adult cardiomyocytes are terminally differentiated, so cell loss is detrimental to cardiac function. Additionally, when cardiomyocytes are lost, deposition of collagen occurs. Ultimately, this decreases heart compliance, increases cardiomyocyte wall stress, and impairs ventricular relaxation. The debilitating loss of cardiomyocytes in DCM and heart failure is considered to occur mainly from apoptosis rather than from necrosis [152-154].

Apoptosis is considered as a vital component of various processes including normal cell turnover, proper development and functioning of the immune system, hormone-dependent atrophy, embryonic development and chemical-induced cell death [155]. The mechanisms of apoptosis are highly complex and sophisticated, involving an energy-dependent cascade of molecular events (Fig. 3). Some cells apoptose

through extrinsic pathways that involve death receptors such as fatty acid synthetase (Fas) or tumor necrosis factor (TNF) receptors; others have a default death pathway (known as the intrinsic or mitochondrial) that must be blocked by a survival factor such as a hormone or a growth factor (the withdrawal from serum is a classic way to initiate this pathway) [155] (Fig. 3).

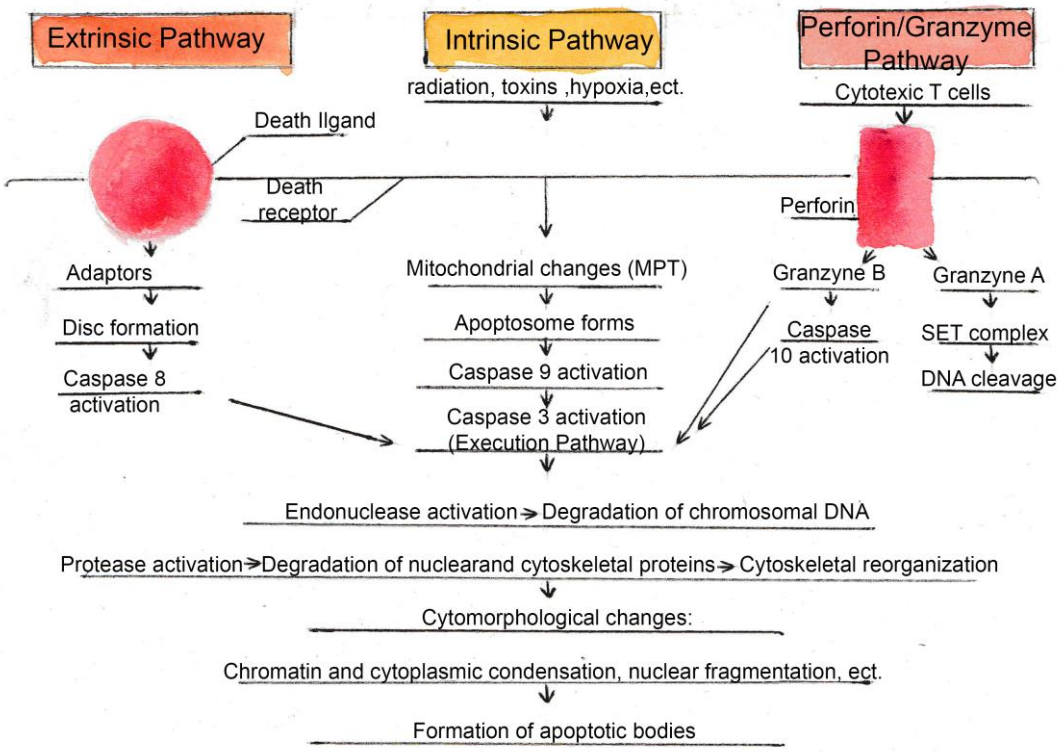


**Fig. 3. Mechanisms of apoptosis. A schematic view of the three main apoptotic pathways: the intrinsic pathway, the extrinsic pathway and the granzyme pathway. See text for explanation. Adapted from [155].**

However, it is also known, that the two pathways are linked and that molecules in one pathway can influence the other [156]. In the end, apoptosis is an energy-dependent process that involves the activation of a group of cystine proteases called "caspases" and involves a complex cascade of events that link the initiating stimuli to the death of the cell [155]. The two main regulatory mechanisms used by extracellular signals are either by targeting mitochondrial functionality (intrinsic pathway) or by directly transducing the signal via adaptor proteins to the apoptotic mechanisms (extrinsic pathway). There is an additional pathway mediating T-cell-induced cytotoxixity and perforin-granzyme (a serine protease) A or B-dependent killing of the cell (Fig. 3).

The mitochondrial pathway involves an increased mitochondrial permeability resulting in release in the cytosol of cytochrome c and small mitochondria-derived activators of caspases (SMACS) that bind to and deactivate inhibitors of apoptosis proteins (IAPs), repressing the caspases. Mitochondrial permeability is regulated positively or negatively by 25 members of the Bcl-2 family of proteins [157], under the control of the tumor suppressor protein p53. Cytochrome c and ATP released from the mitochondrial intermembrane space form the apoptosome consisting of ATP, apoptosis protease-activating factor (APAF), cytochrome c and caspase-9, which becomes activated by autoproteolytic cleavage and activates the execution caspase-3.-6 and -7, which leads to the collapse of cellular infrastructure [158]. The extrinsic pathway involves binding of trimeric ligands to their receptors which cluster (FasL to the FasR or TNFa to the TNR1). Binding of FasL to FasR recruits the adapter protein Fas-associated death domain (FADD), while binding of TNFα to the TNR1 recruits the adapter protein TNF receptor-associated death domain (TRADD). TRADD then recruits FADD and receptor-interacting protein (RIP). FADD forms a death-inducing signalling complex (DISC) with procaspase-8 resulting in its autocatalytic activation [155] and triggering of the "execution phase".

The extrinsic, intrinsic and granzyme B pathways converge on the same terminal "execution" pathway. This pathway is initiated by the cleavage of caspase-3 by caspases -8, -9 or -10 and results in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, cross-linking of proteins, formation of apoptotic bodies, expression of ligands for phagocytic cell receptors and finally uptake by phagocytic cells. The granzyme A pathway activates a parallel, caspase-independent cell death pathway via single stranded DNA damage [159].

Studies from human heart biopsies reveal that greater cell loss is observed in the early stages of cardiomyopathy, suggesting that anti-apoptotic pathways are up-regulated after cell loss has been initiated [160]. It follows that tight regulation of apoptosis is required for proper cardiac adaptation and that disruption of these pathways can have irreversible consequences in the heart. On the other hand, inflammation-initiated apoptosis plays an extremely important and positive role in the clearing of inflammatory infiltrates when they have completed their function [161]. Other authors marked the presence of apoptosis in inflammatory myocarditis or DCM

to be a problematic issue [162, 163]. Additionally to apoptosis, intramyocardial inflammation activates members of the matrix metalloproteinase (MMP) family, zinc-dependent endopeptidases, that impairs cell membrane permeability and cleaves extracellular matrix (ECM) resulting in increased levels of extra-myocardial apoptotic molecules, chemokines and cytokines [164-166].

### *3.9. The role of extracellular matrix in functioning of myocardium*

The heart relies on a complex network of cells to maintain appropriate function. The contracting cells in the heart (cardiomyocytes) exist in a three-dimensional network of endothelial cells, vascular smooth muscle, and an abundance of fibroblasts as well as transient populations of immune cells. The contraction of individual cardiomyocytes is coordinated electrochemically by gap junctions. The connections of cardiomyocytes to the ECM transduce the force and coordinate the overall contraction of the heart. Intracellularly, repeating units of actin and myosin form the backbone of sarcomeric structure, the basic functional unit of the cardiomyocyte. The sarcomere itself consists of around 20 proteins; however, more than 20 other proteins form connections between the myocytes and the ECM and regulate muscle contraction. Given the complexity of the coordinated efforts of the many proteins that exist in multimeric complexes, dysfunction occurs when these interactions are disrupted [19]. Although the heart may functionally tolerate a variety of pathological insults, adaptive responses that aim to maintain function eventually fail, resulting in a wide range of functional deficits or cardiomyopathy.

Cardiac fibrosis is associated with disruption of the normal myocardial structure by excessive deposition of extracellular matrix. The term fibrosis encompasses several processes including fibroblast proliferation, collagen synthesis and degradation as well as conversion of fibroblasts into a contractile “myofibroblast” phenotype. The development of interstitial and perivascular fibrosis is a hallmark of pathology in the heart. Focal fibrosis composed primarily of collagen types I and III, occurs in the early stages of cardiomyopathy, but over time fibrosis increase and directly compromises the function of cardiomyocytes. The presence of collagen-rich regions in the myocardium cause disruption of excitation–contraction coupling between cardiomyocyte and increased stiffness of the myocardium, which leads to decreased

contractility in the heart [15]. Fibrosis is primarily produced by resident fibroblasts in the heart however, there is evidence for collagen production also by cardiomyocytes [167].

The key player in regulating the normal myocardial function and in adverse myocardium remodeling is a cardiac fibroblast (Fig. 4).

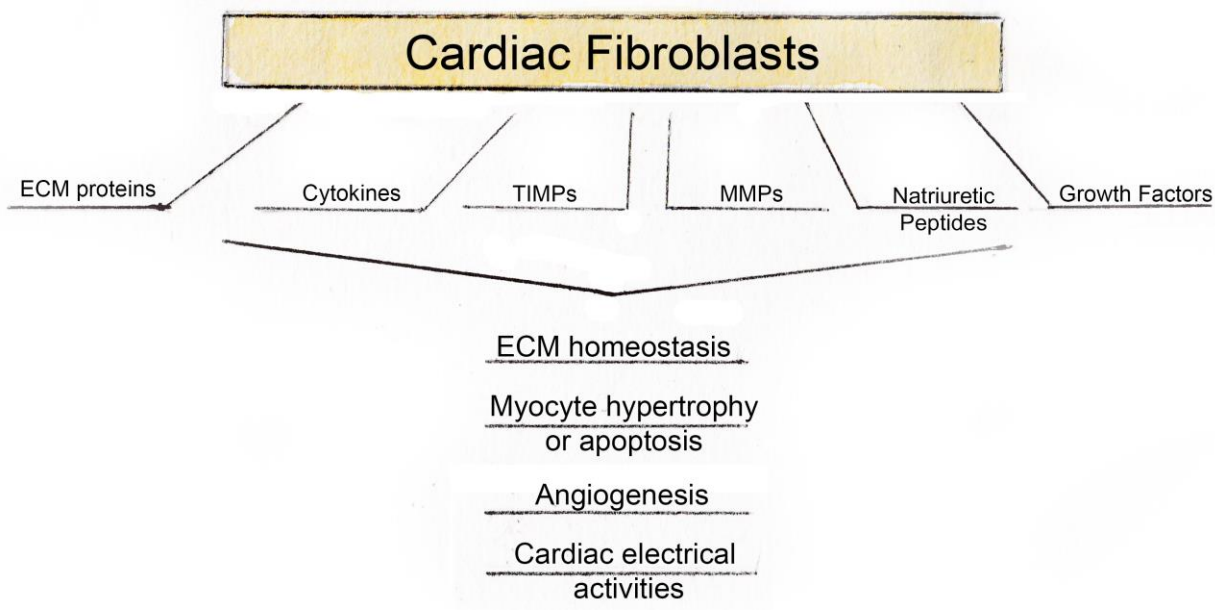


**Fig. 4. Pluripotent cardiac fibroblasts impact different aspects of cardiac structure and function. Cardiac fibroblasts can produce a number of active peptides (for example, cytokines, growth factors, peptides), extracellular matrix (ECM) proteins (collagens, elastin, fibronectin, and so forth), and ECM-regulatory proteins, matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs). As such, cardiac fibroblasts can impact molecular and cellular events that collectively determine cardiac structure and function. Adapted from [168].**

This most prevalent cell in the heart can transform in to myofibroblast, proliferate, secrete certain cytokines and growth factors and thus can alter extracellular matrix turnover through changes in matrix protein synthesis and degradation. While these changes in fibroblast function are an important adaptive response to altered environment that can aid myocardial recovery, they can also become maladaptive leading to pathological remodeling, fibrosis and heart failure. The cardiac extracellular matrix is composed of a collagen network that consists mainly of collagen type I (Col I) (85 %) and type III (Col III) (11 %) [169]. It provides architectural support for the muscle cells and also plays an important role in myocardial function and cardiac remodeling [170-172]. Collagen is synthesized by cardiac fibroblasts and is composed

of a triple helix, which generally consists of two identical chains (α1) and an additional chain that differs slightly in its chemical composition (α2) (Fig. 5).

Several studies have shown, that alteration of the collagen network, such as a differential increase in Col I or Col III, may play an important role in myocardial dysfunction in the failing heart [173, 174]. It was also noted, that in DCM collagen content was characterized primarily by an increase in the percentage of Col I [175, 176]. Col I represents a stiff fibrillar protein which provides tensile strength, and thus the increase in Col I protein levels may impose increasing myocardial stiffness, compromising diastolic and systolic ventricular function in DCM [177]. Col III forms an elastic network which stores kinetic energy as elastic recoil [178].

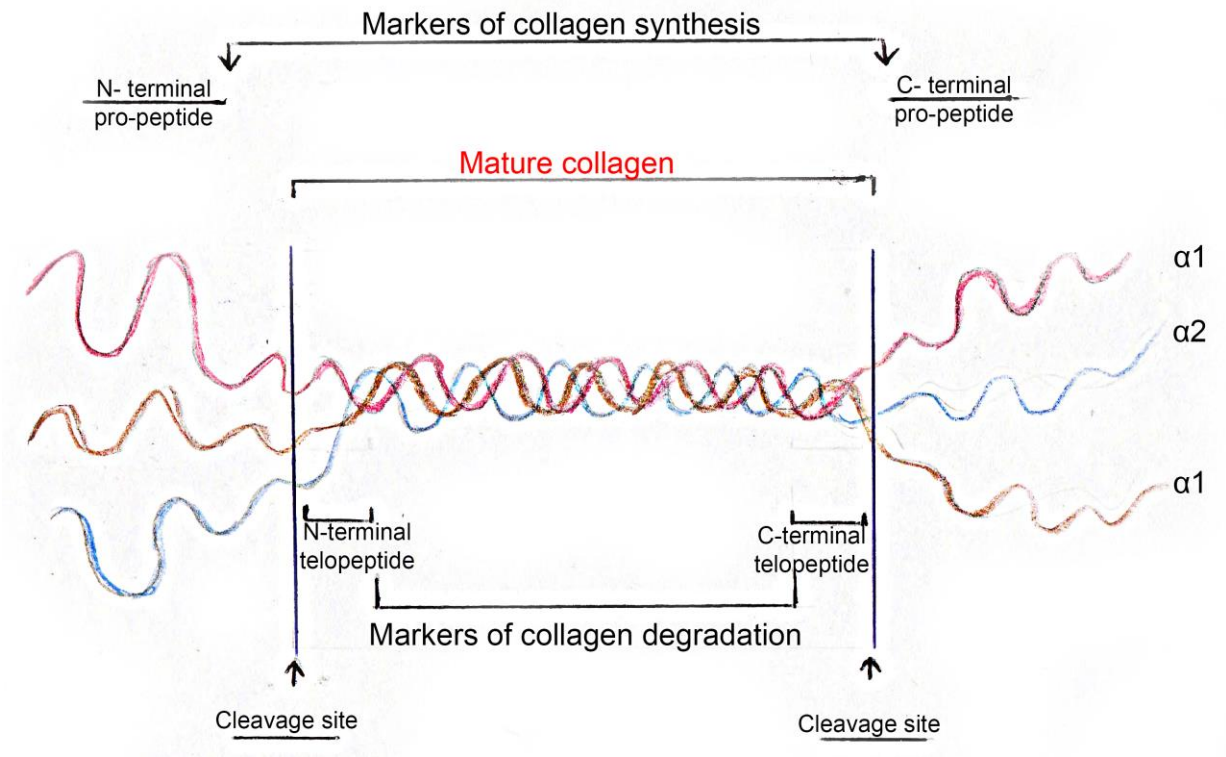


**Fig. 5. Structure of collagen molecule. Pro-collagen is comprised of two alpha-1 chains and one alpha-2 chain intertwined into a triple helix. Pro-peptide domains at the carboxy-terminals and amino-terminals are cleaved, resulting in formation of mature collagen. When collagen is degraded, during physiological turnover or pathological adverse remodeling, telopeptides (from the amino-terminals or carboxy-terminals) are cleaved and released into the plasma. Adapted from [168].**

There are many markers that can be used to characterize collagen synthesis or destruction during cardiac failure. For example, N-terminal (PINP), C-terminal (PICP) propeptides are markers of collagen I synthesis and positively correlate with proper

functioning of cardiac collagen [179, 180], whereas the C-terminal telopeptide (CITP) is a marker of collagen I degradation and is usually released during collagen cleavage by MMP1, MMP2, MMP9 and other endopeptidases in chronic heart lesions [181]. Propeptides of collagen III (PIIICP and PIIINP) are not completely cleaved during the conversion of procollagen III to collagen III and remain in the final fiber [182]. The composition of extracellular matrix, however, basically depends on a proper balance between collagen deposition and degradation and its dysfunction has been shown to correlate with myocardial fibrosis and development of heart failure [183].

The net effect of cardiac fibrosis is exaggerated by the increased tissue stiffness, impaired contraction due to myocyte slippage (separation), disrupted electrotonic connectivity and tissue hypoxia [184]. It is well known, that fibrosis and certain histological changes in the myocardium impact heart function and even survival [185, 186]. For these reasons, cardiac fibrosis and ECM biology remains an important target of therapy. However, the mechanistic basis of fibrotic cardiac remodeling in response to injurious stimuli that do not result in cardiomyocyte death still remains poorly understood.

### *3.10. Regulation of myocardial fibrogenesis by matrix metalloproteinases*

Maladaptive myocardial matrix remodeling and fibrosis are known to facilitate the structural and functional changes and appear to play a pivotal role in the development of ventricular dilatation and heart failure [187-189]. Matrix metalloproteinases, zinc-dependent endopeptidases, which are readily present in the myocardium and are capable of degrading all the matrix components in the heart, are the driving force behind myocardial matrix degradation during remodeling.

The MMP family consists of more than 20 enzymes, involved in the regulation of the extracellular matrix during physiological (development and organogenesis and pathological processes (inflammation and tissue injury) [190]. The MMPs can be divided into five classes according to their substrate specificity: 1. Collagenases (MMP-1, MMP-8 and MMP-13) cleave fibrillar collagens types I, II and III into smaller polypeptides, the gelatin; 2. Gelatinases (MMP-2 and MMP-9) further degrade gelatin, as well as collagen IV in the basement membrane; 3. Stromelysins (MMP-3

and MMP-10) degrade non-collagen matrix proteins, including proteoglycans, fibronectin, laminin and some other types of collagen; 4. Matrilysins (MMP-7, MMP-26 and MMP-11) apart from matrix components can also digest cell surface molecules; 5. Fifth group contains the new MMPs where substrate specificity is not yet clarified [191]. The expression of MMPs can be regulated at transcriptional level by multiple stimuli: inflammatory cytokines, growth factors, mechanical movement and phagocytosis. At the post-transcriptional level, secreted MMPs are synthesized and excreted as pro-enzymes (zymogens), which remain bound to matrix components until cleaved in order to be activated [192]. The complex interaction of MMP is presented in Fig. 6.

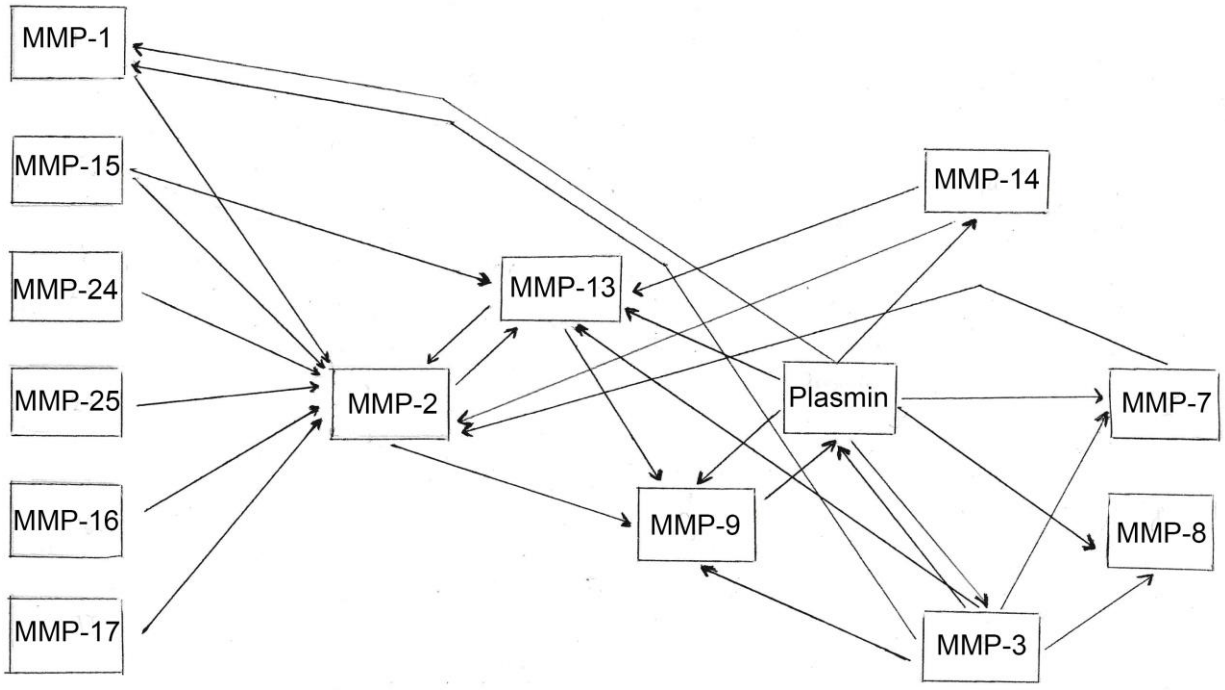


**Fig. 6. Mutual activation of MMP. Adapted from [193].**

The activity of MMPs is counterbalanced by their physiological tissue inhibitors of MMPs (TIMPs), which bind to their catalytic domain in a 1:1 relationship and thus block their action [191]. The interplay between MMPs and TIMPs therefore determines the progression of both ventricular dilatation and fibrosis in the diseased hearts. The increased levels of MMPs and decreased levels of TIMPs within the myocardium resulting ECM degradation have been shown in idiopathic DCM [194, 195]. There have been a large number of studies in various cardiac disease states that have showed abnormalities in MMPs and TIMPs expression within the myocardium.

These studies have identified that changes can occur in all known classes of MMPs, within the myocardium, leading to severe LV dysfunction [188, 189, 195-197]. The abnormalities in the expression and activity of myocardial MMPs have also been identified, as well as an association with the progression of LV remodeling. One of the first reports of abnormalities in MMP profiles in DCM was done by Gunja-Smith et al. [194], whereby increased MMP zymographic activity was associated with abnormalities in collagen cross-linking and overall matrix structure. Furthermore, increased plasma levels of collagen telopeptides indicating an increased matrix turnover have been reported in patients with DCM [198]. Finally, a number of past studies have showed the increased levels of certain MMPs such as the gelatinases, the matrilysins and the membrane-type MMPs in DCM [195, 199]. While associative, these past clinical studies have suggested that changes in myocardial MMP profiles can potentially accelerate the LV remodeling process, particularly LV dilation which is the architectural milestone in DCM. However, it must be recognized that induction of myocardial MMPs is not uniform, and that specific MMP sub-types specific to the underlying aetiology can be induced in LV remodeling process. For example, different profiles of MMPs exist in viral myocarditis as opposed to idiopathic DCM [195, 199].

### *3.11. Automated evaluation of cardiac fibrosis*

Evaluation of the extent of fibrosis, including semi-automated and semi-quantitative methods has been introduced earlier, however, detailed literature on methodological and technical aspects of quantification of fibrosis is scarce [200]. Most previous studies explored liver and kidney fibrosis [201-205], but up to now only a few have attempted to automatically quantify cardiac fibrosis [206-212]. Another limitation of recent publications is that the evaluations of cardiac fibrosis mostly have been done on animal models (mice, rats, dogs, pigs) and only few studies are on human hearts [213, 214]. Moreover, the majority of such studies lack data validation to an appropriate criterion standard and the reference values are obtained by semi-quantitative visual evaluations rather than by more direct quantitative estimates.

Significant drift towards automation and quantification in pathology has occurred during the last decade [215-217]. Digital imaging in pathology provides users with similar functionalities of a microscope, but with numerous additional benefits and

consequently, replaces subjective visual evaluation by presumably more objective and reproducible digital analyses [218-221]. Several applications of image analysis have recently received clearance from US Food and Drug Administration, indicating that automated quantification may provide more reliable and reproducible results than visual evaluation [215, 222]. Numerous recent studies show that advanced computer image analyses can be successfully introduced in clinical practice and research [223-225]. Meanwhile, the interpretation of histomorphometric parameters in clinical routine and research is still primarily based on human visual scoring, which is hugely subjective [220, 221]. Many factors affect human vision including: contrast, borders and color – all these impacts may be easily illustrated using a number of optical illusions. Semi-quantitative scoring not only involves a substantial workload on a pathologist, but also has several limitations inherent to the traditional pathology, such as significant intra- and inter-observer variation along with low efficiency [226].

Segmentation of stained tissue images is a complex problem, because of a large variability of the tissue samples (shape, size, color and architecture) [227]. Growing numbers of virtual slides that must be processed, transmitted and analyzed create a clear need of additional image correction and standardization algorithms [228]. Automatic selection of slides, application of appropriate thresholds and also a reliable selection of the slide areas containing the most significant information (regions of interest (ROI)) to deriving the diagnosis is becoming of major importance in virtual pathology [229]. Only a complete set of these computerized algorithms can eventually replace the pathologist's unique work [217, 230].

The most common practice of implementing a new digital algorithm is to compare the results obtained with the pathologist's visual evaluation, that is, to validate it against the best clinically accepted method. This perception, however, is no longer valid: why should one calibrate a potentially more accurate and precise tool against a variable and semi-quantitative evaluation method? To estimate the accuracy of a new method, a criterion standard has to be obtained from an independent source measured in the most possible objective way. In this regard, stereology grid count, rather than the pathologist's visual impression should be used [231-233]. Therefore, we performed this study on evaluating the accuracy of digital image analysis tools and the pathologist's visual scoring for the measurement of fibrosis extent (ie: area fraction) in

human myocardial biopsies, based on reference data obtained by point counting performed on the same images.

# 4. MATERIALS AND METHODS

## *4.1. Secreted and intramyocardial biomarkers in DCM*

### 4.1.1. Inclusion and Exclusion Criteria

Study subjects were 32 consecutive patients (25 males, 7 females, mean age 43.14 ± 11.86 years), admitted to a tertiary referral Centre with clinically suspected DCM and post-myocarditis cardiomyopathy during the time period of July, 2010 to February, 2013.

***Inclusion criteria:*** 1) Newly-onset heart failure (HF) of two weeks' duration associated with a normal-sized or dilated left ventricle (LV) and hemodynamic compromise; 2) Newly-onset HF of two weeks' to three months' duration associated with a dilated LV and new ventricular arrhythmias, second- or third-degree heart block, or failure to respond to usual care within one to two weeks; 3) Echocardiographic or angiographic evidence of global or regional LV dysfunction and/or LV dilation and/or LV reduced systolic LV ejection fraction less than 45 %; 4) Angiographic exclusion of significant coronary artery disease, defined as evidence of a proximal stenosis of one or more main coronary arteries of 50 % or greater, in one or more main coronary arteries.

***Exclusion criteria:*** 1) Known causes of heart failure, such as hypertension, significant coronary artery disease, valvular heart diseases, although not relative mitral regurgitation, endocrine disease, significant renal disease or drug or alcohol abuse; 2) Acute myocarditis and history of myocardial infarction; 3) Patients were excluded if they did not give written informed consent to EMB.

All patients above the age of 16 years (the oldest patient was 67 years old) who met the previously mentioned criteria were included in the study. There was no upper age limit for inclusion.

All patients were subjected to coronary angiography, right heart haemodynamic evaluation and endomyocardial biopsies. The same basic medical treatment scheme was applied to all patients.

### 4.1.2. Basic medical examinations

All patients were interviewed about their medical history and underwent a careful physical examination, as well as selected laboratory studies, including test of thyroid function, serum electrolytes (sodium, potassium), high sensitivity C-reactive protein (hsCRP), glucose, hemoglobin A1c (HbA1c), cholesterol, triglyceride, high-density lipoprotein (HDL), low-density lipoprotein (LDL), cardiac enzymes: creatine kinase (CK), creatine kinase MB (CK-MB), aspartate aminotransferase (AST), high-sensitivity troponin T (hsTnT), urea, creatinine, uric acid, coagulation tests: prothrombin time (PT), activated partial thromboplastin time (aPTT), blood count (hemoglobin, haematocrit, red blood cells (RBC), white blood cells (WBC) and platelet (PT) count.

On admission, each patient underwent anthropometrical and blood pressure measurement, as well as electrocardiography, echocardiography, MRI, Holter monitoring and spiroergometry. All laboratory measurements were done according to the routine clinical protocols.

### 4.1.3. Cardiac catheterization and endomyocardial biopsy

Before EMB, each patient underwent coronary angiography to exclude coronary artery disease as well as right heart catheterization to assess haemodynamic parameters: mean pulmonary artery (PA) pressure, pulmonary capillary wedge pressure (PCWP), pulmonary vascular resistance (PVR) and cardiac index (CI).

Right ventricular EMB was obtained using a flexible bioptome via the right femoral vein [234]. Biopsies were taken from the right inter-ventricular septum at three different levels (upper, medial and lower) from patients with confirmed absence of ischemia and cardiovascular pathology (stenosis and occlusion). Collected heart tissue biopsies were immediately inserted into clean cryovials, carefully labeled and registered. At least three EMBs from different septum levels were subjected to conventional histological and immunohistochemical evaluation, three EMBs to DNA and RNA extraction for the amplification of viral genomes and two EMBs were stored at -70°C in a biobank as retained biosamples and further processed for appropriate studies. Biopsy specimens were investigated within 24 hours.

Before measurements tissue samples were lysed in 100 µl of RIPA lysis buffer (Thermo Scientific Inc., USA), supplemented with protease and phosphatase mini

inhibitor tablets, 1 mM PMSF, 1 mM Na2VO4, 25 mM NaF according to the manufacturer's suggestion (Thermo Scientific Inc., USA). Biopsy samples were sonicated at 10 mV for 2 x 5 s on ice using a Bandelin Sonopuls sonicator, kept 30 min on ice, centrifuged at 12,000g for 15 min, aliquated and stored at -70°C.

### 4.1.4. Collection and preparation of blood samples

Three serum-separating (SST II) 8.5ml tubes (BD **Vacutainer**®) were collected for serum sampling from each patient at the same time as the EMB. Collected blood tubes were kept at room temperature for 30-45 min (no longer than 60 min) to allow clotting. Samples were centrifuged for 15 min at the manufacturer's recommended speed (1,000-2,000 RCF). The upper layer was carefully aspirated, checked for turbidity, aliquated into cryovials, labeled and stored at -70°C.

Before measurement, all serum samples were thawed on ice, centrifuged at 12,000 RCF for 5 min and, if necessary, appropriately diluted.

### 4.1.5. Detection of viral genome on endomyocardial biopsy

Genomic DNA and total RNA were extracted from endomyocardial biopsies using ZR-Duet™ DNA/RNA Miniprep kit (Zymo Research, Irvine, CA, USA). RNA (1 μg) was reversely transcribed in 20 μl reaction volumes using random hexamers and First Strand cDNA Synthesis Kit (Thermo Fisher, Vilnius, Lithuania) according to the vendor's recommendations and diluted up to 100 μl with deionized water after reaction. Nested PCR primers for the detection of adenovirus [235], Herpes simplex viruses 1 and 2, varicella zoster virus, Epstein-Barr virus, cytomegalovirus, parvovirus B19, hepatitis C virus, enterovirus, rubella virus [236], human herpes virus 6 (HHV-6A and HHV-6B, GenBank accession no. **NC001664.2** and **NC000898.1,** respectively), Kirsten rat sarcoma viral oncogene homolog (KRAS, GenBank accession no. **NM033360**) and ubiquitin C (UBC, GenBank accession no. **NM021009**) genes (Table 2) were synthesized by Metabion (Martinsried, Germany). Forward primers of the second round PCR were labeled with 6-carboxyfluorescein (FAM) at the 5′ end.

**Table 2 Oligonucleotides used in nested PCRs.**

| *Target* | *Primer* | *Sequence* | *Size (bp)* |
|---|---|---|---|
| HHV-6 | HHV6-N1Fw | ACCCGAGAGATGATTTTGCGTG | 128 |
| | HHV6-N1Rev | GCAGAAGACAGCAGCGAGATAG | |
| | HHV6-N2Fw | CATAGCAACCTTTTCTAGCTTTGAC | |
| | HHV6-N2Rev | TCTATAACATAAATGACCCCTGGGA | |
| UBC | UBC-N1Fw | TTCTTTCCAGAGAGCCGAAC | 150 |
| | UBC-N1Rev | CCCATCTTCCAGCTGTTTTC | |
| | UBC-N2Fw | TGGGTCGCAGTTCTTGTTTG | |
| | UBC-N1Rev | CCTTCCTTATCTTGGATCTTTGCC | |
| KRAS | KRAS-N1Fw | CTTTGGAGCAGGAACAATGTCT | 160 |
| | KRAS-N2Fw | AATCCAGACTGTGTTTCTCCCT | |
| | KRAS-N1Rev / N2Rev | TACACAAAGAAAGCCCTCCCC | |

All PCRs were run on a TProfessional Standard thermocycler (Biometra, Göttingen, Germany) as described [237]. KRAS and UBC detection was used to validate extraction of nucleic acids and was performed in parallel according to the conditions for DNA and RNR viruses, respectively. First round PCRs for DNA and RNA viruses were performed in a 50 µl reaction volume containing Maxima Probe qPCR Master Mix (Fermentas), 0.2 µM final concentration of each first-round primer, and 10 µl extracted DNA or cDNA solution. Uracil-DNA glycosylase (Fermentas) was added into each reaction mix (0.4 units) to prevent PCR cross-contamination. Amplification conditions for DNA viruses included uracil-DNA glycosylase treatment at 50 °C for 2 min; initial denaturation step at 95 °C for 10 min; 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 60 s; the final extension step was 72 °C for 7 min. Amplification conditions for RNA viruses included uracil-DNA glycosylase treatment at 50 °C for 2 min; initial denaturation step at 95 °C for 10 min; 35 cycles of denaturation at 95 °C for 45 s, annealing at 57 °C for 45 s, extension at 72 °C for 60 s; the final extension step was 72 °C for 7 min. Each primer pair was tested in a reaction setup without DNA sample (negative template control). Second round PCRs were performed in a 50 µl reaction volume containing Maxima Hot Start PCR Master Mix (Fermentas), 0.2 µM final concentration of each second-round primer, and 2 µl of first-round product. Amplification conditions for DNA viruses were the same as for the first round except excluded uracil-DNA glycosylase treatment step. Amplification conditions for RNA viruses included initial denaturation step at 95 °C for 10 min; 35 cycles of denaturation

at 95 °C for 20 s, annealing at 57 °C for 20 s, extension at 72 °C for 20 s; the final extension step was 72 °C for 7 min. Ubiquitin C PCR detection was used to validate extraction and was performed in parallel according the conditions for DNA viruses.

Final PCR products were 10-fold diluted and analyzed by capillary electrophoresis on a Genetic Analyzer 3130*xl* using GeneScan™ 600 LIZ™ Size Standard and Gene Mapper Software v4.1 (Applied Biosystems, Foster City, CA, USA) for sizing PCR fragments. In the case of positive result, the genomic DNA or RNR specimens extracted from whole blood samples were tested to exclude biopsy contamination with viruses circulating in the blood.

### 4.1.6. ELISA assays

Apoptotic and collagen synthesis/degradation protein levels in endomyocardial biopsies and serum samples were measured by specific ELISA assays. The following molecules were assessed: Bcl-2, Caspase-9, Caspase-8 (Novus Biologicals Europe, Cambridge, UK); Bax (Elabscience Biotechnology Co., Ltd, China); Caspase-3, TGF β1, matrix metalloproteinase-9 (MMP9), tissue inhibitor of metalloproteinase-1 (TIMP1), APO1/Fas/CD95, Fas ligand (FasL) (Invitrogen, Paisley, UK); Procollagen I C-Terminal Propeptide, PICP (Bio-Medical Assay Co., Ltd., China); Cross-linked Carboxy-terminal telopeptide of type I collagen, ICTP (Shanghai BlueGene Biotech Co., Ltd, China); Heat Shock Protein-60 HSP60 (AssayPro, Saint Charles, Missouri, USA).

Protein in serum and biopsy samples was measured using a modified Lowry Protein Assay kit according to the manufacturer's recommendations (Thermo scientific Inc., USA). Absorbance was measured with a spectrophotometer (Asys UVM 340 Microplate Reader UK - Biochrom Ltd.) set at 750 nm. A bovine serum albumin (BSA) standard curve was made to determine the protein concentration of each unknown sample. Protein concentration was expresses as μg/ml. Final concentration of searching molecules was expressed as ng/mg of protein.

### 4.1.7. Additional biochemical measurements

The pro-inflammatory cytokine TNFa, IL-6 and IL-1β in serum samples were assayed by solid-phase, chemoluminescent immunometric assays using IMMULITE/Immulite 1000 systems (Immulite, Siemens) according to manufactures

instructions: TNFa (Catalog No: LKNFZ (50 test), LKNF1 (100 tests); IL-6 (Catalog No: LK6PZ (50tests), LK6P1 (100 tests); IL-1β (Catalog No: LKL1Z (50 tests), LKL11 (100 tests).

Adiponectin was measured by Millipore Adiponektin assay according manufacturers' recommendations (Milipore, USA).

The myocardial necrosis marker, a high-sensitivity troponin T (hsTnT) was measured in serum using an Elecsys 2010 analyzer (Roche Diagnostics, Indianapolis, Indiana) and expressed as μg/ml.

Brain natriuretic protein (BNP) was measured by a two-step immunoassay in human plasma using CMIA technology and protocols referred as Chemiflex. Briefly, sample and anti-BNP coated paramagnetic particles were combined. After incubation, samples were washed and combined with an anti-BNP acridinium-labeled conjugate. Samples were incubated, washed again and the chemoluminescence initiating mixture was added. Resulting chemoluminescent reaction was measured as relative light units (RLU) by a chemoluminometer.

Galectin-3 in serum samples was estimated using an in vitro diagnostic device of enzyme linked immunosorbent assay (ELISA) on a micro plate according to the manufacturers' instructions (BG Medicine, Inc.).

### 4.1.8. Histology and immunohistochemistry of endomyocardial biopsies

EMB samples for histological analysis were fixed in 10 % buffered formalin and subsequently paraffin-embedded in a tissue processor. 3 μm-thick sections were used through the study. The EBM sections were stained with Haematoxylin and Eosin (H&E) according to the standard protocol for the routine histological evaluation. The experienced pathologist evaluated: endocardium (thickness, subendocardial fat, fibrosis and inflammation); myocardium (muscle fibre number, size and damage); interstitium (fibrosis, fat, edema and inflammation) and intramural vessels (size, signs of inflammation, damage and luminal stenosis). To estimate the extent of fibrosis the EMB specimens were stained with Masson's trichrome connective tissue stain according to a standard protocol. Keratin and muscle fibers stained red, whereas collagen stained blue.

Immunohistochemical staining was performed on fixed, paraffin-embedded material using antibodies: anti-CD3 (DAKO Hamburg, Germany), anti-CD45Ro (DAKO Hamburg, Germany) and anti-CD68 (DAKO Hamburg, Germany), anti-α smooth muscle actin (α-SMA) (Biocompare, USA); anti-MMP1 (Spring Bioscience Corp., USA); anti-MMP2 (Leica Biosystems Newcastle Ltd, UK); anti-MMP9 (Leica Biosystems Newcastle Ltd, UK), anti-MMP13 (Novus Biologicals Europe, Cambridge, UK), anti-HLA-DR (DAKO Hamburg, Germany), anti-PICP (EMD Millipore, Temecula, USA).

### 4.1.9. Evaluation of histochemical and immunohistochemical stainings in endomyocardial biopsies

Inflammatory infiltrates in the biopsies were immunohistochemicaly classified on tissue sections, according to expression of $CD3^+$ (T lymphocytes), $CD45Ro^+$ (active-memory T lymphocytes) and $CD68^+$ (macrophages). The number of positively stained cells in each biopsy sample was scored by a highly experienced pathologist and expressed as number of positive cells/$mm^2$. According to the World Health Organization / International Society and Federation of Cardiology Task Force on the Definition and Classification of Cardiomyopathies, EMB were considered to be inflamed after immunohistochemical detection of focal or diffuse mononuclear infiltrates with >14 leukocytes per 1 $mm^2$ ($CD3^+$ T lymphocytes and/or $CD68^+$ macrophages) in the myocardium [13, 50, 51].

Additionally myocardial necrosis was estimated by routine histochemical staining of fixed biopsy samples with Haematoxylin and Eosin (H&E). Normal myofibres had peripheral nuclei, intact sarcolema and non-fragmented nuclei. Pyknosis of muscle fibers nuclei, edema, and beginning of leuco-diapedesis from the capillaries suggested that these myocardial cells reached the stage of necrosis. A pathologist scored the number of necrotic myofibres on at least three independent tissue sections.

Digital images from the experimental glass slides were obtained using ScanScope Digital Slide Scanner (Aperio, Vista, CA) at x20 magnification and archived on a devoted Spectrum Server 11.1.0.751 (Aperio). Quality control of the scanned images and all further analysis were performed using ImageScope V11.1.2.760 (Aperio) and WebScope V11.1.0.756 (Aperio).

Genie algorithm was used to measure the extent of fibrosis, to calculate immunostained cells and to evaluate the area of immunohistochemically stained elements in the myocardium. Genie (GENetic Imagery Exploration [238]) is a pattern recognition algorithm that distinguishes spatial and morphological features based on structures (classes) provided by the user. The algorithm was run for the whole slide, ignoring the number of overlapped tissue sections on it – making the process fully automated. Specific Genie classifiers were developed for this study:

1. Myocardial fibrosis identification was made by using basic tissue recognition Genie classifier v1 algorithm. Total cardiac fibrosis (including interstitial and perivascular) was assessed. For better identification fibrosis, we used only spatial recognition, disabling the detection of morphological features. For this purpose, the Genie system was trained to distinguish the myocardium, fibrous tissue (fibrosis) and glass (Fig. 7). Total cardiac fibrosis percentage was adjusted to a total tissue area in the image analyzed, ignoring the glass.

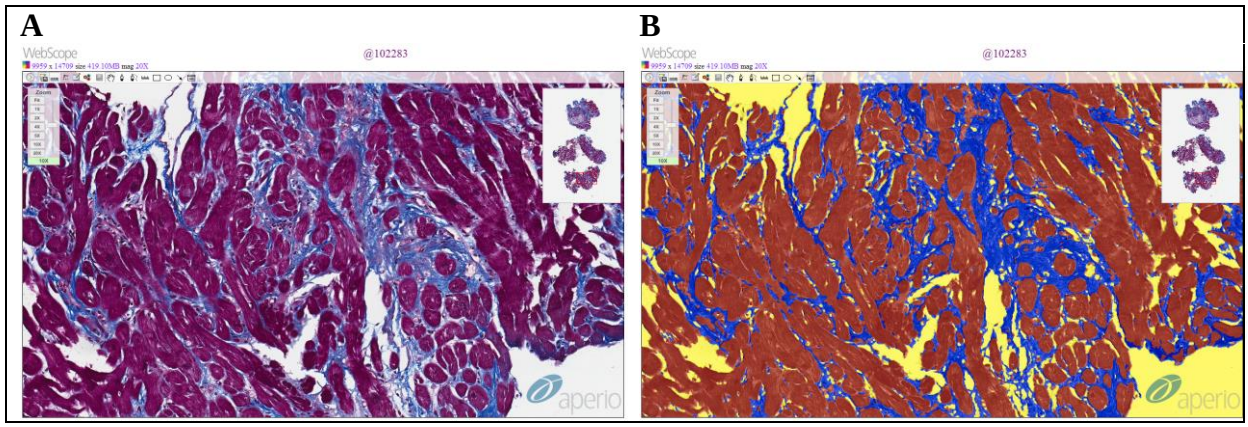


**Fig. 7 Fibrosis mark-up on digitized slide at x10 magnification. (A) Masson trichrome original staining, (B) Genie algorithm.**

2. For counting activated immune cells (HLA-DR) and cardiac fibroblasts (α-SMA) in myocardium tissue Genie Nuclear v9.0 algorithm was used. This algorithm distinguishes and counts immunostained positive and negative cells (Fig. 8). Depending on parameters it can mark different size and shape cells. Thus, in order to get accurate analysis, for different immunocell types, different Nuclear v9.0 parameters were set. After analysis the results were shown as total amount of immunopositive cells in the tissue area measured and expressed as cells/mm$^2$.

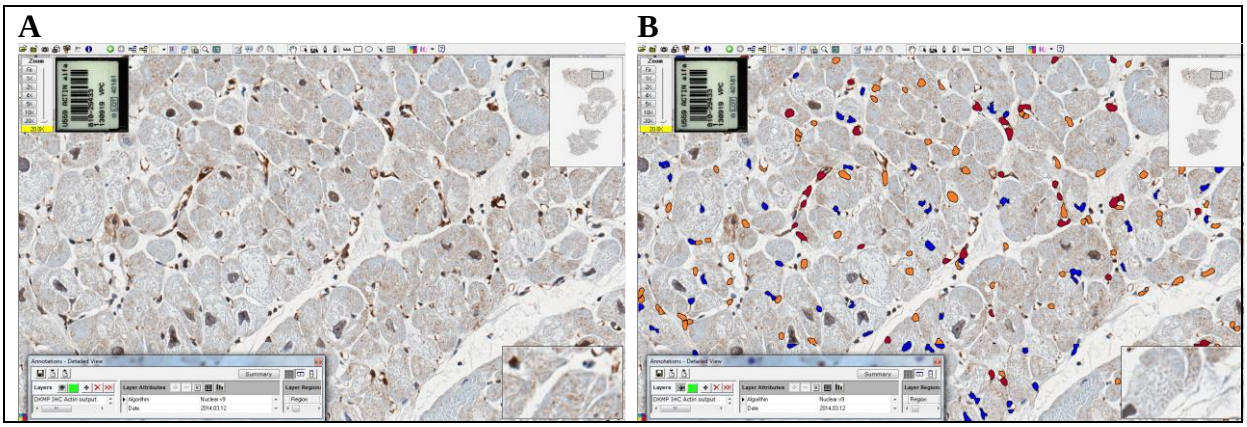


**Fig. 8 Immunopositive cell mark-up on digitized slide at x20 magnification. (A) α-SMA staining, (B) Genie algorithm.**

3. For measuring immunostained proteins (MMP1, MMP2, MMP9, MMP13 and PICP) in myocardial biopsies Genie pixel counting Algorithm was used. Firstly on digital slide the region of interest (myocardial biopsy) was marked and after that the algorithm was ran in this area. The results were shown as total amount of weak/ moderate/ strong positive and negative pixels (Fig. 9). For each protein a sum of weak, moderate and strong positive pixels was calculated as "positive". After that the percentage of positive pixels were counted manually and adjusted to a total tissue area.

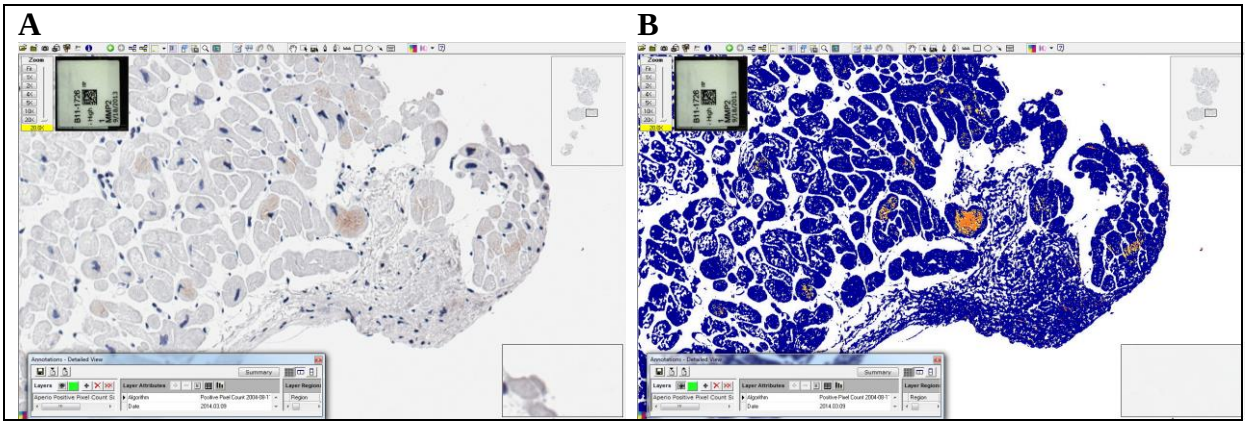


**Fig. 9 Immunostained protein mark-up on digitized slide at x20 magnification. (A) MMP2 staining, (B) Genie algorithm.**

## *4.2. Quantification of myocardial fibrosis by digital image analysis and interactive stereology*

### 4.2.1. Experimental model

The study was conducted on endomyocardial biopsy (EMB) material from 38 patients (29 males, 9 females, mean age 42.3 ± 12.2 years) diagnosed with inflammatory dilated cardiomyopathy. All EMB specimens were collected between July, 2010 and February, 2013. Before EMB, each patient underwent coronary angiography to exclude coronary artery disease. Right ventricular EMB was obtained using a flexible bioptome via the right femoral vein [22]. At least 3 EMBs were subjected to histological evaluation. All specimens were included in the study to provide a full range of fibrosis.

Tissue samples for histological analysis were fixed in 10 % neutral buffered formalin with subsequent routine paraffin embedding. 3 μm-thick sections were used through the study. Sections were stained with Masson's trichrome according to a standard protocol. Whole slide images (WSI) from the experimental glass slides were obtained at a resolution of 0.5 μm using a digital microscopic scanner (ScanScope® XT, Aperio Technologies, Vista, CA, USA) at a 20x objective magnification and stored in a tiled Tiff format on a devoted WSI server (Spectrum 11.1.0.751, Aperio) (Fig. 10). One section was later randomly chosen from the slide for all subsequent analyses. Aperio Colocalization and Genie algorithms were used for image analysis.

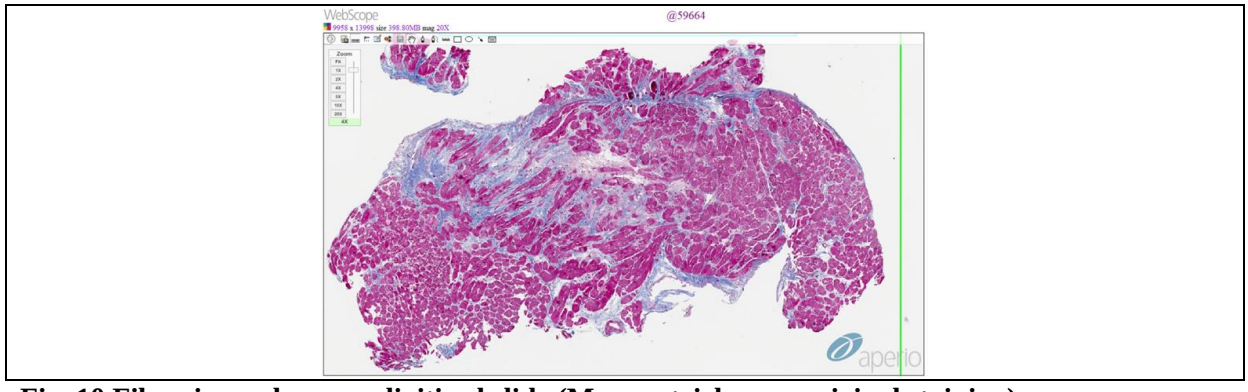


**Fig. 10 Fibrosis mark-up on digitized slide (Masson trichrome original staining).**

### 4.2.2. Colocalization algorithm

Colocalization uses the color deconvolution [239] to separate the stains and classifies each pixel according to the number of stains present. For Colocalization, the

threshold for each stain is specified for a required stain (e.g. Masson's trichrome) and the algorithm reports the percentage of total tissue area for which each stain combination is detected: 1, 2, 3, 1+2, 1+3, 2+3, 1+2+3, or none (up to 3 stains are supported). The algorithm also provides an eight-color mark-up image for the visualization of the colocalized stains. The total percentage of cardiac fibrosis in biopsy samples was calculated according to the sums of the following stain combinations: 3, 2+3 and 1+3 (Fig. 11).

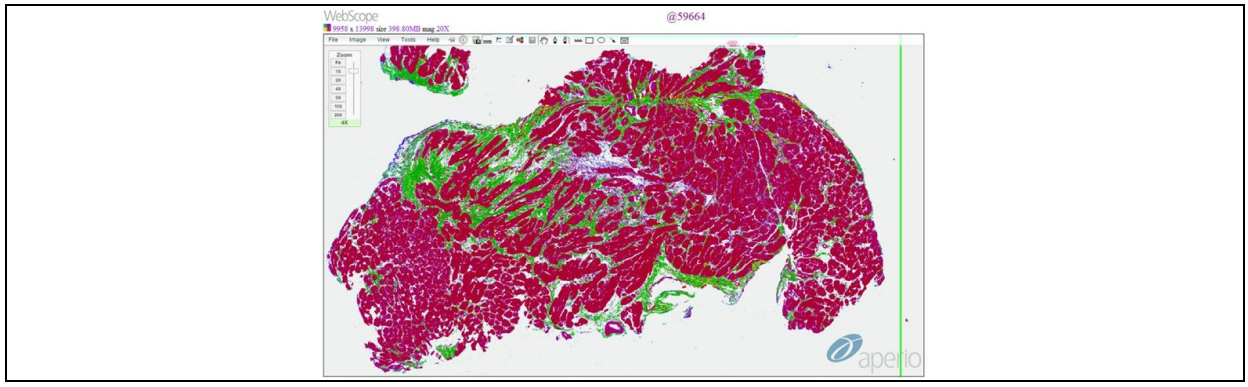

**Fig. 11 Fibrosis mark-up on digitized slide (Colocalization algorithm).**

### 4.2.3. Genie algorithm

Genie (GENetic Imagery Exploration [238]) is a pattern recognition algorithm that distinguishes spatial and morphological features based on structures (classes) provided by the user. A specific Genie classifier was developed as follows: 1. New Genie project and training set created; 2. Digital slides added to a training set; 3. The classes of interest defined and marked in the digital slides in the training set (Fig. 12); 4. Training montage created by running Genie Training v1 algorithm (1000 training iterations set) on user-selected tissue sub-regions (the algorithm estimated the training accuracy at 99.4 %); 5. Based on the training macro, Genie Classifier v1 algorithm was used to create the specific Classifier to be tested and used (Fig. 13). After testing the classifier the classes can then be selected for subsequent analysis using specific task algorithms. For better identification of cardiac fibrosis, we used only spatial recognition, disabling the detection of morphological features. For this study, the Genie system was trained to distinguish the myocardium, fibrous tissue (fibrosis) and glass (Fig. 13). Total cardiac fibrosis percentage was adjusted to a total tissue area in the image analyzed, ignoring the glass (Fig. 14).

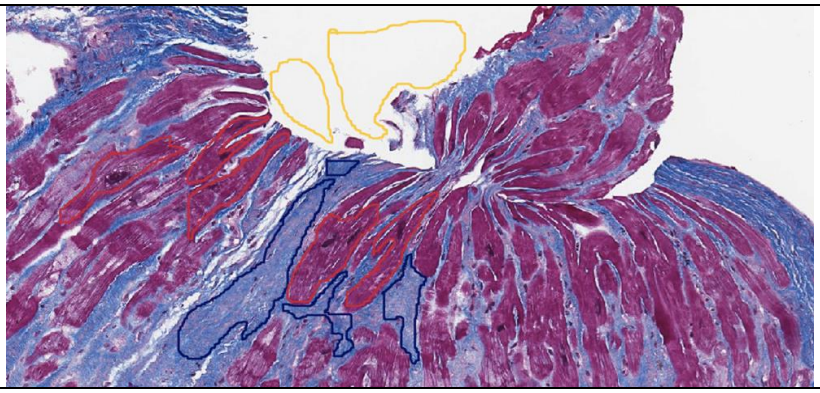

**Fig. 12 Training and using of the Genie. Defining and marking the classes of interest.**

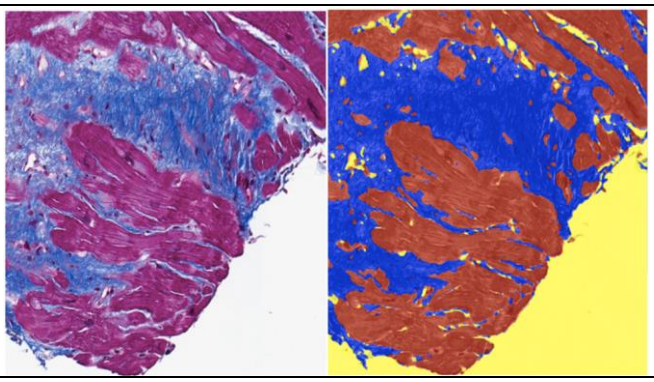

**Fig. 13 Training and using of the Genie. Testing and using the new specific classifier.**

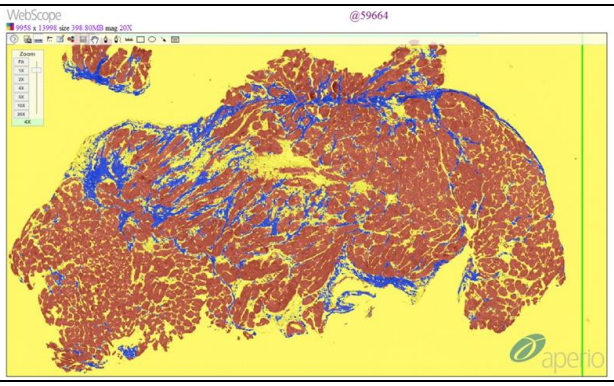


**Fig. 14 Fibrosis mark-up on digitized slide (Genie algorithm).**

### 4.2.4. Stereology

Stereology is an interdisciplinary field for volume estimation of three-dimensional structures by their planar sections. We performed the study on tissue sections of 3 μm, thus the stereology was performed on a projection rather than on ideal 2D plane. A point counting grid was used to estimate the fraction area [240]. "Stereology toolkit 4.2.0" from ADCIS (Saint Contest, France) was used in this study. This stereology module allows defining a ROI and a grid that overlay an area of a virtual slide. Then the type, the spacing and the pattern size of the grid must be adjusted (Fig. 15). 150-200 test points are recommended for acceptable analysis

precision [241, 242]. The grid of point counting, with the sampling interval of 200 pixels and a pattern size of 20 pixels was chosen to evaluate the area fraction of myocardial tissue and cardiac fibrosis. These adjustments of the stereology grid ensured a minimum of 500 test points in the smallest myocardial biopsies and higher counting precision. The structures of interest: glass, fibrosis, myocardium, other (including inflammation, necrosis, glass areas inside the myocardium) were manually highlighted by the observer (Fig. 16). The total percentage of cardiac fibrosis was counted using the number of points ignoring the "glass" and "other" category. The area fraction, equivalent to the volume fraction of cardiac fibrosis was then estimated as the ratio between the number of test marked as fibrosis and the total number of test points included in the ROI, points ignoring the "glass" and "other" categories. The results were expressed as percentages together with the corresponding uncertainty computed according to Weibel [241].

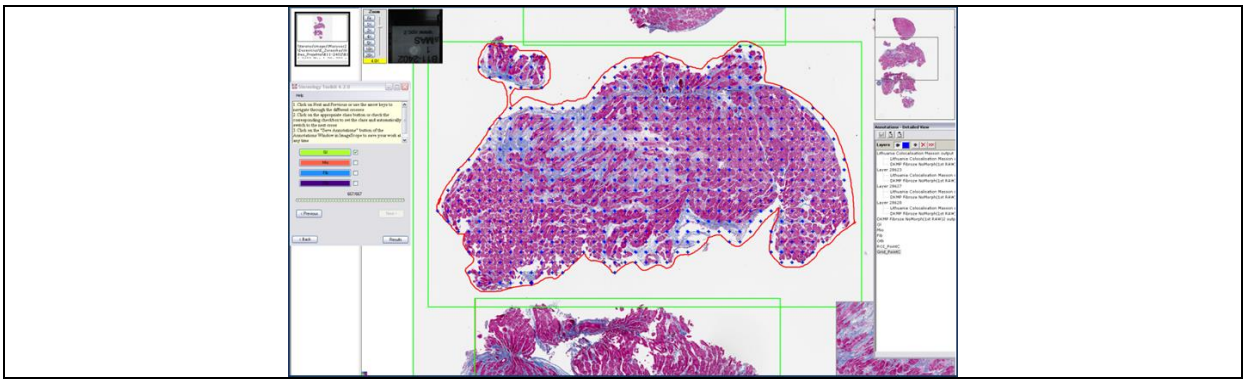

**Fig. 15 Fibrosis mark-up on digitized slide using Stereology protocol. ImageScope V11 view incorporating grid (sampling step of 200 pixels and size of the pattern 20 pixels).**

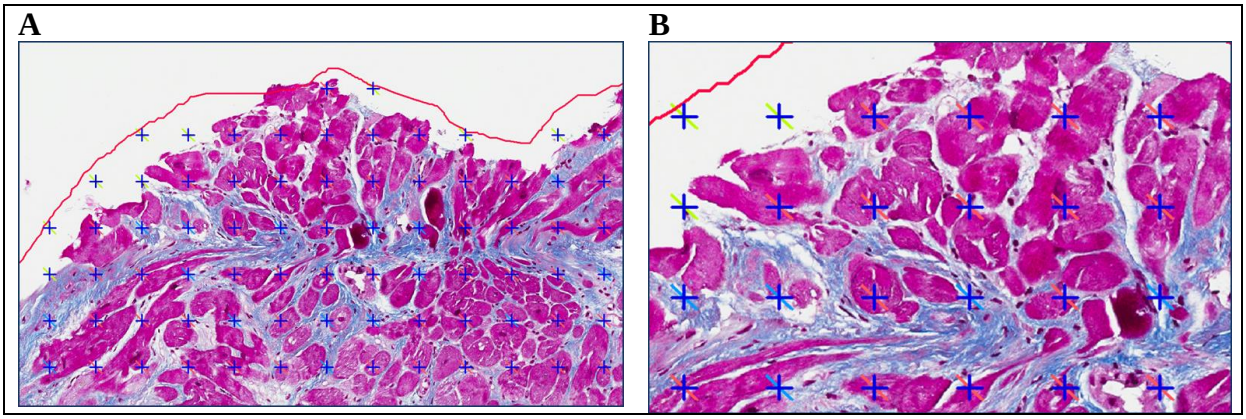


**Fig. 16 Fibrosis mark-up on digitized slide using Stereology protocol. Structures of interest (glass, fibrosis, myocardium, other) manually highlighted by observer. (A) View at x10 magnification, (B) View at x20 magnification.**

### 4.2.5. Pathologist's visual scoring

The extent of total cardiac fibrosis in the samples was also evaluated as a percentage of the sample area by a highly-experienced pathologist using a light microscope. Two evaluations were performed with the time interval of two weeks.

## *4.3. Statistical analyses*

Data are presented as the mean and standard error of mean (Mean ± SEM). All statistical analyses of data were performed using the SPSS package (version 19.0 for Windows; SPSS Inc., Chicago, IL, USA) at 5 % significance level.

### 4.3.1. Analysis of DCM data

The normality of the data distribution was tested by the Shapiro-Wilk test. Differences in parameters of virus-negative and virus-positive and also inflammation-negative and inflammation-positive patient groups were tested by Student's *t* test or the Wilcoxon–Mann–Whitney rank sum nonparametric test (specified in figure legends and table titles). For comparative purposes Pearson's correlation coefficient was used. Pearson's Chi-square test was used when appropriate to compare categorical variables.

### 4.3.2. Analysis of fibrosis quantification data

For the statistical comparison of data, the Pearson's correlation coefficient, Friedman's test with post hoc (Wilcoxon signed-rank with a Bonferroni correction applied) and scatter-dot graphs (with $R^2$, intercept and slope) were used. To enable a standard approach to the data, a natural logarithmic transformation of all measurements was performed before drawing scatted-dot plots. The agreement between fibrosis measurement methods was tested with Bland-Altman plots [243], using the stereology estimation as a reference method for the X axis [244].

## *4.4. Ethical approval*

The study was approved by the Vilnius Regional Biomedical Research Ethics committee (License Nr.158200-09-382-103). All patients gave written informed consent to include their data in the study for each investigational procedure.

The investigation conforms to the principles outlined in the *Declaration of Helsinki*.

# 5. RESULTS

## *5.1. Virus-positive DCM patient study group*

### 5.1.1. Identification of viral genome in endomyocardial biopsy

From a total of 32 patient included in this study, viral genome was detected in the myocardium of 14 subjects (43.8 %). The following virus species were detected: parvovirus B19 (PVB19) (n = 11; 34.4 %), human herpes virus type 6 (HHV6) (n = 4; 12.5 %), enterovirus (EV) (n = 1; 3.1 %), hepatitis C virus (HCV) (n = 1; 3.1 %), Epstein-Barr virus (EBV) (n = 1; 3.1 %), Varicella-zoster virus (VZV) (n = 1; 3.1 %). Among the 3 (9.38 %) patients with double infections: co-detection of PVB19 and HHV6 prevailed in 2 EMB samples, whereas 1 EMB sample had PVB19 and HCV co-infection. There was one patient with triple infection with PVB19, HHV6 and EV.

All patients were subdivided into two groups: virus-negative (n = 18) and virus-positive (n = 14) according to detection of virus genomes in the EMB analysis.

### 5.1.2. Basic clinical parameters

Patient baseline characteristics for the study groups are shown in Table 3. No significant differences in distribution of sex, age and heart failure symptoms between groups were observed.

Heart failure is described as the symptomatic syndrome, graded according to the New York Heart Association (NYHA) functional classification [245]. Most of the patients enrolled to the study had moderate NYHA III class symptoms (11 (61 %) patients in virus negative group, 12 (85 %) patients in virus positive group). There were more patients with severe symptoms of cardiac insufficiency (NYHA IV class) in virus negative group (6 (33 %) patients) than in virus positive group (1 (7 %) patient), however the difference was not significant (p = 0.075).

The prevalence of atrial fibrillation (AF) or left bundle branch block (LBBB) on ECG did not significantly differ between the groups (AF 3 (17 %) versus 1 (7 %), p = 0.360; LBBB 5 (28 %) versus 2 (14 %), p = 0.360) and was even lower in virus-positive patient group.

Generally, main parameters of echocardiography demonstrated characteristic signs of DCM: reduced left ventricular ejection fraction (LVEF), dilatation of left

ventricular. However, there were no significant differences in these parameters between the groups: LVEF 23.11 ± 1.6 % versus 24.93 ± 1.39 % (p = 0.413), left ventricular end-diastolic diameter (LVEDD) 6.87 ± 0.24 cm versus 6.64 ± 0.18 cm, left ventricular end-diastolic diameter index (LVEDDI) 3.66 ± 0.12 cm/m$^2$ versus 3.54 ± 0.12 cm/m$^2$.

**Table 3 Baseline characteristics of patients.**

| ***Variable*** | ***Virus-negative group*** | | ***Virus-positive group*** | | |
|---|---|---|---|---|---|
| | No. of pts. | Value | No. of pts. | Value | p Value |
| **Sex (male/female)** | 18 | 16 (89 %) / 2 (11 %) | 14 | 9 (64 %) / 5 (36 %) | 0.095 |
| **Age (years)** | 18 | 45.11 ± 3.18 | 14 | 40.79 ± 2.29 | 0.279 |
| **NYHA** | | | | | |
| II | 18 | 1 (6 %) | 14 | 1 (7 %) | 0.854† |
| III | 18 | 11 (61 %) | 14 | 12 (85 %) | 0.125† |
| IV | 18 | 6 (33 %) | 14 | 1 (7 %) | 0.075† |
| **Cardiac parameters** | | | | | |
| LBBB (%) | 18 | 5 (28 %) | 14 | 2 (14 %) | 0.360† |
| Permanent AF (%) | 18 | 3 (17 %) | 14 | 1 (7 %) | 0.419† |
| LVEF (%) | 18 | 23.11 ± 1.6 | 14 | 24.93 ± 1.39 | 0.413 |
| LVEDD (cm) | 18 | 6.87 ± 0.24 | 14 | 6.64 ± 0.18 | 0.460 |
| LVEDDI (cm/m$^2$) | 13 | 3.66 ± 0.14 | 14 | 3.54 ± 0.12 | 0.522 |
| Mean Ao (mmHg) | 11 | 91.73 ± 3.26 | 10 | 84.6000 ± 3.44 | 0.149 |
| Mean RAP (mmHg) | 15 | 17.00 ± 2.22 | 11 | 9.00 ± 1.54 | 0.011* |
| Mean PCWP (mmHg) | 16 | 29.00 ± 2.62 | 12 | 17.75 ± 2.18 | 0.004* |
| Mean PAP (mmHg) | 16 | 40.19 ± 3.45 | 12 | 25.42 ± 2.6 | 0.003* |
| CI (L/min/m2) | 13 | 2.08 ± 0.2 | 8 | 2.41 ± 0.17 | 0.309 |

* Significant at p < 0.05 level. † Chi-square test. Data are presented as means ± SEM. Abbreviations: NYHA – New York Heart Association functional class; LBBB – left bundle branch block; AF – atrial fibrillation; LVEF - left ventricular ejection fraction; LVEDD – left ventricular end-diastolic diameter; LVEDDI – left ventricular end-diastolic diameter index; Ao – aortic; RAP – right atrial pressure; PCWP – pulmonary capillary wedge pressure; PAP – pulmonary artery pressure; CI – cardiac index.

Cardiac catheterization was performed to evaluate hemodynamic impairment, to confirm pulmonary hypertension (PH) diagnosis or to access severity of PH (PH is confirmed when the mean pulmonary artery pressure (PAP) is ≥25 mmHg at rest [246], mean pulmonary capillary wedge pressure (PCWP) is necessary for differential diagnosis of PH due left heart impairment [247]. In both groups these hemodynamic parameters were elevated, which confirms PH diagnosis due to left heart disease. However hemodynamic parameters (PAP, PCWP, RAP) were significantly higher in virus negative group (mean PAP 40.19 ± 3.45 versus 25.42 ± 2.6, p = 0.003; mean

PCWC 29.00 ± 2.62 versus 17.75 ± 2.18, p = 0.004; mean RAP 17.00 ± 2.22 versus 9.00 ± 1.54, p = 0.011). The difference among the groups shows more advanced PH in virus negative group and is associated with worse prognosis [248]. Cardiac index (CI) was reduced in both groups, although, did not differ significantly (2.08 ± 0.2 versus 2.41 ± 0.17, p = 0.309). The better hemodynamic situation in virus-positive patients compared to the virus-negative ones might be explained by reduced myocardial fibrosis and decreased levels of heart contraction regulating proteins. The persistent presences of virus in myocardium can swift hemodynamic parameters to the expansion direction indicating worse prognosis.

Additionally, the inverse correlation between LVEF with hsCRP and BNP (R= -0.803, p = 0.005; R = -0.630, p = 0.016, respectively) also revealed that further presence of virus in myocardium might increase inflammation, fibrosis and subsequent release of ventricular natriuretic protein that may unbalance proper functioning of myocardium and induce heart failure (Table 3 and Table 5).

Both patient groups received the same basic treatment according to guidelines (Table 4) [249-251]. No significant difference in prescribed medication was observed between the groups.

**Table 4 Basic treatment for the study patient groups (according ESC guidelines).**

| ***Conventional treatment of heart failure*** | ***Virus-negative group*** | ***Virus-positive group*** |
|---|---|---|
| ACE inhibitors | 9 (50 %) | 7 (50 %) |
| β-blockers | 16 (89 %) | 13 (93 %) |
| Digitalis (in atrial fibrillation) | 3 (17 %) | 6 (43 %) |
| Diuretics | 18 (100 %) | 14 (100 %) |
| Anticoagulation (atrial fibrillation, EF < 40 %) | 14 (78 %) | 7 (50 %) |
| Antiarrhythmics (class III: amiodarone) | 5 (28 %) | 2 (14 %) |
| **Interventions** | | |
| Implantable cardiac defibrillator (ICD) | 0 (0 %) | 0 (0 %) |
| Cardiac resynchronization therapy (CRT) | 3 (17 %) | 1 (7 %) |

### 5.1.3. Estimation of inflammation and cardiomyocyte death in virus-positive and virus-negative DCM samples

Intramyocardial viral infection has been suggested to be mainly an inflammatory-related process leading to myocardial cell death. However, the data show that levels of inflammation and apoptosis were reduced both in serum and biopsy samples. The decrease of inflammatory cytokines IL-6 and hsCRP in sera was

statistically significant (Fig. 17), whereas changes of apoptotic markers were significant only in biopsies (Fig. 18 and Fig. 19 $p < 0.05$).

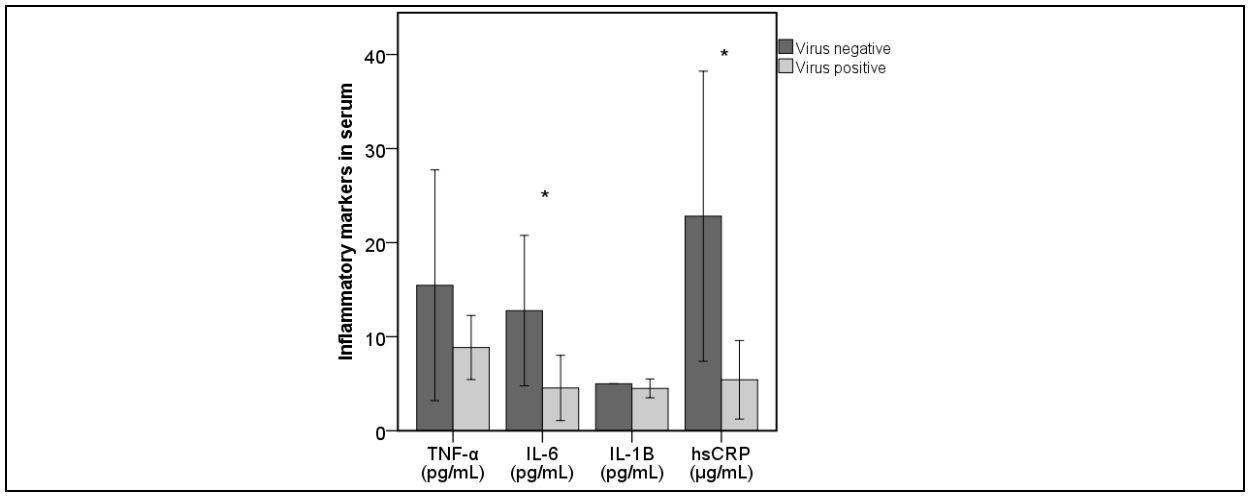


**Fig. 17 Levels of the inflammatory markers in serum. ELISA data are presented as means ± SEM. Data were considered significant at *p < 0.05.**

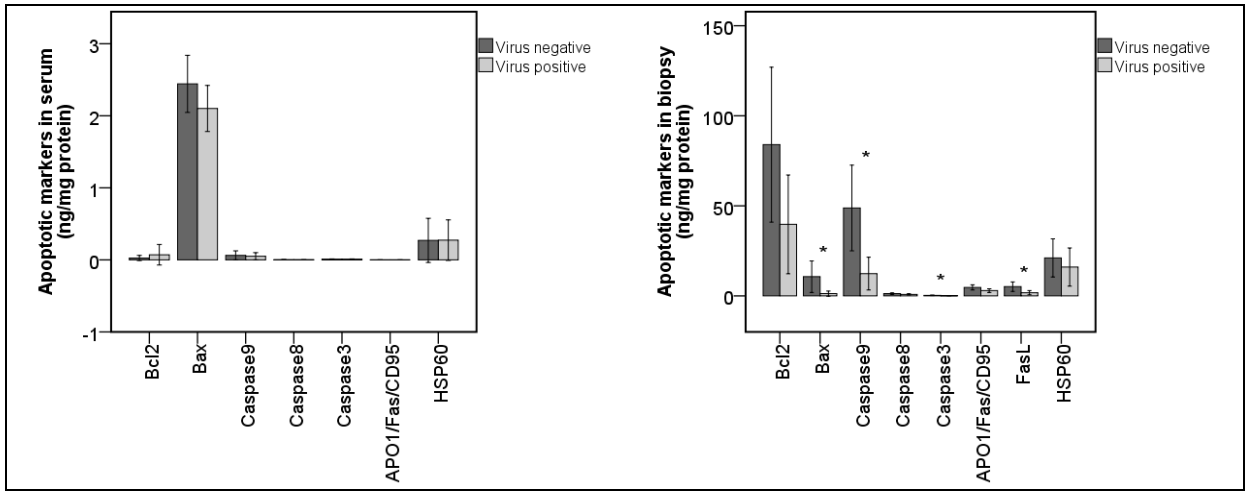


**Fig. 18 Levels of the apoptotic markers in serum. ELISA data are presented as means ± SEM.**




**Fig. 19 Levels of the apoptotic markers in biopsy. ELISA data are presented as means ± SEM. Data were considered significant at *p < 0.05.**

The level of inflammatory infiltrates, $CD3^+$, $CD45Ro^+$ and $CD68^+$, in virus-positive biopsies was also depressed, confirming the absence or low level of intramyocardial inflammation (Fig. 20).

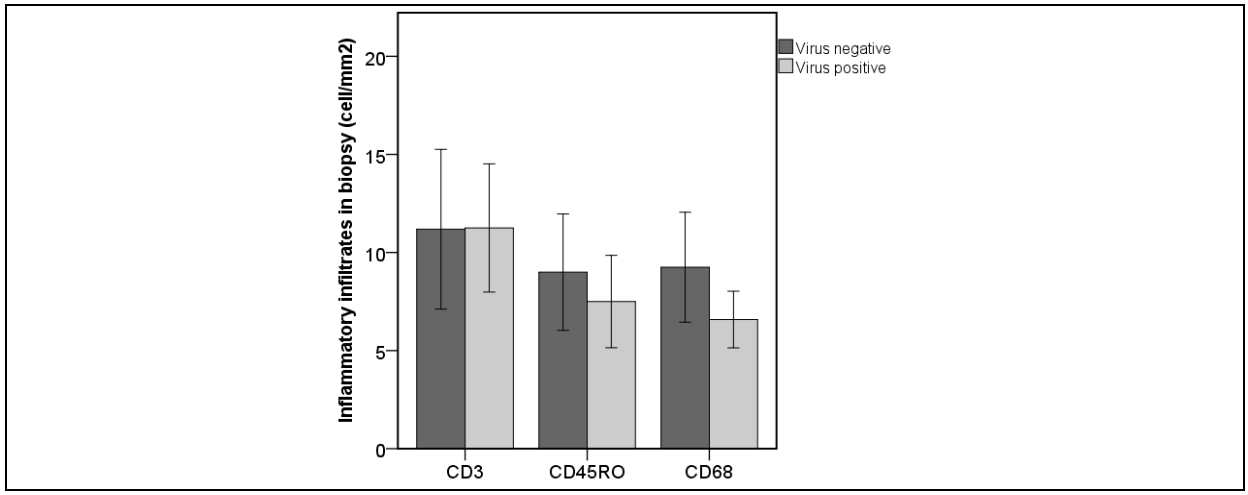


**Fig. 20 Levels of the inflammatory infiltrates in biopsy. Immunohistochemically positive stained cell count data are presented as means ± SEM from at least three independent locations by highly experienced pathologist.**

Additionally, the biomarker of myocardial necrosis, the high-sensitivity troponin T (hsTnT), was also 2.6 fold down-regulated in virus positive serum samples, revealing the absence of necrosis (Table 5). The absence of necrosis was also confirmed by histological analysis of biopsies stained with hematoxylin and eosin.

**Table 5 Summary data of estimated proteins in virus-negative and virus-positive DCM serums and biopsies.**

| ***Variable*** | ***Virus-negative group*** | | ***Virus-positive group*** | | |
|---|---|---|---|---|---|
| | No. of pt. | Value | No. of pt. | Value | p Value |
| **ELISA assays of proteins in serums** | | | | | |
| MMP9 (ng/mg protein) | 18 | 1.4886 ± 0.0648 | 14 | 1.4320 ± 0.0630 | 0.536 |
| TIMP1 (ng/mg protein) | 18 | 6.1611 ± 0.2028 | 14 | 5.7538 ± 0.2945 | 0.266 |
| MMP9/TIMP1 | 18 | 0.2428 ± 0.0084 | 14 | 0.2529 ± 0.0108 | 0.465 |
| PICP (ng/mg protein) | 18 | 0.1589 ± 0.0321 | 14 | 0.0792 ± 0.0180 | 0.039* |
| ICTP (ng/mg protein) | 18 | 0.0177 ± 0.0023 | 14 | 0.0148 ± 0.0033 | 0.495 |
| PICP/ICTP | 18 | 11.2537 ± 2.1036 | 14 | 8.6832 ± 3.9652 | 0.573 |
| TGFβ (pg/mg protein) | 18 | 13.9515 ± 1.9427 | 14 | 13.7339 ± 2.1101 | 0.940 |
| **ELISA assays of proteins in biopsies** | | | | | |
| MMP9 (ng/mg protein) | 18 | 2.9149 ± 1.0740 | 13 | 1.3321 ± 0.3952 | 0.089¡ |
| TIMP1 (ng/mg protein) | 18 | 9.6116 ± 1.4578 | 13 | 5.8772 ± 1.6243 | 0.266 |
| MMP9/TIMP1 | 18 | 0.3434 ± 0.0838 | 13 | 0.4107 ± 0.1461 | 0.694 |
| PICP (ng/mg protein) | 18 | 4.2779 ± 2.3323 | 13 | 1.0708 ± 0.5718 | 0.312¡ |
| ICTP (ng/mg protein) | 18 | 0.0733 ± 0.0337 | 13 | 0.2636 ± 0.1184 | 0.196¡ |
| PICP/ICTP | 18 | 14.6047 ± 6.3008 | 13 | 3.4117 ± 2.1170 | 0.154 |
| TGF-β1 (pg/mg protein) | 18 | 122.0955 ± 82.6902 | 13 | 34.3392 ± 29.0622 | 0.622¡ |

| | | | | | |
|---|---|---|---|---|---|
| **Immuno- and histochemical assays of proteins in biopsies** | | | | | |
| MMP1 (%) | 15 | 6.6747 ± 1.5248 | 14 | 14.6564 ± 22.9998 | 0.022* |
| MMP2 (%) | 15 | 2.8940 ± 0.3412 | 14 | 7.0350 ± 3.9725 | 0.292 |
| MMP9 (%) | 15 | 10.9707 ± 4.2136 | 14 | 9.6871 ± 1.5709 | 0.783 |
| MMP13 (%) | 15 | 12.2860 ± 2.5354 | 14 | 12.6650 ± 2.6789 | 0.919 |
| α-SMA (%) | 15 | 24.1400 ± 4.6102 | 14 | 21.4821 ± 2.5839 | 0.620 |
| HLA-DR (%) | 15 | 0.8253 ± 0.21629 | 14 | 0.8886 ± 0.21432 | 0.837 |
| **Other determinations in serums** | | | | | |
| Adiponectin (µg/mL) | 18 | 28.3389 ± 3.4627 | 14 | 15.0143 ± 2.7360 | 0.005* |
| BNP (pg/mL) | 18 | 1841.7944 ± 341.4525 | 14 | 936.4786 ± 260.1920 | 0.044* |
| hsCRP (µg/mL) | 17 | 22.8118 ± 7.7080 | 10 | 5.4170 ± 2.0884 | 0.043* |
| hsTnT (pg/mL) | 18 | 74.0878 ± 29.8031 | 13 | 28.8277 ± 4.9154 | 0.151 |
| Galectin-3 (ng/mL) | 18 | 12.8344 ± 0.8536 | 14 | 11.0579 ± 1.0107 | 0.190 |
| **Cardiac fibrosis (%)** | **18** | **17.9724 ± 2.2076** | **14** | **10.4818 ± 1.6854** | **0.012*** |

Data are presented as the mean ± SEM. * Significant at 0.05 level; ¡ Wilcoxon–Mann–Whitney rank sum nonparametric test. Abbreviations: Bcl-2 – B-cell lymphoma 2 protein; Bax – Bcl-2–associated X protein; MMP – matrix metalloproteinases; TIMP – tissue inhibitors of matrix metalloproteinases; PICP – type I procollagen carboxy-terminal propeptide; ICTP – type I collagen carboxyterminal telopeptide; TNF-a – tumour necrosis factor-alfa; IL-1β – interleukin 1 beta; IL-6 – interleukin 6; hsCRP – high sensitivity C-reactive protein; hsTnT – high sensitivity troponin T; TGF-β1 - transforming growth factor β1; BNP – brain natriuretic protein.

### 5.1.4. Impact of virus on contraction proteins in myocardial tissue

The absence of inflammation (Fig. 21), apoptosis and necrosis in virus-positive myocardium, stimulated to investigate contraction properties and proteins mostly regulating it.

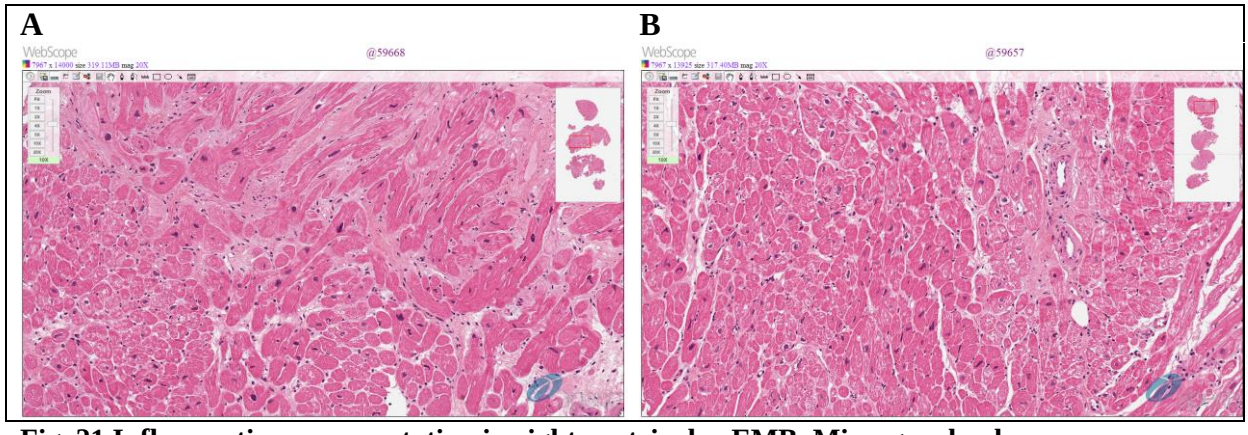


**Fig. 21 Inflammation representation in right ventricular EMB. Micrographs show one representative picture from one patient of each group. (A) virus-negative; (B) virus-positive. Hematoxylin and Eosin staining. Magnification: x10.**

Data presented in Table 5, Fig. 22 and Fig. 23 show lower intensity of fibrosis in virus-positive myocardium.

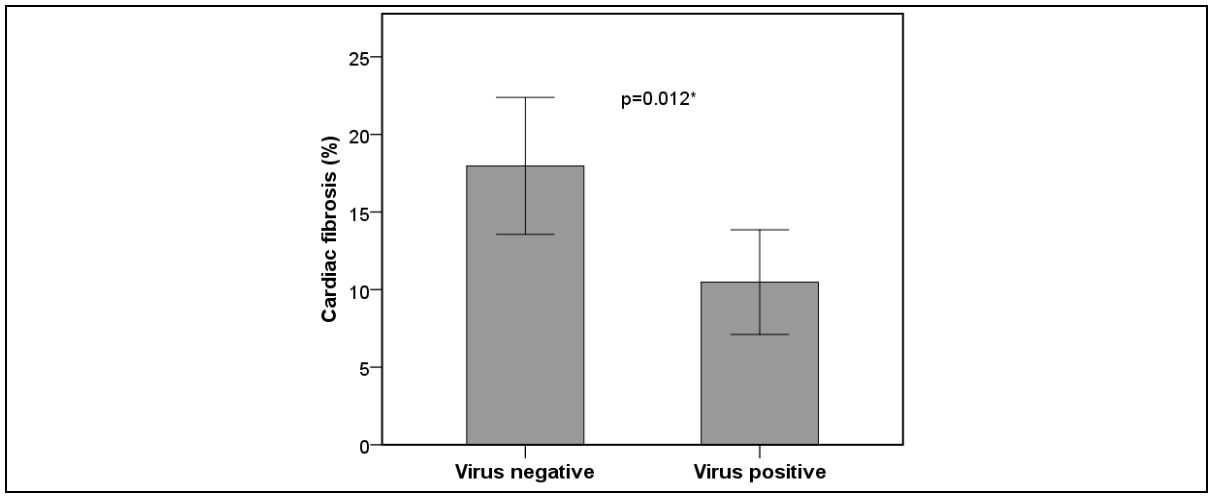


**Fig. 22 Levels of fibrosis in biopsies. Data are presented as means ± SEM from at least three independent measurements. Data were considered significant at *p < 0.05.**

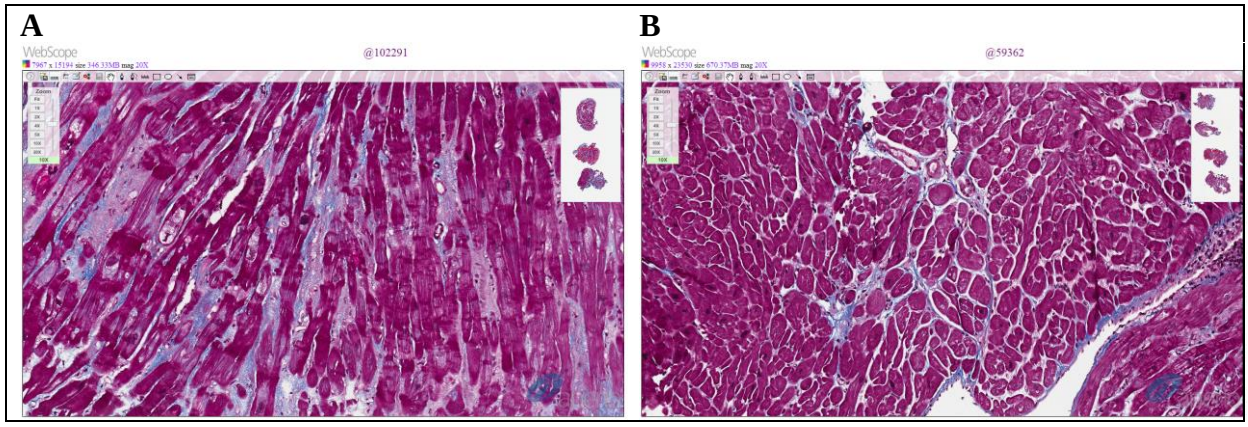


**Fig. 23 Fibrosis in right ventricular EMB. Micrographs show one representative picture from one patient of each group. (A) virus-negative patient (digitally quantified fibrosis – 21.20 %); (B) virus-positive patient (digitally quantified fibrosis – 9.79 %). Masson trichrome staining for cardiac fibrosis (collagen is colored blue). High amounts of collagen reflect a high level of fibrosis. Magnification: x10.**

Additionally, the carboxy-terminal propeptide of procollagen type I (PICP), a marker of collagen I biosynthesis in biopsies was reduced four-fold, whereas carboxy-terminal telopeptide of collagen type I (ICTP), a marker of collagen I degradation, was increased 3.6 fold (Fig. 24, Fig. 25 and Fig. 26). Change of ICTP in myocardial tissue was not confirmed immunohistochemicaly due to the absence of appropriate antibodies.

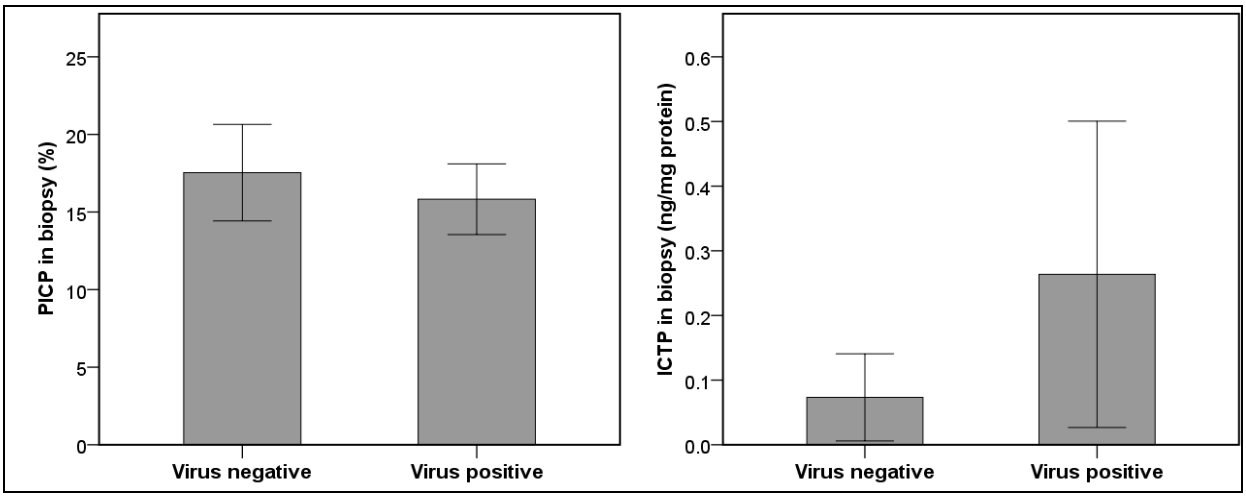


**Fig. 24 Levels of the collagen I synthesis (type I procollagen carboxy-terminal propeptide (PICP)) biomarker. Data are presented as means ± SEM from at least three independent measurements.**




**Fig. 25 Levels of the collagen I degradation (type I collagen carboxyterminal telopeptide; ICTP) biomarker in virus-negative and virus-positive serum samples. Data are presented as means ± SEM.**

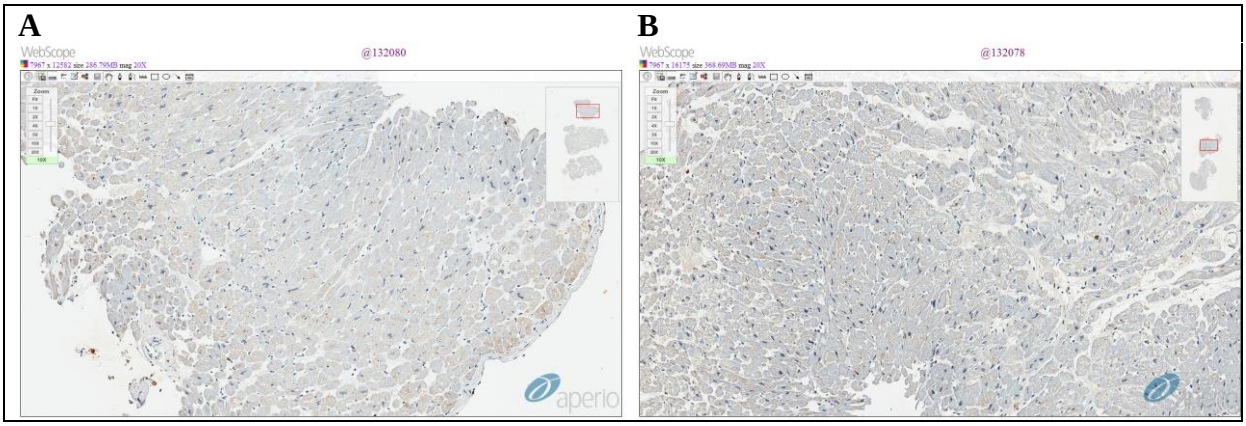


**Fig. 26 Collagen I synthesis (type I procollagen carboxy-terminal propeptide (PICP)) biomarker in right ventricular EMB. Micrographs show one representative picture from one patient of each group. (A) virus-negative patient (digitally quantified PICP – 13.70 %); (B) virus-positive patient (digitally quantified PICP – 16.43 %). Immunohistochemical staining was performed using anti-PICP antibodies (brown color represents a positive staining for PICP). Magnification: x10.**

We also found that myocardial fibrosis in virus-positive biopsies significantly correlated with the PICP/ICTP ratio and the general serum inflammatory marker hsCRP, suggesting activation of inflammation and fibrosis in further presence of intramyocardial virus (Fig. 27).

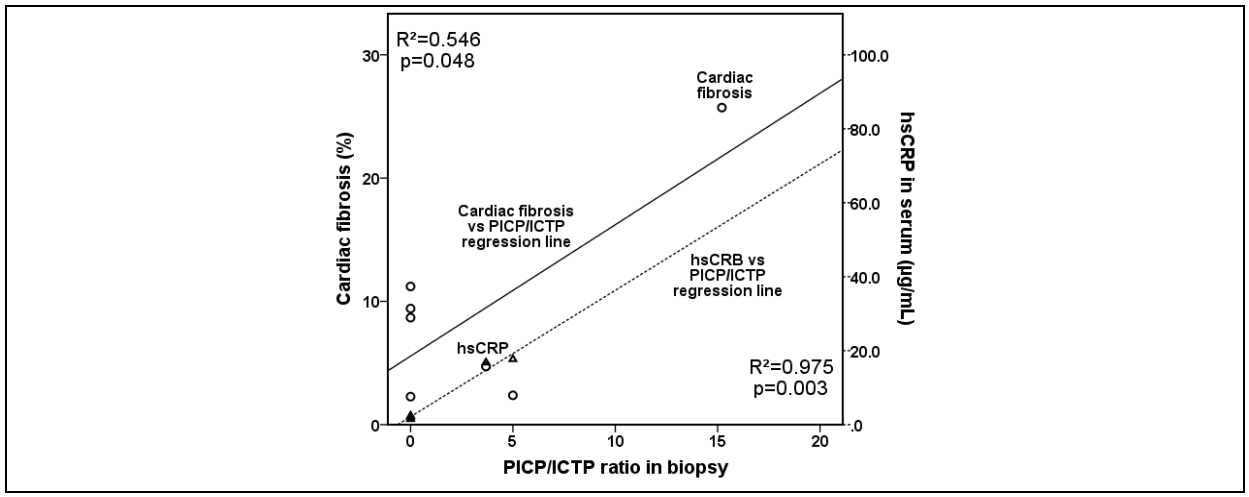


**Fig. 27 Correlation analysis of additional markers in virus-positive patient serum samples. Correlation of collagen synthesis/degradation biomarkers (PICP/ICTP) ratio in biopsy (by ELISA) with cardiac fibrosis and the general inflammatory biomarker high sensitivity C-reactive protein (hsCRP) in serum. Correlation was significant at a level of $p < 0.05$. Linear regression line is presented within 95 % confidence interval. Regression coefficients ($R^2$) are shown in the graphs.**

A more detailed correlation analysis, presented in Table 6, show that the marker of collagen I synthesis (PICP) as well as MMP9 level in virus-positive biopsies were mostly associated with secreted inflammatory cytokine IL-6. Moreover, the turnover of collagen I, and MMP9 activation significantly depended on proper functioning of the mitochondrial outer membranes: the release of mitochondrial outer membrane stabilizing protein Hsp60 strongly correlated with serum markers of collagen I synthesis (PICP) and degradation (ICTP), matrix metalloproteinase 9 (MMP9) and inflammation (IL-6) (Table 6). The correlation data suggest that further presence of virus in myocardium will activate inflammation leading to increased release of Hsp60 and MMP9 activation. Our data show that chronic intramyocardial viral infection is able to induce processes initiating heart failure.

**Table 6 Correlation of collagen I turnover markers in biopsy and serum samples.**

| | PICP in biopsy | ICTP in biopsy | MMP9 in biopsy | IL-6 in serum | hsCRP in serum |
|---|---|---|---|---|---|
| ICTP in biopsy | **0.806**** | | | | |
| MMP9 in biopsy | 0.283 | 0.385 | | | |
| IL-6 in serum | **0.572*** | 0.489 | **0.584*** | | |
| hsCRP in serum | **0.913**** | **0.933**** | 0.527 | 0.576 | |
| HSP60 in serum | **0.641*** | **0.641*** | **0.646*** | **0.756**** | 0.591 |

Two tailed significance: *p < 0.05; **p < 0.01. Significant correlations are in bold phase type. Abbreviations: PICP – type I procollagen carboxy-terminal propeptide; ICTP – type I collagen carboxyterminal telopeptide; MMP9 – matrix metalloproteinase 9; IL-6 – interleukin-6; hsCRP – high sensitivity C-reactive protein ; Hsp60 – heat shock protein 60.

### 5.1.5. Changes of released contraction-regulating proteins in virus-positive serums

The data show that the release of most tested intramyocardial apoptotic, inflammatory, and contraction-regulating proteins such as TGF-β1, MMP9, TIMP1, hsTnT, galectin-3 and PICP/ICTP in virus-positive serums were reduced Table 5.

The decrease of collagen I synthesis biomarker (PICP) in serum was even significant (Fig. 28, $p < 0.05$). However, the level of collagen I synthesis (PICP) in biopsy inversely correlated with its level in serum, showing that further presence of viral infection will impair myocardial permeability (Fig. 29).

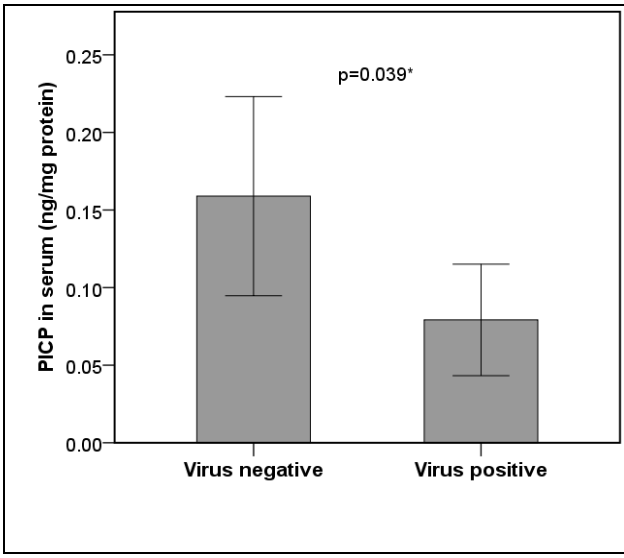


**Fig. 28 Levels of collagen I synthesis biomarker (type I procollagen carboxy-terminal propeptide; PICP) in virus-negative and virus-positive serum samples. Data are presented as means ± SEM and considered to be significant at *p < 0.05.**

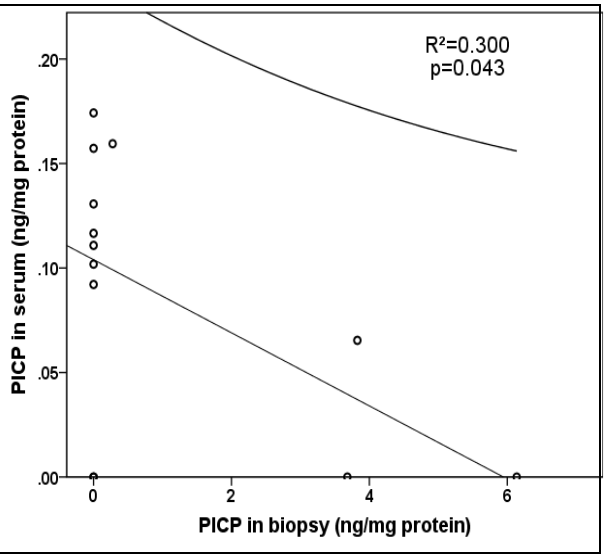


**Fig. 29 Inverse correlation between distribution of collagen synthesis biomarker PICP (type I procollagen carboxy-terminal propeptide) in virus-positive patient group serum and biopsy samples. Correlation was significant at a level of p <0.05. Linear regression line is presented within 95 % confidence interval. Regression coefficients ($R^2$) are shown in the graphs.**

Additional correlation analysis showed that markers of apoptotic pathways, the intrinsic (Bcl-2 and caspase-9) and the extrinsic (FasL and caspase-8) also were strongly associated with the release of collagen I turnover biomarkers (Fig. 30 and Fig. 31).

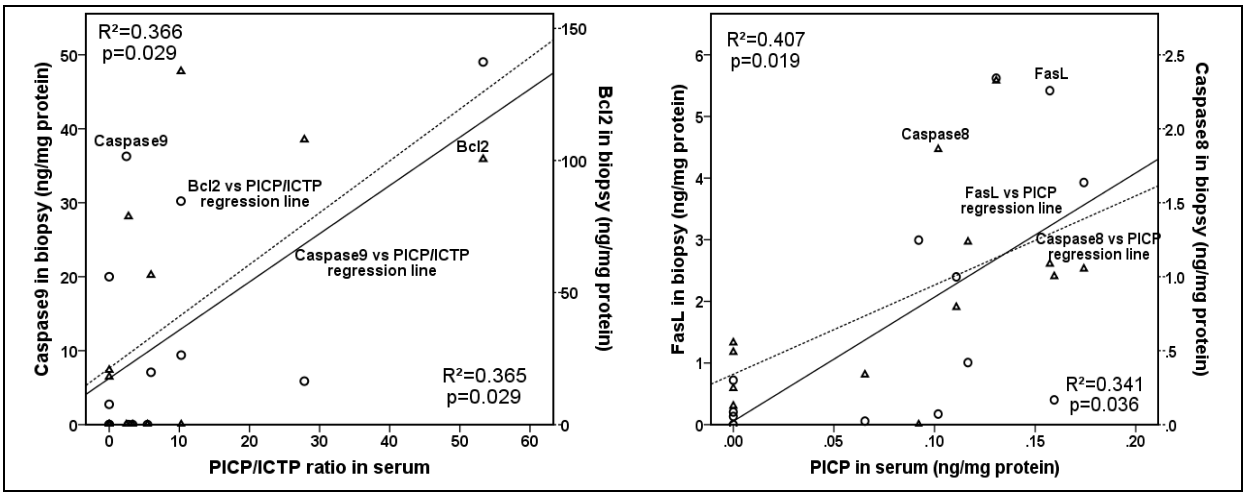


**Fig. 30 Correlation analysis of contraction-regulating proteins in virus-positive patient group. Correlation of collagen synthesis/degradation biomarkers (PICP/ICTP) ratio in serum with the markers of intrinsic apoptotic pathway (BcL-2 and caspase-9) in biopsies. Correlation was significant at a level of p <0.05. Linear regression line is presented within 95% confidence interval. Regression coefficients ($R^2$) are shown in the graphs.**

**Fig. 31 Correlation analysis of contraction-regulating proteins in virus-positive patient group. Correlation between the collagen I synthesis biomarker PICP with markers of the extrinsic apoptotic pathway (Fas ligand and caspase-8). Correlation was significant at a level of p < 0.05. Linear regression line is presented within 95% confidence interval. Regression coefficients ($R^2$) are shown in the graphs.**

Data in Fig. 32 show that release of intracellular fibrosis-related proteins cardiac high-sensitivity troponin T (hsTnT) and galectin-3 into serum was also reduced. Moreover, galectin-3 significantly correlated with macrophage-secreted cytokines TNF-α and IL-6, suggesting possible its co-localization and secretion by infiltrated macrophages (Fig. 33).

The lower level of infiltrated macrophages and secreted cytokines TNF-α and IL-6 in tested samples can be a reason of lower serum level of galectin-3 (Fig. 20). The detection of intracellular proteins in plasma indicates the loss of integrity of cell membrane due to the acute myocardium and cardiovascular injuries. Considering this, data show that viral infection in tested samples is not acute but chronic and does not impair cell membrane permeability. However, the correlation analysis shows that prolonged intramyocardial viral infection will activate inflammation, cardiomyocyte apoptotic death and fibrosis leading to heart failure.

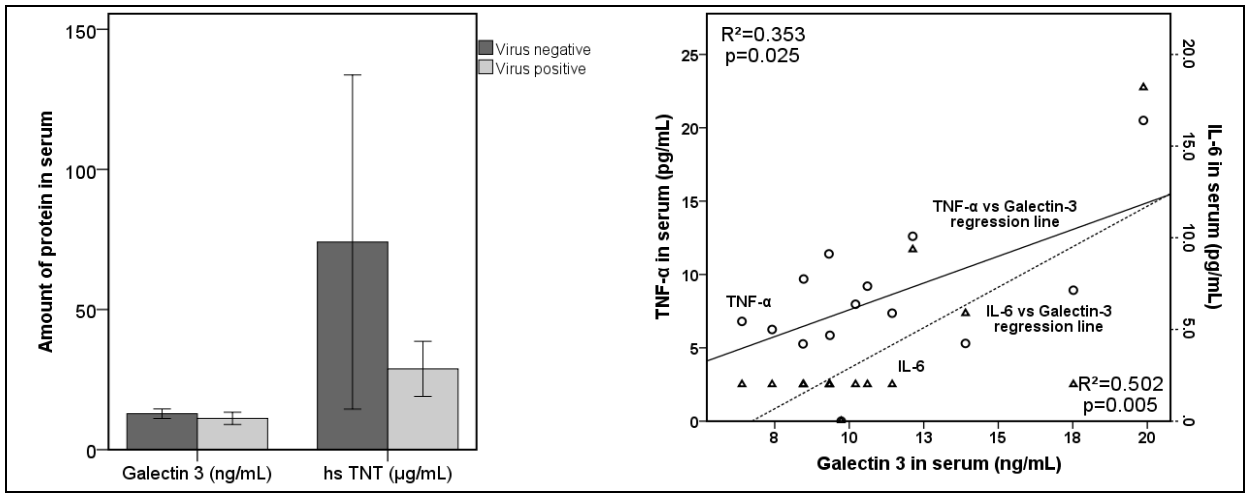


**Fig. 32 Levels of the contraction-regulating proteins: cardiac high-sensitivity troponin T (hsTnT) and galectin-3 in serum samples. Data are presented as means ± SEM.**




**Fig. 33 Correlation of galectin-3 in serum with pro-inflammatory cytokines: tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) in serum. Correlation was significant at a level of $p < 0.05$. Linear regression line is presented within 95 % confidence interval. Regression coefficients ($R^2$) are shown in the graphs.**

### 5.1.6. Other mechanisms regulating progression of viral DCM

In the last experiments, it was found that the serum levels of adipocytokine adiponectin and BNP in virus-positive serums were significantly (almost two fold) lower as compared to the virus-negative ones (Fig. 34 and Fig. 35; Table 4).

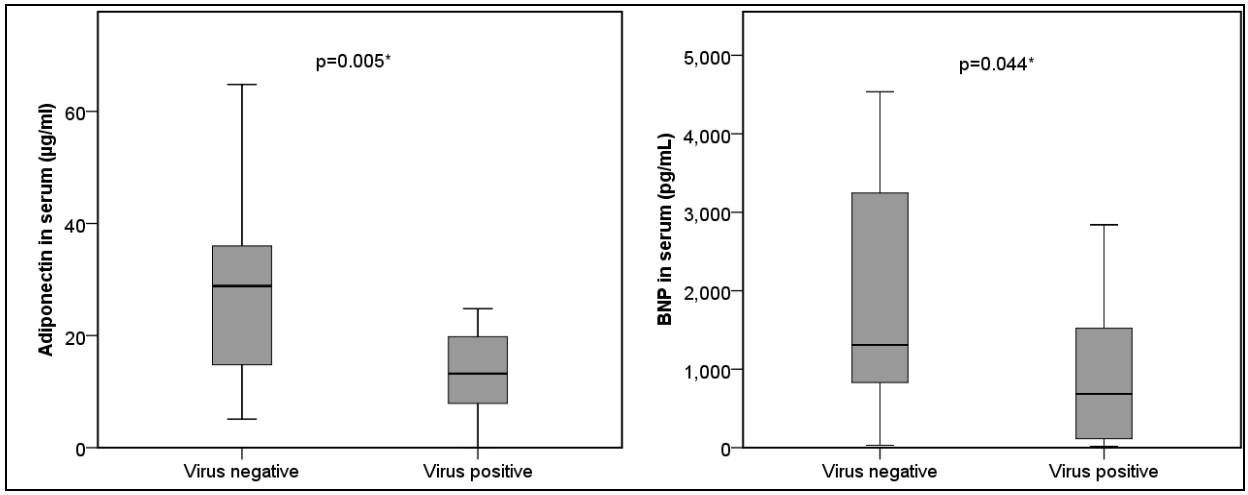


**Fig. 34 Levels of adiponectin in virus-negative and virus-positive serum samples. Data are presented as means ± SEM and considered to be significant at *$p < 0.05$.**




**Fig. 35 Levels of brain natriuretic protein B (BNP) in virus-negative and virus-positive serum samples. Data are presented as means ± SEM and considered to be significant at *$p < 0.05$.**

A correlation analysis revealed that serum adiponectin significantly correlated with main regulator of collagen I transformed growth factor- β1 (TGF-β1) (Fig. 36) and brain natriuretic protein (BNP) (Fig. 37).

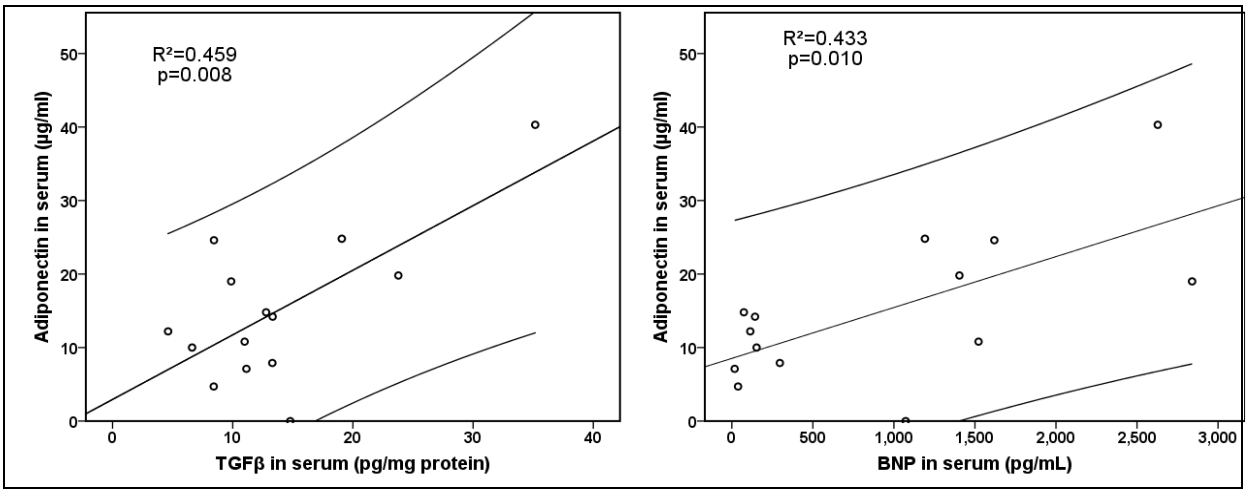


**Fig. 36 Correlation analysis of additional markers in virus-positive patient serum samples. Correlation between adiponectin and transforming growth factor β1 (TGF- β1). Correlation was significant at a level of $p < 0.05$. Linear regression line is presented within 95 % confidence interval. Regression coefficients ($R^2$) are shown in the graphs.**




**Fig. 37 Correlation analysis of additional markers in virus-positive patient serum samples. Correlation between adiponectin and brain natriuretic protein B (BNP). Correlation was significant at a level of $p < 0.05$. Linear regression line is presented within 95 % confidence interval. Regression coefficients ($R^2$) are shown in the graphs.**

Additionally, serum adiponectin significantly correlated with macrophages ($CD68^+$) (Fig. 38), whereas BNP correlated with memory T cells ($CD45Ro^+$) and T lymphocytes ($CD3^+$) (Fig. 39). Serum TGF-β1 also showed strong correlation with intramyocardial macrophages ($CD68^+$, $R = 0.649$; $p = 0.022$) and MMP9 ($R = 0.689$; $p = 0.009$).

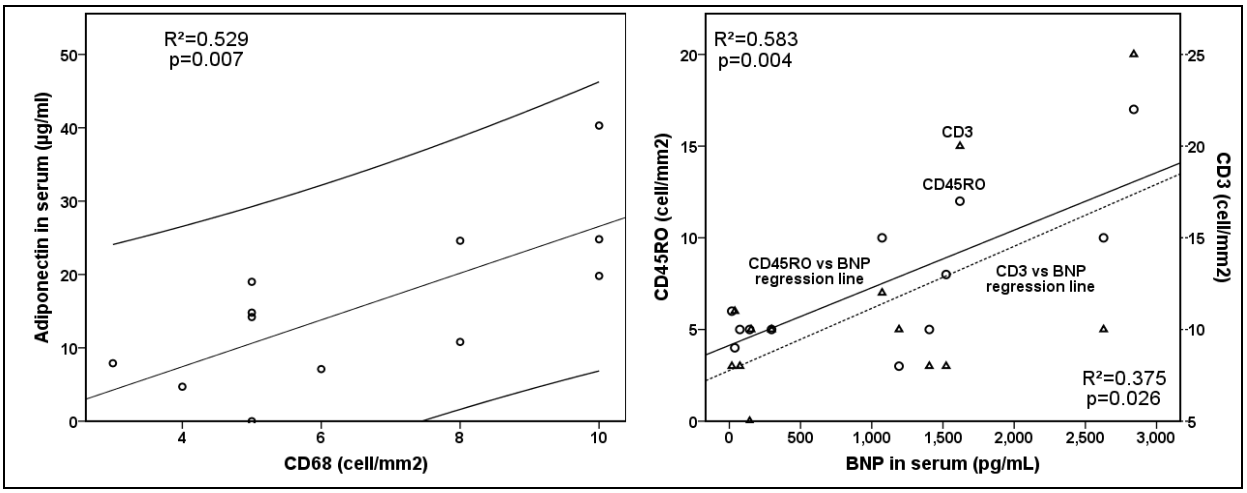


**Fig. 38 Correlation analysis of additional markers in virus-positive patient group. Correlation between adiponectin in serum and infiltrated macrophages ($CD68^+$) in cardiac biopsy. Correlation was significant at a level of $p < 0.05$. Linear regression line is presented within 95 % confidence interval. Regression coefficients ($R^2$) are shown in the graphs.**

**Fig. 39 Correlation analysis of additional markers in virus-positive patient group. Correlation of BNP in serum with T cells ($CD3^+$) and T memory cells ($CD45Ro^+$) in cardiac biopsies. Correlation was significant at a level of $p < 0.05$. Linear regression line is presented within 95 % confidence interval. Regression coefficients ($R^2$) are shown in the graphs.**

Data of this section shows that increased intramyocardial inflammations due to sustained presence of viruses will upregulate levels of serum adiponectin and BNP leading to further activation of TGF-β1, collagen synthesis and fibrosis. The main collagen I synthesis regulator, TGF-β1, can be also activated directly by inflammation infiltrates, particularly macrophages. The sustained presence of the virus in the heart will increase inflammation leading to activation of fibrosis and heart failure.

## *5.2. Inflammation-positive DCM patient study group*

### 5.2.1. Detection of inflammatory infiltrate in endomyocardial biopsy

Not only can the persistent presence of virus influence the inflammatory processes in myocardium. In most of the cases DCM is caused by inflammatory processes of unknown origin. Since is always the goal to determine the origin of inflammation, (which is not always achieved), in this part of our study we have explored the molecular mechanisms dominating in the inflammatory DCM of unknown origin. The inflammatory process in the myocardium was determined by the presence of inflammatory infiltrates. From the immunohistochemically assessed inflammatory cells in EMB T lymphocytes ($CD3^+$) was the most frequently detected cell (11.28 ± 1.21), followed by active-memory T lymphocytes ($CD45Ro^+$) (8.29 ± 0.91) and macrophages ($CD68^+$) (7.75 ± 0.85).

All patients were subdivided into two groups: inflammation-negative (n = 10) and inflammation-positive (n = 22) according to the presence of inflammatory infiltrate following the World Health Organization / International Society and Federation of Cardiology Task Force on the Definition and Classification of Cardiomyopathies recommendations [13, 50, 51].

### 5.2.2. Basic clinical parameters

Patient baseline characteristics for the study groups are shown in Table 7. No significant differences in distribution of sex, age and heart failure symptoms between the groups.

Heart failure is described as the symptomatic syndrome, graded according to the New York Heart Association (NYHA) functional classification [245]. Most of the patients enrolled to the study had moderate (NYHA III class 7 (70 %) patients in inflammation negative group, 15 (68 %) patients in inflammation positive group) or severe symptoms of cardiac insufficiency (NYHA IV class 2 (20 %) patients versus 7 (32 %) patients).

**Table 7 Baseline characteristics of patients.**

| *Variable* | *Inflammation-negative group* | | *Inflammation-positive group* | | |
|---|---|---|---|---|---|
| | No. of pts. | Value | No. of pts. | Value | p Value |
| **Sex (male/female)** | 10 | 8 (80 %) / 2 (20 %) | 22 | 17 (77 %) / 5 (33 %) | 0.863¿ |
| **Age (years)** | 10 | 46.7 ± 5.87 | 22 | 42.36 ± 2.07 | 0.389 |
| **NYHA** | | | | | |
| II | 10 | 1 (10 %) | 22 | 0 (0 %) | 0.132¿ |
| III | 10 | 7 (70 %) | 22 | 15 (68 %) | 0.918¿ |
| IV | 10 | 2 (20 %) | 22 | 7 (32 %) | 0.491¿ |
| **Cardiac parameters** | | | | | |
| LBBB (%) | 10 | 3 (30 %) | 22 | 5 (22.7 %) | 0.659¿ |
| Permanent AF (%) | 10 | 2 (20 %) | 22 | 0 (0 %) | 0.000¿* |
| LVEF (%) | 10 | 24.10 ± 2.28 | 22 | 23.05 ± 1.35 | 0.678 |
| LVEDD (cm) | 10 | 6.89 ± 0.17 | 22 | 6.89 ± 0.19 | 0.998 |
| LVEDDI (cm/m$^2$) | 10 | 3.68 ± 0.21 | 22 | 3.71 ± 0.09 | 0.847 |
| Mean Ao (mmHg) | 10 | 92.00 ± 3.95 | 22 | 86.06 ± 2.71 | 0.291 |
| Mean RAP (mmHg) | 10 | 16.22 ± 3.19 | 22 | 11.44 ± 1.74 | 0.164 |
| Mean PCWP (mmHg) | 10 | 25.00 ± 2.79 | 22 | 23.45 ± 2.70 | 0.731 |
| Mean PAP (mmHg) | 10 | 34.89 ± 4.33 | 22 | 32.95 ± 3.24 | 0.734 |
| CI (L/min/m2) | 10 | 2.38 ± 0.33 | 22 | 2.2 ± 0.14 | 0.573 |

Data are presented as the means ± SEM. * Significant at 0.05 level. ¿ Chi-square test. Abbreviations: NYHA – New York Heart Association functional class; LBBB – left bundle branch block; AF – atrial fibrillation; LVEF – left ventricular ejection fraction; LVEDD – left ventricular end-diastolic diameter; LVEDDI – left ventricular end-diastolic diameter index; Ao – aortic; RAP – right atrial pressure; PCWP – pulmonary capillary wedge pressure; PAP – pulmonary artery pressure; CI – cardiac index.

The prevalence of atrial fibrillation (AF) was significantly higher in inflammation negative group compared to inflammation positive 2 (20 %) versus 0 (0 %) respectively, $p < 0.001$, which might be explained by a lower degree of fibrosis (Fig. 50) in the former group (Table 1). There was no significant difference noticed in the prevalence of left bundle branch block on ECG among the groups 3 (30 %) versus 5 (22 %), $p = 0.659$.

Generally, main parameters of echocardiography demonstrated characteristic signs of DCM: reduced left ventricular ejection fraction (LVEF), dilatation of left ventricular. However, there were no significant differences in these parameters between the groups: LVEF 24.10 ± 2.2 % versus 23.05 ± 1.3 %, left ventricular end-diastolic diameter (LVEDD) 6.89 ± 0.17 cm versus 6.89 ± 0.19 cm, left ventricular end-diastolic diameter index (LVEDDI) 3.68 ± 0.21 cm/m$^2$ versus 3.71 ± 0.09 cm/m cm/m$^2$.

Cardiac catheterization was performed to access the severity of hemodynamic impairment. Although, hemodynamic parameters did not differ significantly between the groups, the average values of pulmonary artery pressure (PAP) in both groups: 34.89 ± 4.33 mmHg versus 32.95 ± 3.24 mmHg, p = 0.734) were increased, which confirms pulmonary hypertension (PH) diagnosis (PH is confirmed when the mean PAP is ≥25 mmHg at rest [246]). Elevated mean pulmonary capillary wedge pressure (PCWP) (>15 mmHg) among the groups 25.00 ± 2.79 mmHg versus 23.45 ± 2.70 mmHg, p = 0.731 shows post-capillary PH due to left heart impairment [247]. Elevated mean right atrial pressure (RAP), elevated mean PAP and decreased cardiac index (CI) in both groups indicate a worse prognosis [248].

Both patient groups received the same basic treatment according to guidelines (Table 8) [249-251]. No significant difference in prescribed medication was observed between the groups.

**Table 8 Basic treatment for the study patient groups (according ESC guidelines).**

| ***Conventional treatment of heart failure*** | ***Inflammation-negative group*** | ***Inflammation-positive group*** |
|---|---|---|
| ACE inhibitors | 5 (50 %) | 10 (46 %) |
| β-blockers | 10 (100 %) | 19 (87 %) |
| Digitalis (in atrial fibrillation) | 2 (20 %) | 7 (32 %) |
| Diuretics | 10 (100 %) | 22 (100 %) |
| Anticoagulation (atrial fibrillation, EF < 40 %) | 7 (70 %) | 15 (68 %) |
| Antiarrhythmics (class III: amiodarone) | 1 (10 %) | 5 (23 %) |
| **Interventions** | | |
| Implantable cardiac defibrillator (ICD) | 1 (10 %) | 0 (0 %) |
| Cardiac resynchronization therapy (CRT) | 1 (10 %) | 3 (14 %) |

### 5.2.3. Inflammatory markers in dilated cardiomyopathy

The inflammatory process was monitored by detecting $CD3^+$, $CD45Ro^+$ and $CD68^+$ expression in inflammatory infiltrates by immunohistochemistry. In addition, we determined the inflammatory cytokines TNF-α, IL-6 and IL-1β. Representative immunohistochemical micrographs show expression of $CD3^+$, $CD45Ro^+$ and $CD68^+$ from individual inflammatory DCM patients (Fig. 40, Fig. 41 and Fig. 42).

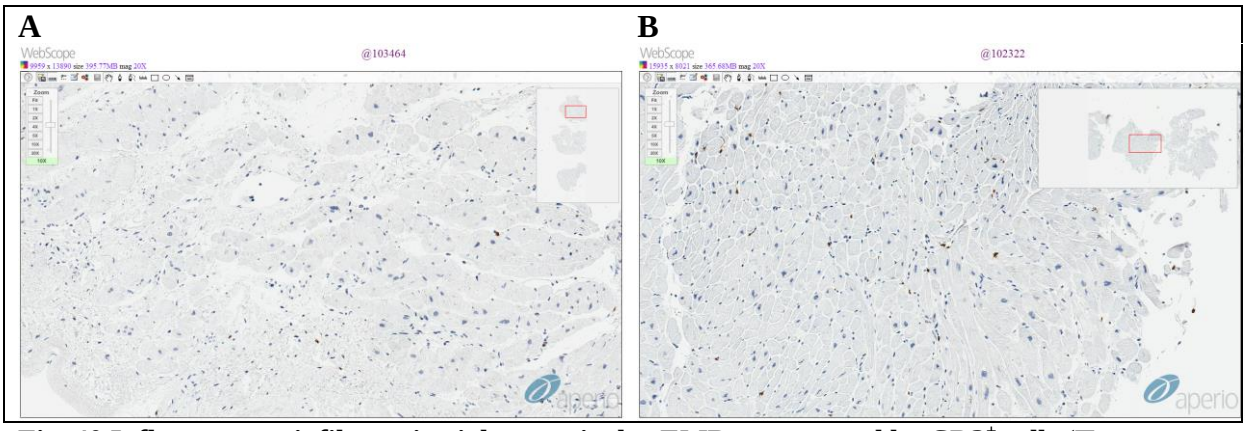


**Fig. 40 Inflammatory infiltrate in right ventricular EMB represented by $CD3^+$ cells (T lymphocytes). (A) inflammation-negative ($CD3^+$ = 3 cell/$mm^2$); (B) inflammation-positive ($CD3^+$ = 20 cell/$mm^2$). Immunohistochemical staining was performed using anti-CD3 antibodies. Magnification: x10.**

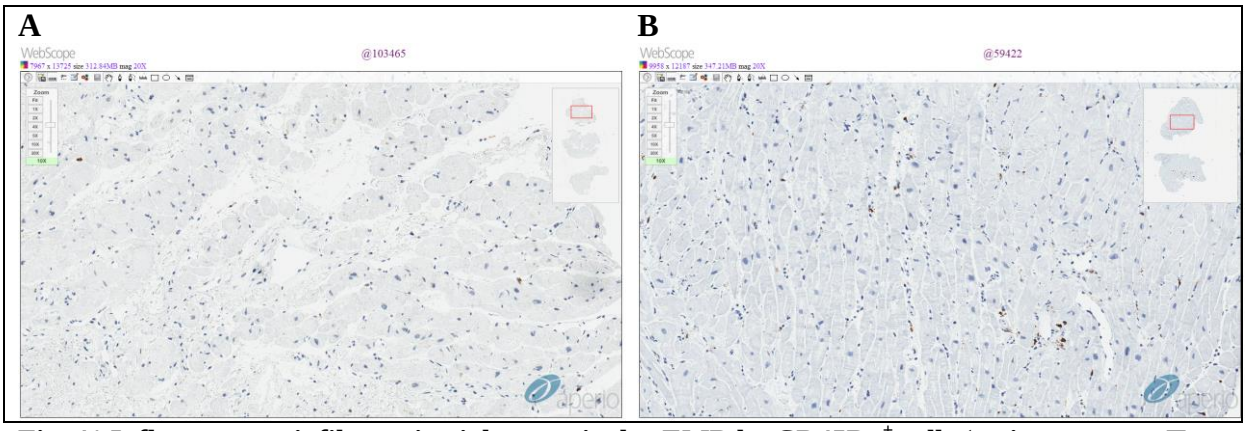


**Fig. 41 Inflammatory infiltrate in right ventricular EMB by $CD45Ro^+$ cells (active-memory T lymphocytes). (A) inflammation-negative ($CD45Ro^+$ = 3 cell/$mm^2$); (B) inflammation-positive ($CD45Ro^+$ = 17 cell/$mm^2$). Immunohistochemical staining was performed using anti-CD45Ro antibodies. Magnification: x10.**

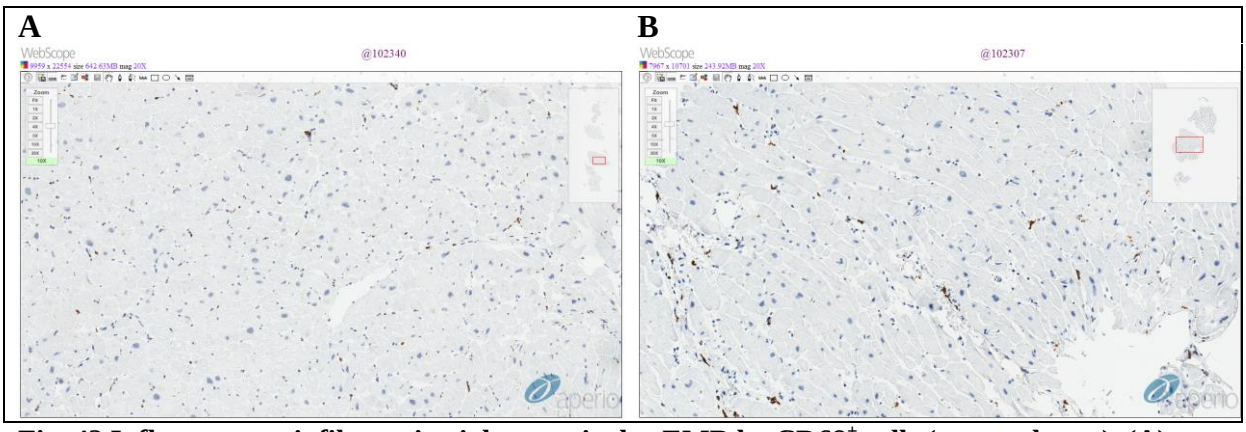


**Fig. 42 Inflammatory infiltrate in right ventricular EMB by $CD68^+$ cells (macrophages). (A) inflammation-negative ($CD68^+$ = 5 cell/$mm^2$); (B) inflammation-positive ($CD68^+$ = 20 cell/$mm^2$). Immunohistochemical staining was performed using anti-CD68 antibodies. Magnification: x10.**

Total expression of cytokines in infiltrates from inflammatory-negative and inflammatory-positive groups is shown in Fig. 43. The inflammatory infiltrates mostly

upregulated in inflammatory DCM patients were: T-lymphocytes ($CD3^+$) and active memory T-lymphocytes ($CD45Ro^+$) (2.38-fold and 2.1; $p < 0.001$ and $p < 0.01$, respectively), whereas the macrophages ($CD68^+$) were only enhanced 1.63 fold (Fig. 44). Significant accession of $CD3^+$ and $CD45Ro^+$ in inflammatory DCM myocardium also suggests increased myocardial micro-vascular permeability.

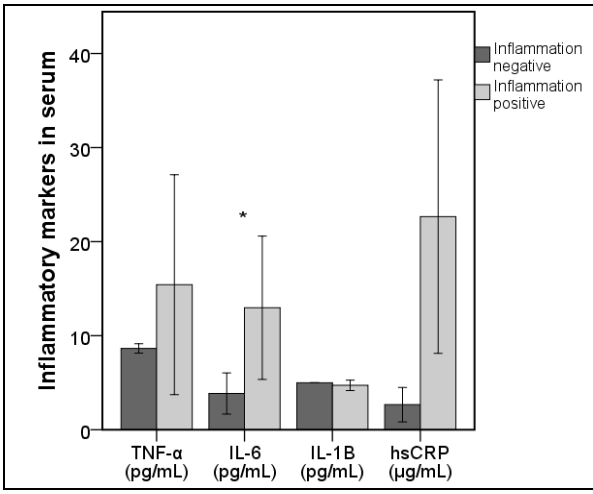


**Fig. 43 Levels of the inflammatory markers in serum. ELISA data are presented as means ± SEM from at least three independent measurements. Data were considered significant at *p < 0.05.**

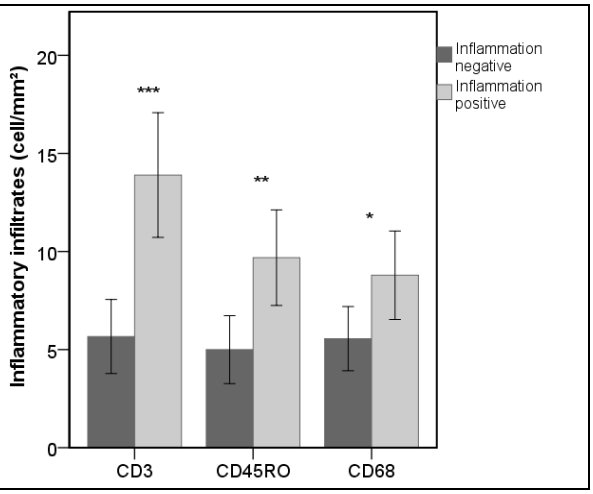


**Fig. 44 Levels of the inflammatory infiltrates in biopsy. Immunohistochemically positive stained cell count data are presented as means ± SEM from at least three independent locations, evaluated by highly experienced pathologist. Data were considered significant at *p < 0.05, **p < 0.01 and ***p < 0.001.**

Data in Table 9 summarize the upregulation of specific and general inflammatory markers interleukin-6 (IL-6) and high sensitivity C-reactive protein (hsCRP), respectively, in inflammatory DCM serum samples (3.45, and 2.76 folds). The mean value of the inflammatory cytokine IL-6 was three times increased in inflammatory DCM serum samples ($p < 0.05$). The tumor necrosis factor alpha (TNF-α) level in inflammatory-positive DCM serum samples was approximately two folds enhanced compared to non-inflammatory DCM patients, suggesting its possible activation of caspase-8 (Table 9). Interleukin-1beta (IL-1β), also known as catabolin, did not show significant changes in serum samples.

### 5.2.4. Changes of apoptotic biomarkers in inflammatory DCM samples

Correlation analysis of inflammatory cytokines and other secreted biomarkers pointed to importance of the intrinsic apoptotic mechanism in inflammatory DCM. Data presented in Fig. 45 show significant correlation between $CD3^+$ and IL-6.

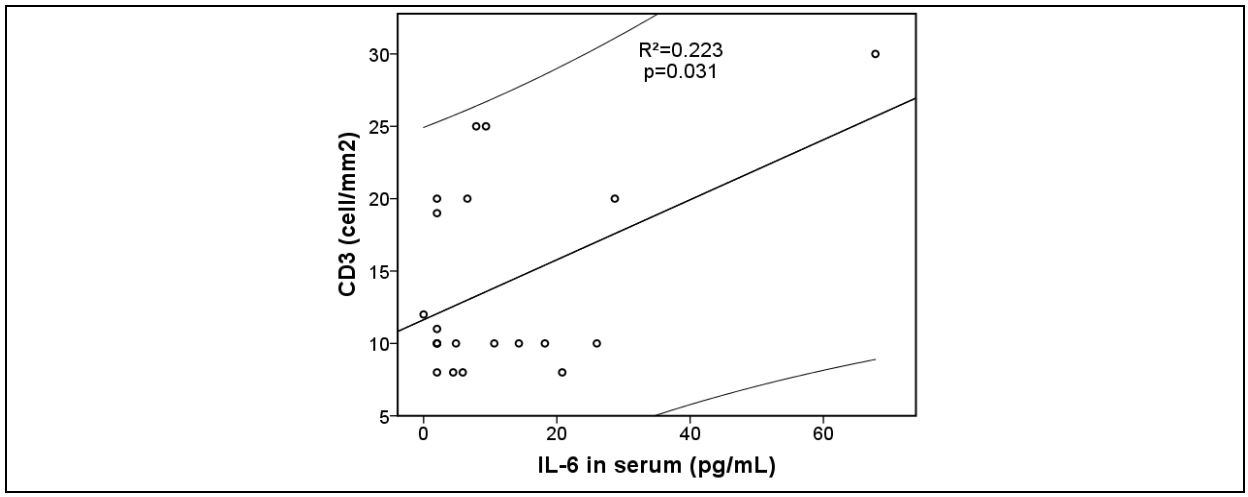


**Fig. 45 Correlation between inflammatory and mitochondrial membrane destabilization markers in inflammation-positive patient group. Correlation between serum inflammatory cytokine IL-6 and $CD3^+$ cell count on EMB. Correlation was significant at a level of $p < 0.05$. Linear regression line is presented within 95 % confidence interval. Regression coefficients ($R^2$) are shown in the graphs.**

Moreover, significant correlation of IL-6 and hsCRP with the mitochondrial chaperonic protein Hsp60 and pro-apoptotic Bax, respectively, in serums suggests that myocardial inflammation mostly affected integrity of mitochondrial membranes and activated Bax-dependent apoptotic pathway (Fig. 46 and Fig. 47).

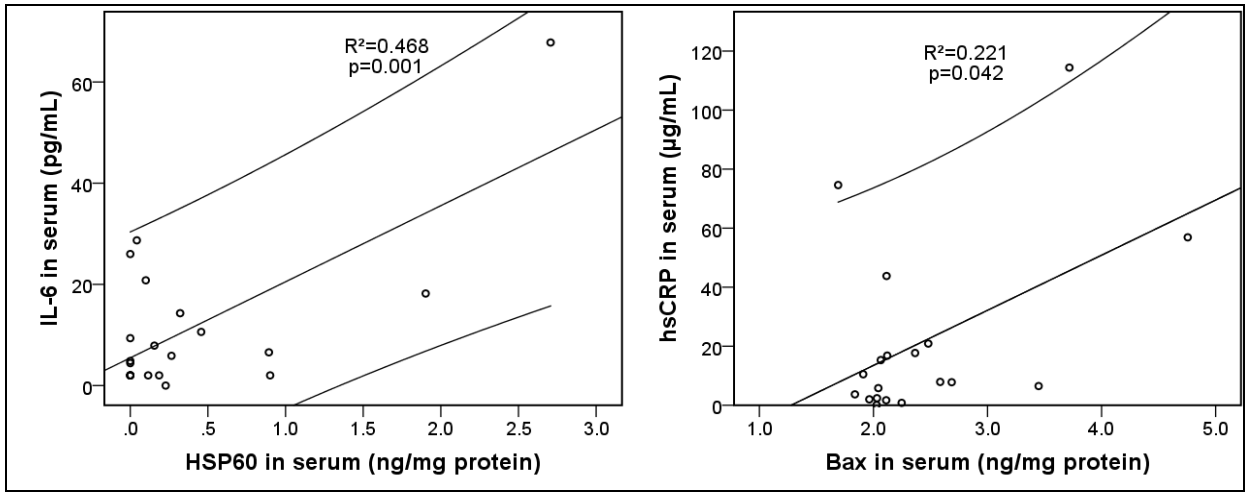


**Fig. 46 Correlation between inflammatory and mitochondrial membrane destabilization markers in inflammation-positive patient group. Correlation between IL-6 and mitochondrial membrane stabilizing chaperone Hsp60 in serums. Correlation was significant at a level of $p < 0.05$. Linear regression line is presented within 95 % confidence interval. Regression coefficients ($R^2$) are shown in the graphs.**

**Fig. 47 Correlation between inflammatory and mitochondrial membrane destabilization markers in inflammation-positive patient group. Correlation between high sensitivity C-reactive protein (hsCRP) and Bax in serums. Correlation was significant at a level of $p < 0.05$. Linear regression line is presented within 95 % confidence interval. Regression coefficients ($R^2$) are shown in the graphs.**

Additionally, the levels of the mitochondrial membrane stabilizing protein Bcl-2 and Hsp60 in inflammatory-positive serums were 1.86 and 8.97 ($p < 0.05$) folds higher, respectively, supporting the theory of increased permeability of both outer mitochondrial and myocardial cell plasma membranes in inflammatory DCM (Table 9). In parallel, Bcl-2 in inflammatory DCM biopsies was depressed 1.34 fold, whereas Bax was 1.22 fold increased, revealing activation of the Bcl-2/Bax-dependent apoptotic pathway (Table 9). Parallelly, the level of APO1/Fas/CD95 (FasR), a main receptor of the extrinsic apoptotic pathway, was only slightly increased in serum and biopsy samples from inflammatory DCM patients, whereas Fas ligand (FasL) in biopsies was even down-regulated (Table 9).

Data presented in Fig. 48 demonstrate statistically significant ($p < 0.05$) increase of caspase-9, -8 and -3 in serums with most prominent expression of caspase-9. Increased expression of the same caspases in endomyocardial biopsy samples (Fig. 49) was not statistically significant due to high variation. However, the basic mean value of caspase-9 in inflammatory DCM patients was 31 and 118 folds higher compared to caspase-8 and -3, respectively, additionally pointing on the higher role of intrinsic apoptotic pathway in inflammatory DCM (Fig. 49).

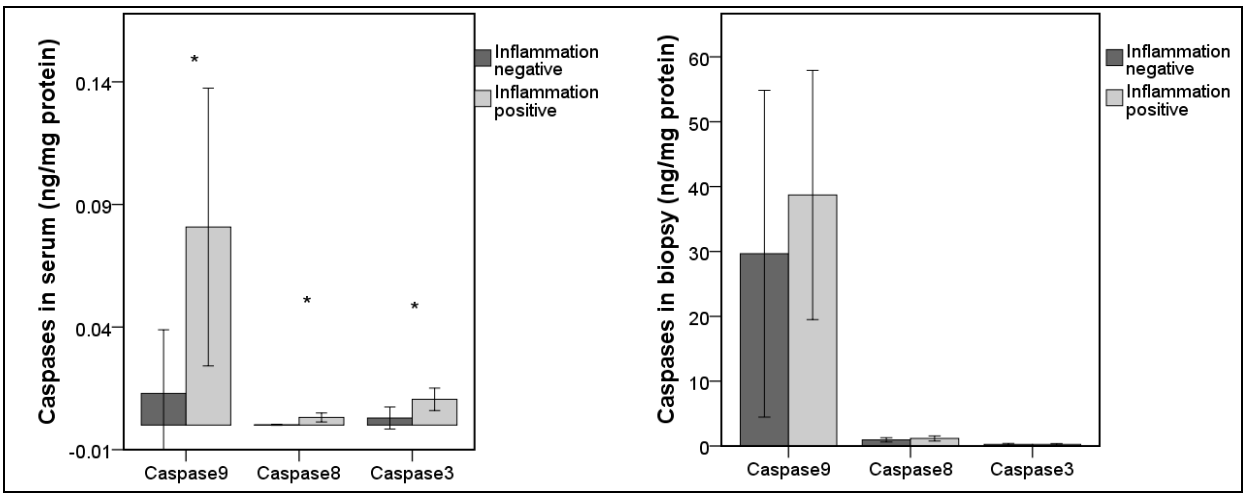


**Fig. 48 Levels of pro-caspases-9, -8, and -3 in serum samples. Data are presented as means ± SEM from at least three independent measurements. Data were considered significant at *p < 0.05.**




**Fig. 49 Levels of pro-caspases-9, -8, and -3 in EMB samples. Data are presented as means ± SEM from at least three independent measurements.**

**Table 9 Summarized data of measured biomarkers.**

| *Variable* | *Inflammation-negative group* | | *Inflammation-positive group* | | |
|---|---|---|---|---|---|
| | No. of pts. | Value | No. of pts. | Value | p Value |
| **Markers of inflammation in serum** | | | | | |
| TNF-α (pg/mL) | 8 | 7.9313 ± 0.5106 | 21 | 14.2819 ± 5.0280 | 0.223 |
| IL-6 (pg/mL) | 8 | 3.3938 ± 0.8554 | 21 | 11.4038 ± 3.3614 | 0.031* |
| IL-1β (pg/mL) | 8 | 5.0000 ± 0.0000 | 21 | 4.7619 ± 0.2381 | 0.329 |
| hsCRP (μg/mL) | 8 | 7.6875 ± 5.0460 | 19 | 21.5563 ± 6.9633 | 0.066¡ |
| **Markers of apoptosis in serum** | | | | | |
| Bcl2 (ng/mg protein) | 10 | 0.0288 ± 0.0288 | 22 | 0.0536 ± 0.0455 | 0.889¡ |
| Bax (ng/mg protein) | 10 | 2.1527 ± 0.2400 | 22 | 2.3354 ± 0.1606 | 0.535 |
| Caspase-9 (ng/mg protein) | 10 | 0.0130 ± 0.0013 | 22 | 0.0808 ± 0.0283 | 0.038* |
| Caspase-8 (ng/mg protein) | 10 | 0.0010 ± 0.0001 | 22 | 0.0031 ± 0.0009 | 0.043*¡ |
| Caspase-3 (ng/mg protein) | 10 | 0.0029 ± 0.0022 | 22 | 0.0105 ± 0.0023 | 0.025* |
| APO1/Fas/CD95 (ng/mg protein) | 10 | 0.0000 ± 0.0000 | 22 | 0.0000 ± 0.0000 | 0.857¡ |
| FasL (ng/mg protein) | 10 | 0.0000 ± 0.0000 | 22 | 0.0000 ± 0.0000 | N.A. |
| HSP60 (ng/mg protein) | 10 | 0.0419 ± 0.0253 | 22 | 0.3760 ± 0.1468 | 0.035* |
| **Markers of apoptosis in biopsy** | | | | | |
| Bcl2 (ng/mg protein) | 10 | 83.5523± 26.2936 | 21 | 63.8790 ± 17.2137 | 0.540 |
| Bax (ng/mg protein) | 10 | 5.6452 ± 2.6905 | 21 | 6.8873 ± 3.7924 | 0.724¡ |
| Caspase-9 (ng/mg protein) | 10 | 29.6575 ± 12.5969 | 21 | 38.7122 ± 9.6108 | 0.950¡ |
| Caspase-8 (ng/mg protein) | 10 | 0.9483 ± 0.1640 | 21 | 1.1611 ± 0.1962 | 0.413 |
| Caspase-3 (ng/mg protein) | 10 | 0.2503± 0.0773 | 21 | 0.2586 ± 0.0649 | 0.935 |
| APO1/Fas/CD95 (ng/mg protein) | 10 | 3.4651 ± 0.6568 | 21 | 4.1921 ± 0.6607 | 0.443 |
| FasL (ng/mg protein) | 10 | 4.5550 ± 1.3594 | 21 | 4.0588 ± 1.1083 | 0.780 |
| HSP-60 (ng/mg protein) | 10 | 24.1262 ± 6.9102 | 21 | 19.2656± 4.5617 | 0.565 |
| **Marker of heart tissue contraction in serum** | | | | | |
| hsTnT (pg/mL) | 8 | 35.4988 ± 9.0908 | 20 | 66.4145 ± 26.9755 | 0.289 |
| **Markers of extracellular matrix degradation in serum** | | | | | |
| MMP9 (ng/mg protein) | 10 | 1.3867 ± 0.0674 | 22 | 1.5261 ± 0.0508 | 0.115 |
| TIMP1 (ng/mg protein) | 10 | 5.9610 ± 0.3597 | 22 | 6.1223 ± 0.1497 | 0.686 |
| MMP9/TIMP1 | 10 | 0.2355 ± 0.0090 | 22 | 0.2511 ± 0.0086 | 0.223 |
| **Markers of extracellular matrix degradation in biopsy** | | | | | |
| MMP9 (ng/mg protein) | 10 | 2.3698 ± 1.1931 | 21 | 2.7630 ± 0.9394 | 0.798 |
| TIMP1 (ng/mg protein) | 10 | 9.4917 ± 1.7605 | 21 | 7.8056 ± 1.4029 | 0.462 |
| MMP9/TIMP1 | 10 | 0.1931 ± 0.0729 | 21 | 0.4760 ± 0.1048 | 0.035* |
| **Other measurements** | | | | | |
| BNP (pg/mL) | 10 | 1277.8500 ± 428.5054 | 22 | 1603.2591 ± 276.3777 | 0.532 |
| Adiponectin (μg/mL) | 8 | 24.1000 ± 3.5914 | 21 | 22.9048 ± 3.5287 | 0.815 |
| Galectin-3 (ng/mL) | 10 | 12.4670 ± 1.9009 | 22 | 12.3895 ± 0.8635 | 0.971 |
| **Cardiac fibrosis (%)** | 10 | 15.8447 ± 2.6319 | 22 | 14.5632 ± 1.9258 | 0.699 |

Data are presented as the means ± SEM. * Significant at 0.05 level. ¡ Wilcoxon–Mann–Whitney rank sum nonparametric test. Abbreviations: TNF-α—tumor necrosis factor α; IL-6 – interleukin-6; IL-1β – interleukin 1β; Bcl-2 – B-cell lymphoma 2 protein; Bax – Bcl-2–associated X protein; Hsp60 – heat shock protein 60; MMP9 – matrix metalloproteinase 9; TIMP1 – tissue inhibitor of matrix metalloproteinase 1; TNF-a – tumor necrosis factor-alfa; IL-1β – interleukin 1 beta; IL-6 – interleukin 6; hs TnT – high sensitivity troponin T; hsCRP – high sensitivity C-reactive protein, N.A. – not available.

### 5.2.5. Induction of necrosis in inflammatory DCM samples

The mean value of the high-sensitivity troponin T (hsTnT), a major structural sarcomeric protein in the heart, was two folds upregulated in serums of inflammatory-positive patient group (Table 9). The release of hsTnT from the myocardium revealed chronic structural and functional degradation of myocardium that corresponded to a decreased level of fibrosis in inflammatory DCM biopsies (Fig. 50 and Fig. 51).

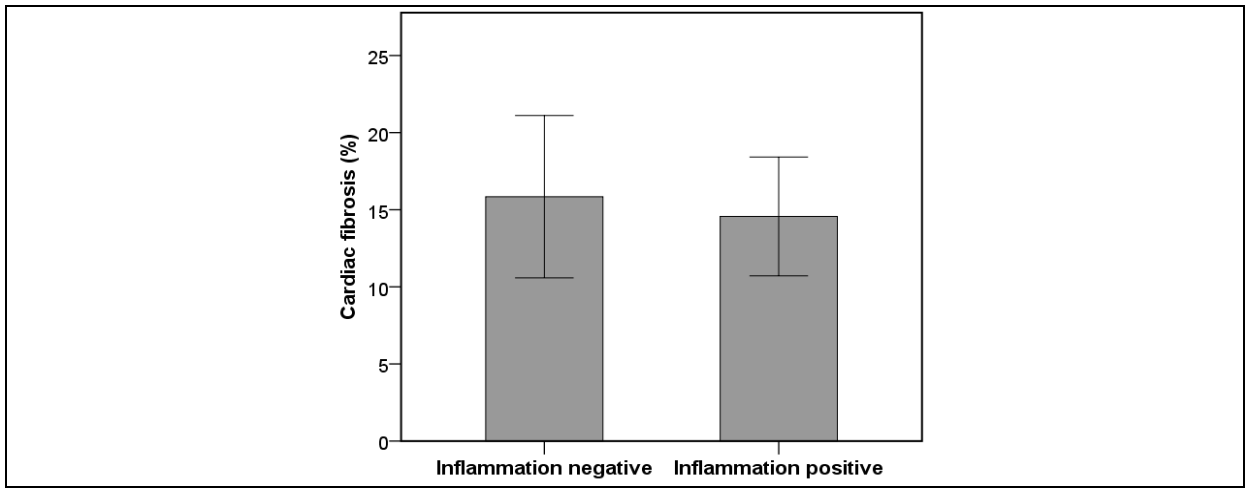


**Fig. 50 Cardiac fibrosis in right ventricular EMB. Data are presented as means ± SEM from at least three independent measurements.**

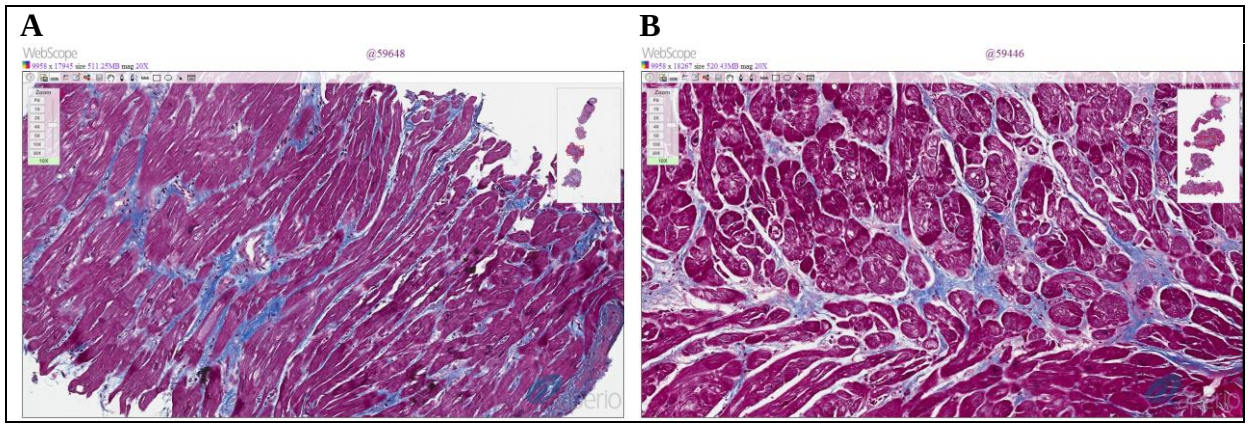


**Fig. 51 Micrographs show one representative picture from one patient of each group. (A) inflammation-negative (quantified fibrosis – 20.34 %); (B) inflammation-positive (quantified fibrosis – 18.65 %). Masson trichrome staining for cardiac fibrosis (collagen is colored blue). High amounts of collagen reflect a high level of fibrosis. Magnification: x10.**

Additionally, there was no necrosis in the samples assayed by histological analysis in myocardial biopsies (Fig. 52). The two fold of hsTnT upregulation in tested inflammatory DCM serums suggests a steady hsTnT release and slow impairment of striated muscle contraction rather than induction of necrosis in inflammatory DCM.

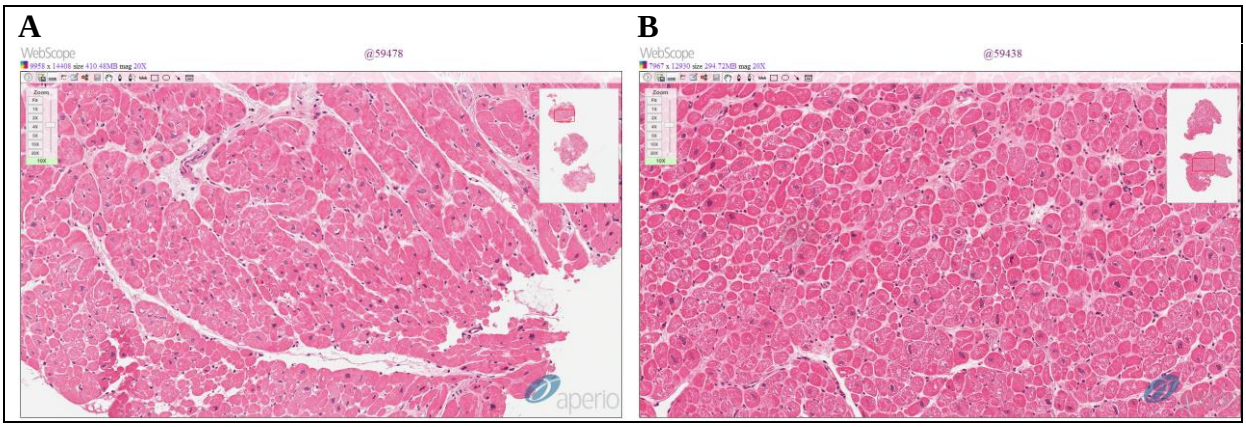


**Fig. 52 Inflammation representation in right ventricular EMB. Micrographs show one representative picture from one patient of each group. (A) inflammation-negative; (B) inflammation-positive. Hematoxylin and Eosin staining. Magnification: x10.**

### 5.2.6. The interaction between apoptotic pathways supporting progression of inflammatory DCM

Data in Fig. 53 demonstrate that caspase-9, a serum cysteine-aspartic acid specific protease, named apoptosis-initiating caspase, strongly correlated with the general inflammatory marker high sensitivity C-reactive protein (hsCRP) additionally confirming sensitivity of intrinsic apoptotic pathway to inflammation.

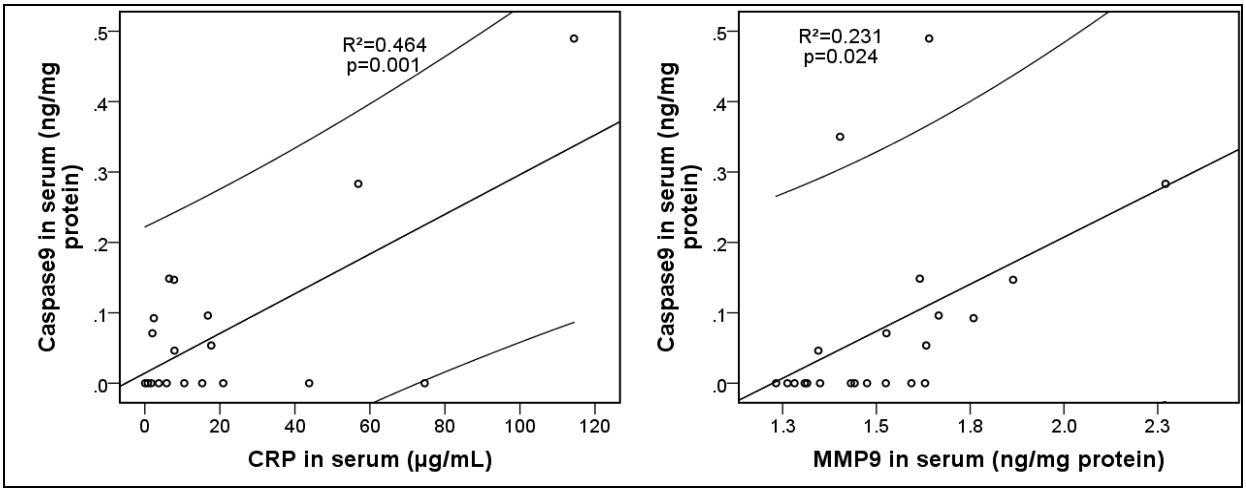


**Fig. 53 Correlation of caspase-9 with biomolecules in inflammation-positive patient group serum samples. Correlation of caspase-9 with high sensitivity C-reactive protein (hsCRP) in serum samples. Linear regression line is presented within 95 % confidence interval. Coefficients of regression ($R^2$) and statistical significance ($p < 0.05$) are shown in the graphs.**




**Fig. 54 Correlation of caspase-9 with biomolecules in inflammation-positive patient group serum samples. Correlation of caspase-9 with matrix metalloproteinase-9 (MMP-9) in serum samples. Linear regression line is presented within 95 % confidence interval. Coefficients of regression ($R^2$) and statistical significance ($p < 0.05$) are shown in the graphs.**

In parallel, the correlation between caspase-9 and MMP9 (Fig. 54) tells us that caspase-9 might either be directly activated by the MMP9 or, alternatively, through other mediators of intrinsic apoptotic pathways, such as Bcl-2 and Bax (Fig. 55 and Fig. 56; $p < 0.05$).

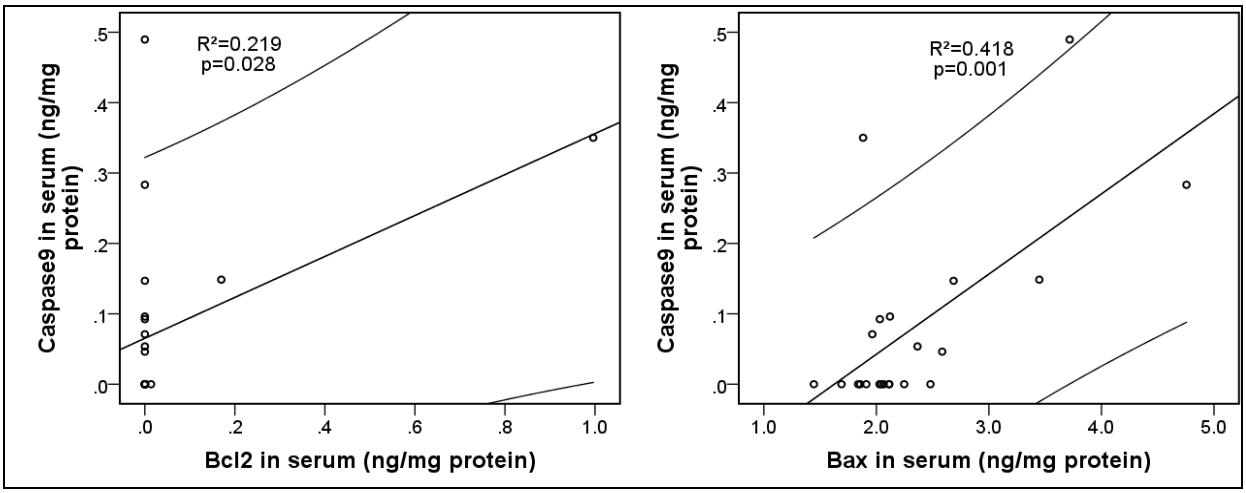


**Fig. 55 Correlation of caspase-9 with biomolecules in inflammation-positive patient group serum samples. Correlation of caspase-9 with B-cell lymphoma 2 protein (Bcl-2) in serum samples. Linear regression line is presented within 95 % confidence interval. Coefficients of regression ($R^2$) and statistical significance ($p < 0.05$) are shown in the graphs.**

**Fig. 56 Correlation of caspase-9 with biomolecules in inflammation-positive patient group serum samples. Correlation of caspase-9 with Bcl-2–associated X protein (Bax) in serum samples. Linear regression line is presented within 95 % confidence interval. Coefficients of regression ($R^2$) and statistical significance ($p < 0.05$) are shown in the graphs.**

Data in Fig. 57 and Fig. 58 also demonstrate that intrinsic apoptotic pathway-initiating caspase-9 is not the only caspase participating in regulation of myocardium destruction. A strong correlation ($p < 0.05$) between caspase-9 and executing caspase-3, and extrinsic apoptotic pathway-initiating caspase-8 suggest an interaction between intrinsic and extrinsic apoptotic pathways.

Furthermore, Fig. 59 shows a statistically significant correlation between main players of extrinsic apoptotic pathway caspase-8 and the APO1/Fas/CD95 in inflammatory DCM serums. The significant correlation between caspase-8 and Bax additionally confirms the intersection of extrinsic and intrinsic pathways at mitochondrial level with particular role of pro-apoptotic Bax in it (Fig. 60).

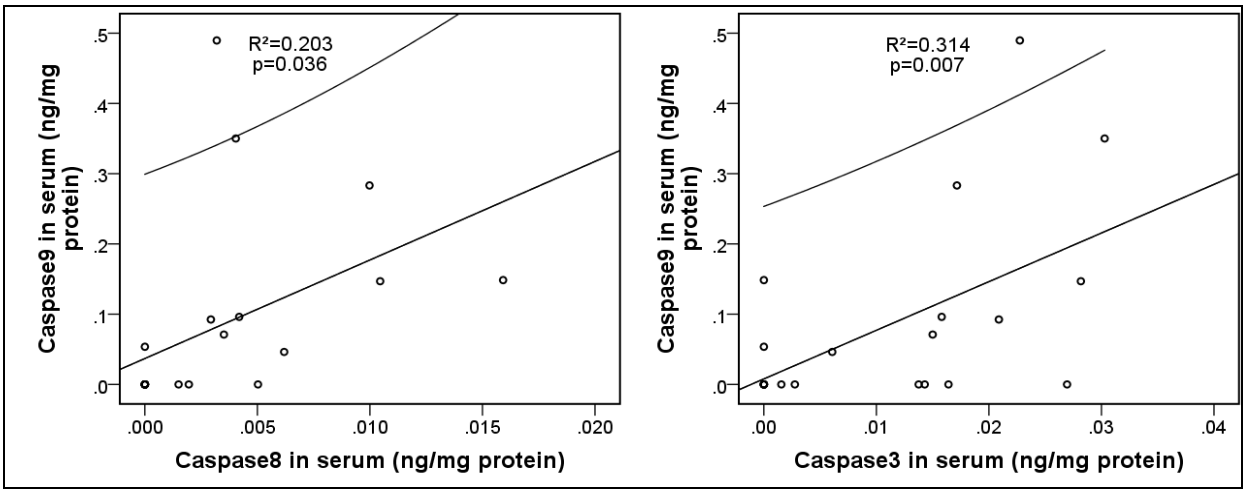


**Fig. 57 Correlation of caspase-9 with biomolecules in inflammation-positive patient group serum samples. Correlation of caspase-9 with Caspase-8 in serum samples. Linear regression line is presented within 95 % confidence interval. Coefficients of regression ($R^2$) and statistical significance ($p < 0.05$) are shown in the graphs.**




**Fig. 58 Correlation of caspase-9 with biomolecules in inflammation-positive patient group serum samples. Correlation of caspase-9 with Caspase-3 in serum samples. Linear regression line is presented within 95 % confidence interval. Coefficients of regression ($R^2$) and statistical significance ($p < 0.05$) are shown in the graphs.**

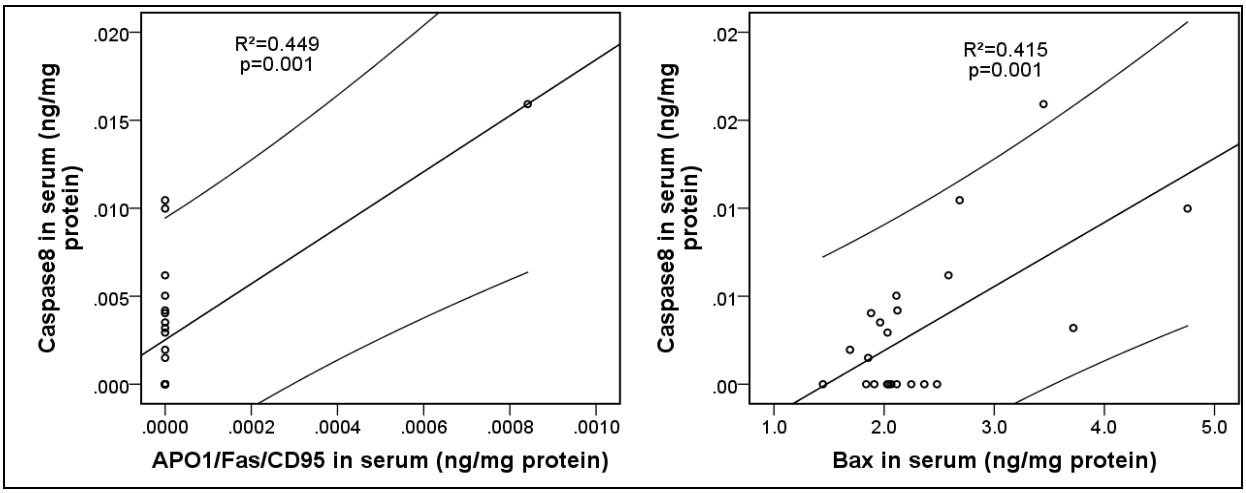


**Fig. 59 Correlation between caspase-8 and biomolecules in inflammation-positive patient group serum samples. Correlation of caspase-8 with Fas receptor (APO1/Fas/CD95) in serum samples. Linear regression line is presented within 95 % confidence interval. Coefficients of regression ($R^2$) and statistical significance ($p < 0.05$) are shown in the graphs.**




**Fig. 60 Correlation between caspase-8 and biomolecules in inflammation-positive patient group serum samples. Correlation of caspase-8 with Bcl-2–associated X protein (Bax) in serum samples. Linear regression line is presented within 95 % confidence interval. Coefficients of regression ($R^2$) and statistical significance ($p < 0.05$) are shown in the graphs.**

Additionally, a correlation between caspase-8 and MMP9, similarly to caspase-9, suggested an involvement of matrix metalloproteinases, in direct pro-caspases' activation and/or in the relocation of proapoptotic proteins into the extracellular space (Fig. 61).

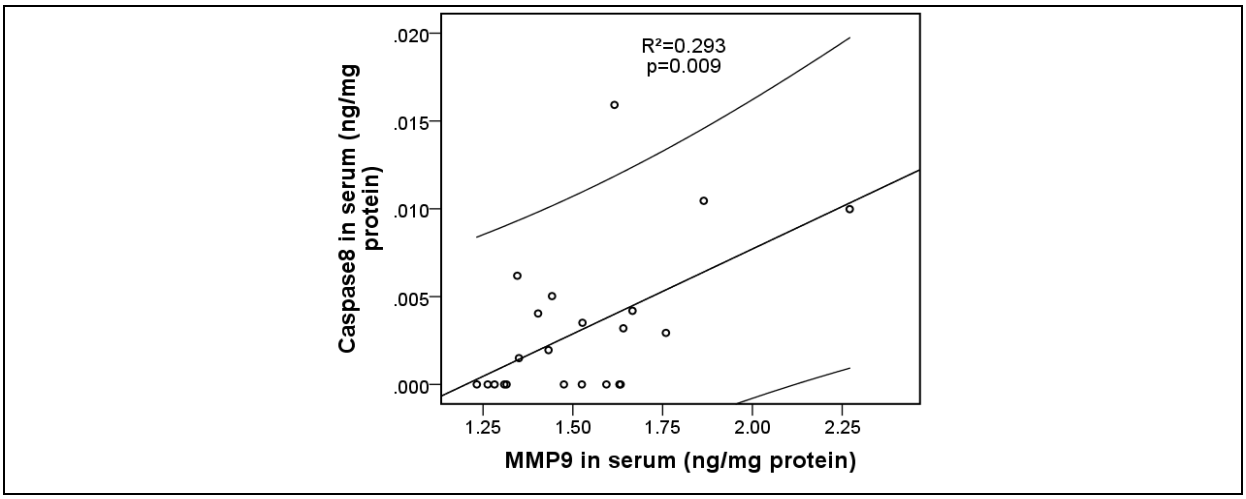


**Fig. 61 Correlation between caspase-8 and biomolecules in inflammation-positive patient group serum samples. Correlation of caspase-8 with matrix metalloproteinase-9 (MMP-9) in serum samples. Linear regression line is presented within 95 % confidence interval. Coefficients of regression ($R^2$) and statistical significance ($p < 0.05$) are shown in the graphs.**

### 5.2.7. The expression of apoptotic biomarkers in myocardial tissue

Next, it was investigated if secretion of apoptotic biomarkers to the blood reflects similar processes in inflammatory DCM myocardium. Caspase-9 in heart tissue, similarly to that in serum, had a high correlation with inflammation, particularly inflammatory cytokine IL-6, whereas caspase-8 showed more prominent correlation with Bax and caspase-3 (see caspase-8 and -9 correlations in Table 10). An executing caspase-3 demonstrated a significant correlation with activators of both intrinsic (Bax, Bcl-2) and extrinsic (APO1/Fas/CD95 and FasL) pathways (see caspase-3 correlation in Table 3). Similarly to serums, members of both apoptotic pathways (Bcl-2, and APO1/Fas/CD95, FasL) in biopsies had significant correlation with MMP9 and its inhibitor TIMP1 (Table 10). We also observed a strong ($p < 0.001$) correlation between changes of Bax in biopsies and Bax in sera (data not shown). Finally, the sarcomeric protein hsTnT in inflammatory DCM sera also strongly correlated with caspases-8, Bax and caspase-3 in biopsies, suggesting the present apoptotic pathway to be mostly involved in caspase-regulated release of hsTnT (see hsTnT correlation in Table 10).

**Table 10 Correlation of apoptotic, necrotic and inflammatory biomarkers.**

| | Caspase 9 in biopsy | Caspase 8 in biopsy | Caspase 3 in biopsy | Bcl2 in biopsy | FasR in biopsy | FasL in biopsy | Bax in biopsy |
|---|---|---|---|---|---|---|---|
| Caspase 8 in biopsy | 0.303 | | | | | | |
| Caspase 3 in biopsy | 0.063 | **0.436*** | | | | | |
| Bcl2 in biopsy | -0.202 | 0.175 | **0.486*** | | | | |
| FasR in biopsy | -0.097 | -0.074 | **0.526*** | **0.739**** | | | |
| FasL in biopsy | -0.046 | 0.007 | **0.442*** | **0.835**** | **0.907**** | | |
| MMP9 in biopsy | -0.229 | 0.024 | 0.419 | **0.764**** | **0.730**** | **0.824**** | |
| TIMP1 in biopsy | -0.012 | -0.205 | 0.213 | **0.517*** | **0.795**** | **0.722**** | |
| Bax in biopsy | 0.283 | **0.584**** | **0.678**** | 0.056 | 0.139 | 0.053 | |
| IL-6 in serum | **0.518*** | -0.016 | -0.011 | -0.262 | -0.202 | -0.154 | 0.131 |
| hsTnT in serum | 0.434 | **0.598**** | **0.563*** | -0.125 | -0.067 | -0.165 | **0.954**** |

Two tailed significance: *p < 0.05; **p < 0.01. Significant correlations are in bold phase type. Abbreviations: IL-6 – interleukin-6; Bcl-2 – B-cell lymphoma 2 protein; FasR—Fas receptor; FasL – Fas ligand; MMP9 – matrix metalloproteinase 9; TIMP1 – tissue inhibitor of matrix metalloproteinase 1; Bax – Bcl-2–associated X protein; Hsp60 – heat shock protein 60; hsTnT – high sensitivity troponin T.

### *5.3. Quantification of myocardial fibrosis by digital image analysis and interactive stereology*

A total of 116 slides were analyzed digitally, by visual scoring and using stereology grids.

The mean result of fibrosis obtained by Colocalization software was 13.72 ± 1.14 % being closest to the reference value of stereology (RVS: 13.21 ± 1.42 %). The mean values obtained by the Genie software (11.60 ± 1.43 %) and the pathologist's score at week 0 (11.20 ± 1.44 %) and week 2 (10.76 ± 1.61 %) indicated a slight underestimation relative to RVS. However, the range of Colocalization software was 73.79 % being the lowest of all tested methods with a difference of around 20 %. The range of the Genie software was 88.22 % and the pathologist's score had the highest range of 100 %. These results were comparable to the range of the RVS (96.50 %), Table 11.

**Table 11 Summary statistics for cardiac fibrosis (%) evaluation methods.**

| | Stereology | Colocalization | Genie | Pathologist week 0 | Pathologist week 2 |
|---|---|---|---|---|---|
| Number of observations | 116 | 116 | 116 | 116 | 116 |
| Mean | 13.21 | 13.72 | 11.60 | 11.20 | 10.76 |
| Median | 8.70 | 11.12 | 7.39 | 5.00 | 5.00 |
| Std. Error of Mean | 1.42 | 1.14 | 1.43 | 1.44 | 1.61 |
| Range | 96.50 | 73.79 | 88.22 | 100.00 | 100.00 |
| Minimum | 0.00 | 1.57 | 0.05 | 0.00 | 0.00 |
| Maximum | 96.50 | 75.36 | 88.27 | 100.00 | 100.00 |

Both the Colocalization and Genie methods correlated very strongly with the RVS cardiac fibrosis estimates, yielding $R = 0.928$ and $R = 0.946$ ($p < 0.001$), respectively. Similarly, the pathologist's visual score strongly correlated with RVS: $R = 0.913$ ($p < 0.001$) at week 0 and $R = 0.929$ ($p < 0.001$) at week 2 (Table 12).

**Table 12 Pairwise correlations between stereology, digital algorithms and pathologist score (Pearson's coefficients, $p < 0.001$, $n = 116$).**

| | Stereology | Colocalization | Genie | Pathologist week 0 |
|---|---|---|---|---|
| Colocalization | 0.928 | | | |
| Genie | 0.946 | 0.973 | | |
| Pathologist week 0 | 0.913 | 0.839 | 0.841 | |
| Pathologist week 2 | 0.929 | 0.853 | 0.856 | 0.965 |

Friedman's test revealed statistically significant differences in the results of tested cardiac fibrosis evaluation methods $\chi^2(3) = 62.405$, $p = 0.000$. Post hoc analysis with Wilcoxon signed-rank tests with a Bonferroni correction (significance level set at $p < 0.0125$) was applied. The differences in the results of Colocalization *versus* RVS were statistically insignificant ($Z = -2.259$, $p = 0.024$) with a mean difference value of 0.50 %. However, post hoc analysis showed significant differences between the results of Genie *versus* RVS ($Z = -5.000$, $p = 0.000$) and the pathologist's mean score *versus* RVS ($Z = -4.422$, $p = 0.000$) with mean difference values of: -1.61 % and 2.24 %. Similarly significant difference of the results between both digital methods (Genie *versus* Colocalization) was noted: $Z = -6.639$, $p = 0.000$ with a variance bias of 2.11 % (Table 13).

**Table 13 Paired comparison of cardiac fibrosis (%) evaluation methods.**

| | | Paired Differences | | *Z** | p Value* |
|---|---|---|---|---|---|
| | | Mean | Std. Error Mean | | |
| Pair 1 | Colocalization – Stereology | 0.50 | 0.56 | -2.259 | 0.024 |
| Pair 2 | Genie – Stereology | -1.61 | 0.47 | -5.000 | 0.000 |
| Pair 3 | Pathologist mean – Stereology | 2.24 | 0.56 | -4.422 | 0.000 |
| Pair 4 | Colocalization – Genie | 2.11 | 0.42 | -6.639 | 0.000 |

* Based on post hoc analysis with Wilcoxon signed-rank tests (Bonferroni correction applied with significance level set at $p < 0.0125$).

Single linear regression model plots demonstrated some advantage of Genie software over the Colocalization software with noticeably better values in both original raw and log-transformed measurements for R-square 0.896 and 0.804 (log) *versus* 0.861 and 0.707 (log); slope 0.956 and 1.222 (log) *versus* 0.745 and 0.639 (log); intercept -1.033 and -0.860 (log) *versus* 3.875 and 0.972 (log) (Fig. 62 and Fig. 63).

The pathologist's mean score correlation with RVS was similar: R-square 0.864 and 0.684 (log), slope 0.994 and 0.838 (log), intercept -2.155 and 0.062 (log); the inter-observer variation at week 0 and week 2 was negligible: R-square 0.931 and 0.824 (log), slope 1.079 and 0.939 (log), intercept -1.328 and -0.020 (log). Surprisingly, both digital methods did not correlate as well as expected with still

acceptable R-square values (0.947 and 0.794 (log)), but high intercept (4.744 and 1.500 (log)) and slope far from ideal (0.773 and 0.486 (log)) (Fig. 64, Fig. 65 and Fig. 66).

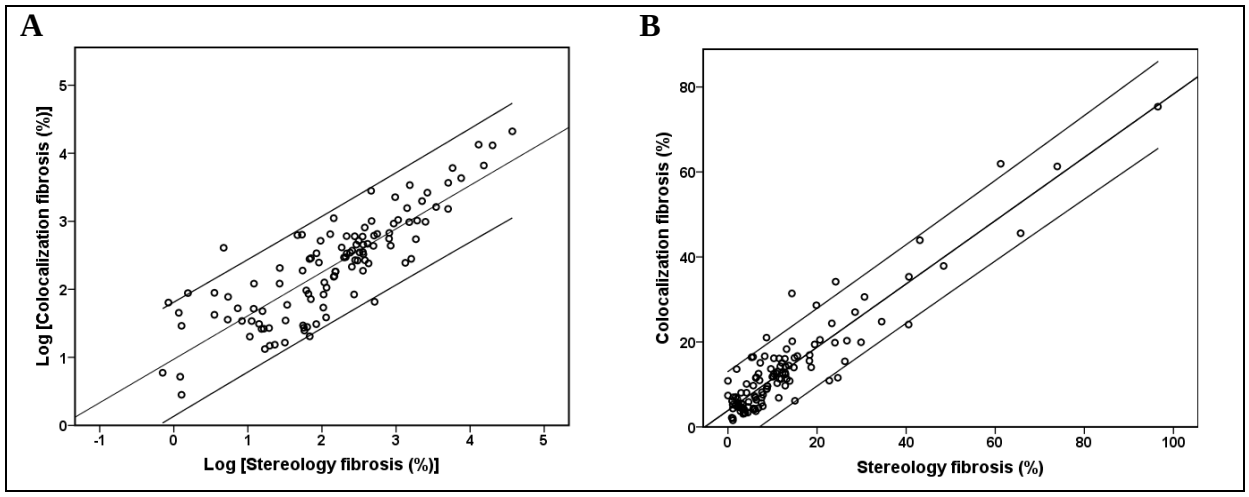


**Fig. 62 Single linear regression models with reference values. Original raw (A) and log-transformed measurements (B) for Colocalization and Stereology. Linear regression line is presented within 95 % confidence interval.**

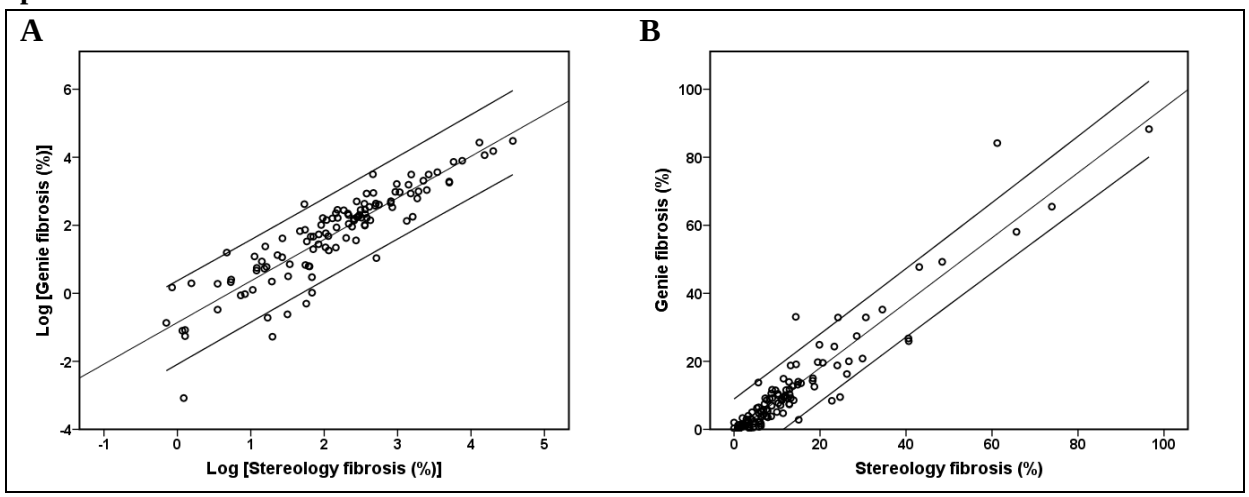


**Fig. 63 Single linear regression models with reference values. Original raw (A) and log-transformed measurements (B) for Genie and Stereology. Linear regression line is presented within 95 % confidence interval.**

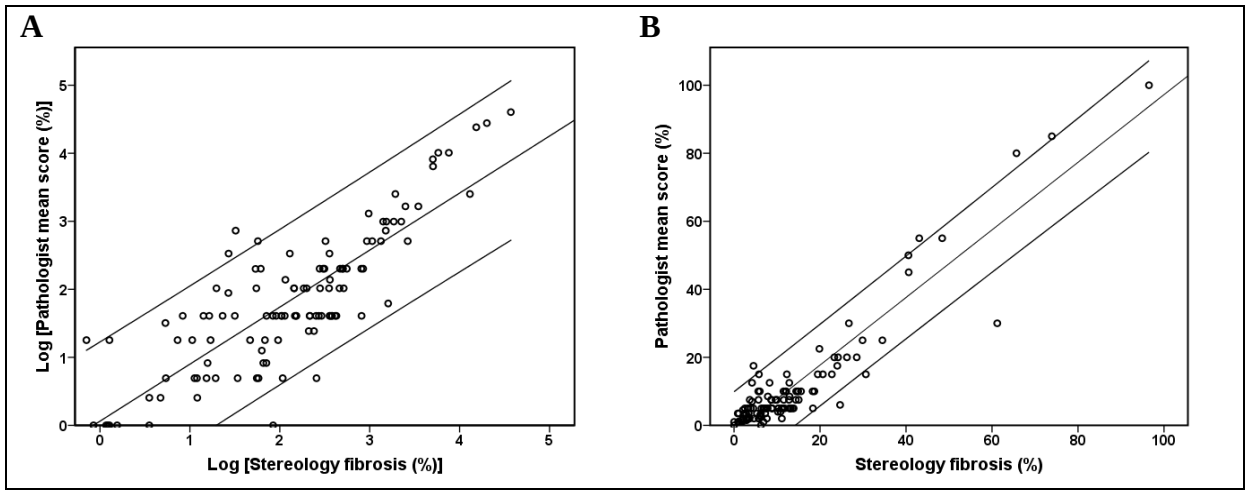


**Fig. 64 Single linear regression models with reference values. Original raw (A) and log-transformed measurements (B) for Pathologist mean score and Stereology. Linear regression line is presented within 95 % confidence interval.**

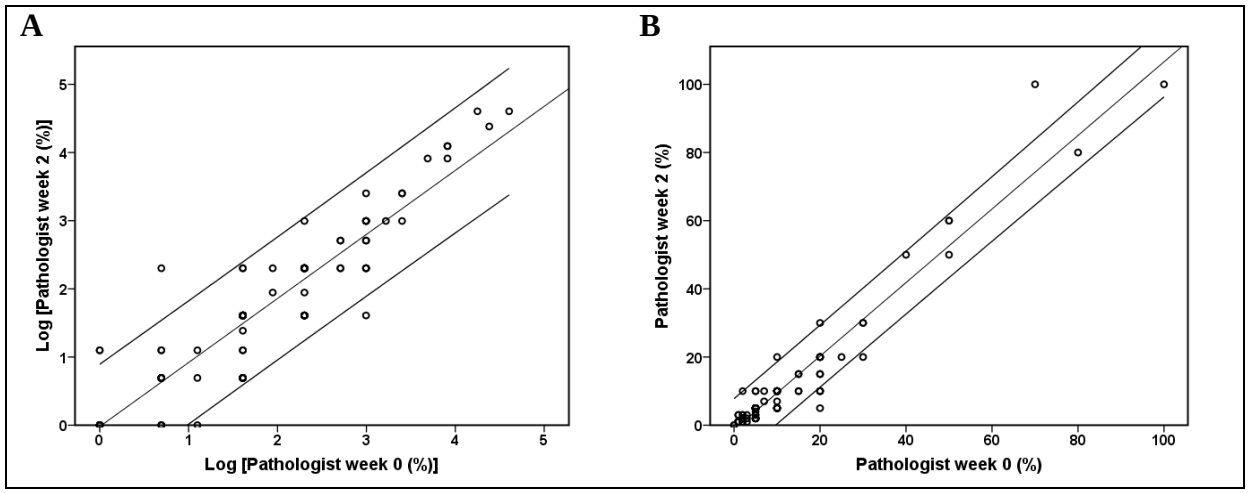


**Fig. 65 Single linear regression models with reference values. Original raw (A) and log-transformed measurements (B) for Pathologist score at week 0 and week 2. Linear regression line is presented within 95% confidence interval.**

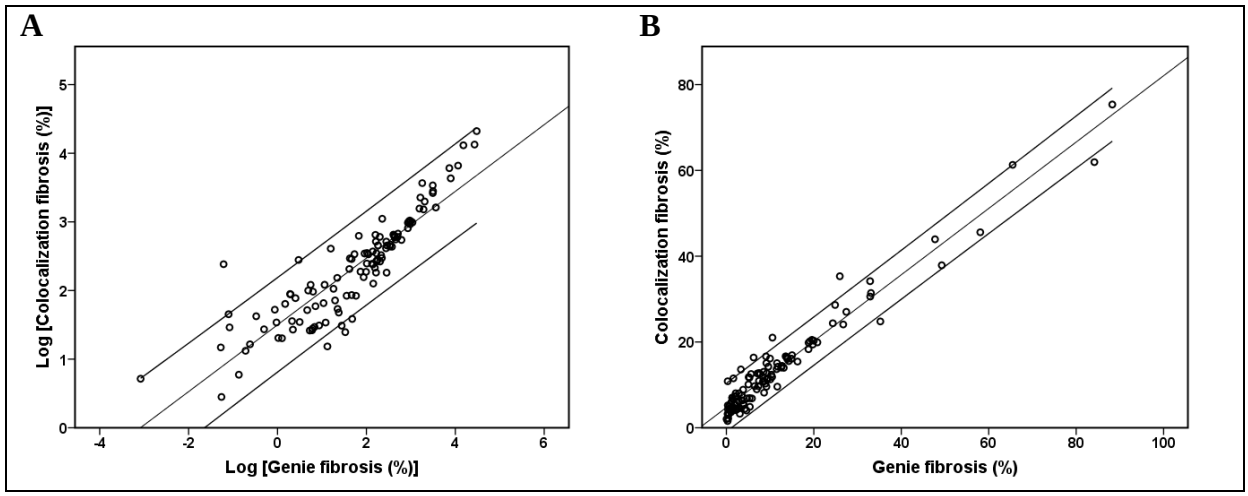


**Fig. 66 Single linear regression models with reference values. Original raw (A) and log-transformed measurements (B) for Colocalization and Genie. Linear regression line is presented within 95 % confidence interval.**

Bland-Altman plots showed a bidirectional bias dependent on the magnitude of the measurement: Colocalization software overestimated the area fraction of fibrosis in the lower end, and underestimated it in the higher end of the RVS scale (Fig. 67).

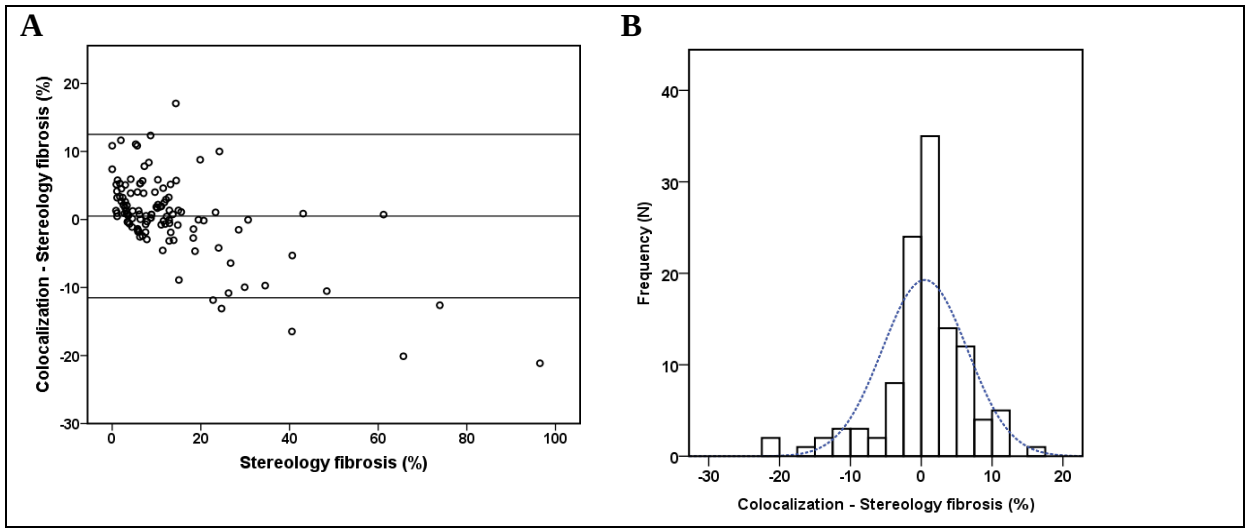


**Fig. 67 Bland-Altman plots (A) and histograms (B) of the method score differences. Colocalization and Stereology. Horizontal line represents mean difference within limits of agreement, which are defined as the mean difference ± 2 standard deviations.**

Meanwhile, Genie software as well as the pathologist's mean score showed more uniform results throughout the complete scale with a slight underestimation in the mid-range for both (Fig. 68 and Fig. 69).

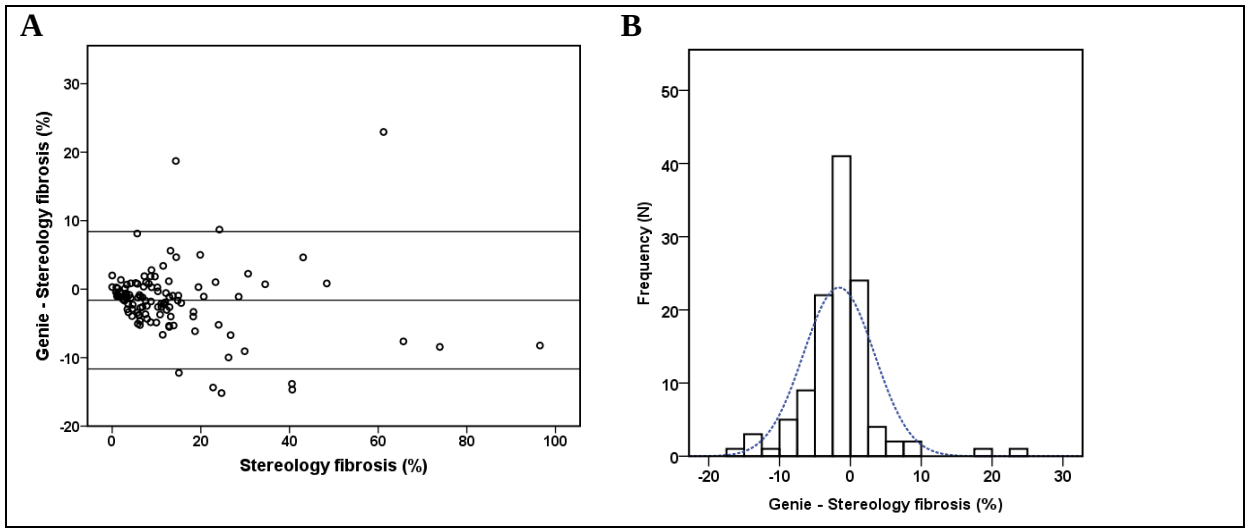


**Fig. 68 Bland-Altman plots (A) and histograms (B) of the method score differences. Genie and Stereology. Horizontal line represents mean difference within limits of agreement, which are defined as the mean difference ± 2 standard deviations.**

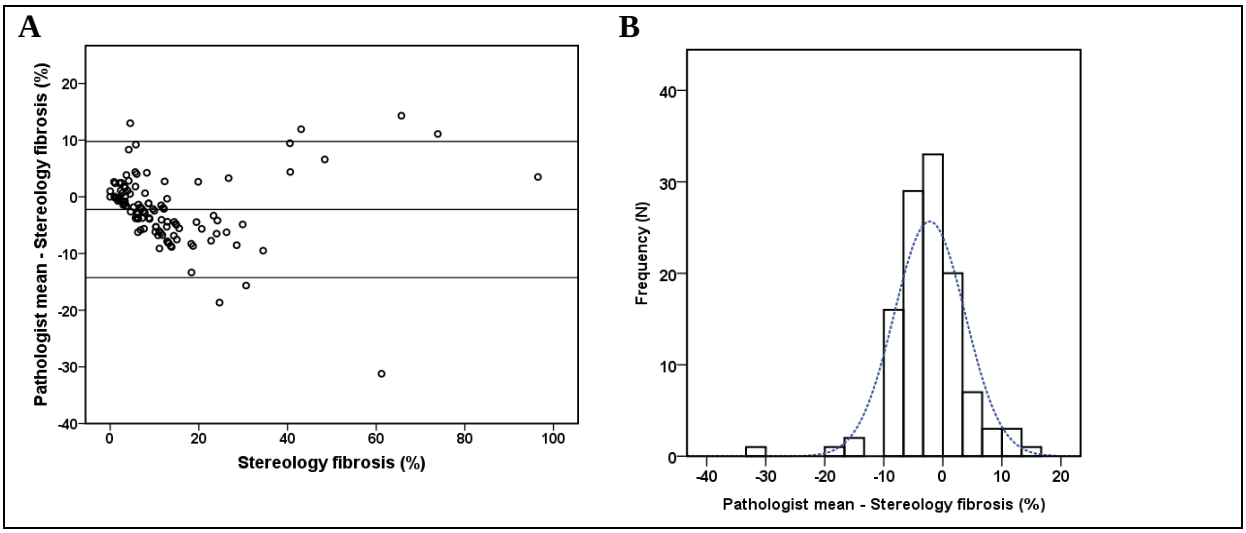


**Fig. 69 Bland-Altman plots (A) and histograms (B) of the method score differences. Pathologist mean score and Stereology. Horizontal line represents mean difference within limits of agreement, which are defined as the mean difference ± 2 standard deviations.**

Presented histograms indicate a normal distribution of the differences for each plot (Fig. 67, Fig. 68 and Fig. 69).

# 6. DISCUSSION

## *6.1. Molecular mechanisms of virus-induced DCM*

The myocardium consists mainly of myocytes, extracellular matrix (ECM) and a capillary microcirculation system, the impairment of which causes various heart problems that demand different treatment strategies. A proper myocyte function is responsible for myocardial tension and contractile functioning, whereas ECM function ensures structural integrity of adjoining myocytes, myofilaments and microcirculation. Which of these processes: cardiomyocyte death or ECM degradation impairs myocardial functioning mostly depends on intensity and duration of the injury. It is important to estimate which part of myocardium from previously mentioned is injured by persistent presence of virus [252, 253].

Data presented in this study show that persistent presence of vira (chronic viral infection) in myocardial tissue do not initiate inflammation, fibrosis and apoptosis. Decreased serum level of the sarcomeric protein hsTnT, a marker of necrotic cell death [254], confirms the absence of necrotic cardiomyocytic death as well. However, chronic viral infection may not be as innocent as it seems because the collagen synthesis/degradation balance (PICP/ICTP ratio) in virus-positive biopsies was found to be reduced 4.3 fold. Since the myocardium up to 85-90 % consists of collagen I and only up to 10-15 % of collagen III and other components [253, 255], the disturbance of collagen I turnover in the tested myocardiums may be one of the most significant factors in development of chronic viral DCM. In addition to the decrease of collagen I turnover in biopsies, α-SMA, an intracellular fibrosis marker, was also slightly down-regulated (1.13 fold). The positive correlation of collagen I synthesis with the general inflammation marker CRP in serum suggests that the sustained presence of vira in the myocardium may further upregulate inflammation and fibrosis. Based on the results, it may be stated that degradation of contracting proteins, particularly collagen I, in a persistently virus-infected myocardium overshadows induction of inflammation and cardiomyocyte death.

One of the main factors strongly implicating collagen I synthesis in myocardium is transforming growth factor β1 (TGF-β1). TGF-β1 plays a key role in heart remodeling

trough mediating cardiomyocyte growth, fibroblast activation and ECM deposition [256]. The TGF-β1 is not only known as fibrogenic, but also as an anti-inflammatory cytokine; therefore its suppression might affect both collagen synthesis as well as the immune system and activate inflammation [257-259]. The data show that TGF-β1 was 3.6 fold down-regulated in virus-positive DCM biopsies, whereas in serums its level was almost non-altered. The mechanism by which TGF-β1 affects collagen synthesis is very complex and not fully known. It was also shown that TGF-β1 can directly activate synthesis of collagen I [260]. Additionally, TGF-β1 can activate collagen synthesis indirectly through the phosphorylation of SMAD proteins, increased endoglin expression, stimulation of TIMP1, activation of lysyl oxidase and other signaling systems [261-264]. The correlation analysis shows that prolonged intramyocardial viral infection will activate inflammation, particularly macrophage activity, with subsequent activation of MMP1, MMP2 and TGF-β1. Similar molecular mechanisms of TGF-β1 activation and collagen I deposition has been shown by other authors [265]. Moreover, the release of MMP9 to serum was significantly associated with Hsp60 (Table 6), showing that untreated viral infection might activate MMP9 and increase mitochondrial outer membrane permeability leading to mechanical myocyte dysfunction. There is also a possibility that part of MMP9 is located in and, therefore, released from virus-damaged mitochondria [266].

In parallel to TGF-β1, secreted galectin-3 may also induce collagen I production. Galectin-3 is a member of β-galactoside-binding animal lectins and is predominantly located in macrophages, whereas its expression in human hearts is limited [132]. It is shown that activated macrophages secrete cardiac galectin-3 that activates fibroblast proliferation, collagen deposition and contributes to the development of heart failure [132]. Galectin-3 can also activate fibrosis by increasing expression of α-SMA, an intracellular fibrosis marker, and collagen I α-1 chain (COL1A1), an extracellular fibrosis marker [267]. Parallely, galectin-3 might also affect fibrosis trough the down-regulation of TIMP1 and MMP9 [267]. The depressed levels of α-SMA, collagen I, TIMP1 and MMP9 observed in the virus-positive biopsies may be a result of reduced serum level of galectin-3 and infiltrated macrophages. On the other hand, activation of MMP1 and MMP2 by intramyocardial viral infection might be enough to remodel myocardial collagen I trough binding to the ά-2 chain [268].

The last ones of the tested mechanisms indirectly affecting collagen turnover might be related to myocardial protecting systems. One of them is immunoregulator adiponectin, the role of which under different toxic conditions seems to be quite paradoxical. The majority of evidences show that over-expression of adiponectin has anti-diabetic, anti-apoptotic and anti-inflammatory effects [110, 111]. It has been also indicated that high adiponectin levels lower the risk for myocardial infarction but increases the risk for heart failure, whereas hypo-adiponectinemia was connected to cardiovascular diseases [112, 118, 269]. Additionally to adiponectin, BNP was also shown to have a cardio-protective effect [270]. However, the significantly and almost two fold decreased serum levels of BNP and adiponectin, and its correlation with anti-inflammatory cytokine TGF-β1 ($R^2$ = 0.459, p = 0.008) might not only show some endothelial dysfunction but also the reduced myocardial protection. Slight upregulation of major histocompatibility agent class II HLA-DR also pointed on endothelial cell injury in viral myocardium. Since levels of pro-inflammatory infiltrates and cytokines in virus-positive myocardium were reduced compared to virus-negative ones, the slight HLA-DR upregulation might be a direct effect of vira. On the other hand, correlation of adiponectin and BNP with pro-inflammatory infiltrates suggests a possible BNP and adiponectin overproduction due to persistent presence of intramyocardial viral infection leading to heart failure. Similar dependence of adiponectin, BNP and inflammation in heart failure were also observed by other authors [271, 272].

### *6.2. Molecular mechanisms dominating in inflammatory DCM*

Myocarditis is a predominant cause of heart failure of young age patients [273]. When the immune system fails to eliminate infections in a timely fashion, a chronic myocardial destruction starts. Thereby, approximately 20 % of myocarditis-affected individuals develop a chronic heart disease leading to inflammatory DCM and only half of the patients survive longer than 5 years [274, 275]. This severe prognosis urges detailed investigations of the molecular mechanisms triggering progression of inflammation into inflammatory DCM and heart failure.

The pathophysiology of DCM is an exceedingly complex process caused by activation of neurohormones and pro-inflammatory cytokines. It is becoming apparent

that inflammatory mediators play a crucial role in the development of DCM, subsequently progressing to heart failure. It is shown that the pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-1β, may act synergistically at both messenger RNA (mRNA) and protein levels inducing cardiac contractile defects [276, 277]. On the other hand, IL-6 also referred to as myokine, activates anti-inflammatory pathways [278, 279]. This dual IL-6 effect might be explained by different durations of IL-6 exposure: a short and acute IL-6 increase has a positive effect, whereas chronic hyperproduction results in a pathological condition [280]. Data of the study revealed a significant upregulation of IL-6 (3.23 fold, $p < 0.05$), much less for TNF-α (1.76 fold) and none for IL-1β (0.88 fold) in inflammatory DCM sera. Since inflammatory DCM has a chronic way of progression, the significant over-production of IL-6 in the tested sera may be a cause of inflammatory DCM rather than its consequence. Additionally, the significantly increased serum level of IL-6 and its correlation with myocardial $CD3^+$ suggest T-lymphocytes to be involved in inflammatory DCM apoptosis, whereas only a slight increase of infiltrated macrophages confirmed the absence of necrosis. The default of pro-interleukin-1 beta (IL-1β) activation in the tested sera might be explained by the low intensity of myocardium inflammation, absence of caspase-1 cleavage-activation and a pyroptotic way of cardiomyocyte death [281].

Recently, a strong and direct influence of IL-6 was shown on mitochondrial function: IL-6 inhibits adipocyte mitochondrial membrane potential, ATP production and increased intracellular reactive oxygen species (ROS) level [282, 283]. Additionally, a general inflammatory biomarker secreted C-reactive protein (CRP), similarly to IL-6, also correlates with poor DCM prognosis, heart failure and mitochondrion-mediated myocyte apoptosis [284-286]. Findings in this study showed that IL-6 and CRP levels in inflammatory-positive DCM serums were not only significantly upregulated but also significantly correlated with the secreted mitochondria-related apoptotic biomarkers Hsp60 and Bax suggesting that the intrinsic apoptotic pathway in myocardium is more sensitive to inflammation than the extrinsic one. The low level of upregulations of the Fas receptor, Fas ligand and caspase-8 also confirmed that extrinsic apoptotic pathway is less important in development of inflammatory DCM.

Many signaling pathways, including the intrinsic and extrinsic apoptotic ones, might have mutual interaction leading to the synergistic implication on final myocardial response. It was shown that the member of the extrinsic apoptotic pathway pro-caspase-8 might cleave the BH3 domain-only protein Bid, which in turn, activates Bax that integrates to mitochondrial membranes, thereby releasing cytochrome C [287, 288]. In agreement with previous observations, data in the present study show a significant correlation between caspase-8 and Bax in inflammatory DCM sera, suggesting that Bax is one of the most important intersection points between the intrinsic and extrinsic apoptotic pathways. However, the approximately 30 folds higher initial level of caspase-9 compared to caspase-8 in inflammatory DCM biopsies suggests the intrinsic apoptotic pathway to be leading in inflammatory DCM. Similarly, other authors also have observed a low impact of the extrinsic apoptotic pathway on cardiomyocyte death [289].

In addition to the intrinsic apoptotic pathway, MMP-9 was also found to be sensitive to inflammation and to participating in the pathogenesis of cardiomyopathy [290]. The intensity of MMP9 activation also indicates the level of heart damage: higher proteolytic MMP9 activity is related to more pronounced heart damage. Data of this study show a slight increase of MMP9/TIMP1 ratios both in biopsies (from 0.25 to 0.35) and in serums (from 0.23 to 0.25) of inflammatory DCM patients. Even if the increase of the MMP9/TIMP1 ratio in inflammatory DCM biopsies was mild and not fully significant, it significantly correlated with markers of both apoptotic pathways (Bcl-2, Fas receptor and Fas ligand, Table 10) supporting the idea of chronic remodeling of the mitochondrial outer membrane and activation of the extrinsic apoptotic pathway. Additional correlation of MMP-9 with caspase-9 and -8 in the tested serums confirmed previous statement.

In parallel to apoptosis, we investigated the necrotic way of myocardial cell death. Necrosis, as a passive and adenosine triphosphate (ATP)-independent process, was shown to be more characteristic to acute and global cardiomyocyte damage than to chronic processes [291]. Another way of cell death, a regulated necrosis (also known as caspase-independent death or necroptosis), is suppressed by activated pro-caspase-8 and therefore absent in tested samples similar to what has been shown by others [292]. Since histological analysis of the samples did not show myocardial necrosis and

fibrosis, the release of serum sarcomeric protein hsTnT is related to decreased myocardium contraction. However, the myofilament degradation and irreversible myocardium damage might be supported by prolonged hsTnT leakage out of myocardium. Additionally, we suggest that the release of hsTnT is a caspase-related process, as the secreted hsTnT significantly correlated with activation of the apoptotic signaling cascade: caspases-8, Bax and caspase-3 in inflammatory DCM biopsies.

### *6.3. Quantification of myocardial fibrosis by digital image analysis and interactive stereology*

To this day a pathologist's visual score is widely accepted as ground truth and, despite already available digital methods, it is still used as a primary method for histomorphometric evaluations. Many attempts to incorporate digital methods into clinical practice face the same issue of proper validation – the digital analysis data are commonly compared to semi-quantitative visual evaluation, while most direct criterion standard yet requires time-consuming procedures.

The early study of Vasiljevic et al. [208] based on human endomyocardial biopsies compared results of semi-quantitative scoring, point-lesion counting (using a grid) to computer-assisted methods. This was the first study to demonstrate strong correlation of different cardiac fibrosis scoring methods, however, due to considerable input by the investigator in computer analysis it still can be considered as subjective to some degree. Particularly since a stereology test grid was not used for RVS. Hadi et al. [211] quantified cardiac fibrosis by automated analysis using ImageJ software and traditional polarization microscopy, with subsequent validation of the results, using stereology data as criterion standard. To our knowledge, it is the only study of cardiac fibrosis that applied stereology procedures to obtain RVS; however, the validation was performed on rat cardiac rather than human samples (the analysis was then tested on a post-mortem tissue samples from a 78 year old man).

In this study, several methods were tested to evaluate the extent of human cardiac fibrosis, which can be readily implemented in clinical practice today. Stereology was used as the most independent and objective RVS available and a modified Bland-Altman plot as the best statistical tool to measure agreement between the tested method and a RVS.

The initial data were somewhat in favor of the Colocalization software: it demonstrated the closest fibrosis mean value to a reference and resulting difference of 0.50 % was statistically insignificant. However, the Colocalization software had a noticeably narrower variation, which was 20 % behind the RVS and the pathologist's range, and also 15 % behind the Genie software. This drawback may be not of great importance in clinical practice, as the range limitation was only evident in the higher range and myocardium fibrosis hardly reaches these values, whereas the lower range was acceptable. Further analysis revealed the superiority of Genie software: the higher correlation with RVS, the better values in single linear regression against the reference and, most importantly, more uniform results in Bland Altman analysis. While the Colocalization software was overestimating at the lower end and underestimating at the higher end, Genie software was only slightly underestimating in a mid-range with the results still exceeding those of the pathologist's mean score. Of note, both digital algorithms produced slightly different results, a fact that might appear surprising. Despite both algorithms are aimed to measure the same feature, namely, the proportion of connective tissue in the myocardium, they are still based on different principles and may result in different measurement errors. While Colocalization classifies each pixel according to its color characteristics, the Genie software is based on a far more complex pattern recognition system, which also refers to spatial aspects of the image. Probably, the only relevant drawback of Genie was the underestimation bias of 1.61 % from the RVS. Overall, the Genie classifier performed best in this study, being closest to the RVS, with almost perfect correlation, adequate range and uniform results throughout the whole scale.

Potential limitations of the Genie software are related to the necessity to train the system to identify the various structures of interest, which is time-consuming and based on the inherent subjectivity of the "human trainer". This fact also makes the Genie software sensitive to inter-laboratory reproducibility issues. However, after the adaptation of Genie software to the clinical needs it can be run fully automated and as a result it can be equally as time-efficient as the Colocalization software is. The Genie software has the possibility of tuning the algorithm, which makes it more flexible in practical maintenance. Even if 2 % is an acceptable error for cardiac fibrosis estimate

in clinical sense, this algorithm may require further adaptation to potential sources of slide quality variation.

The Colocalization software has also proved to be a fully acceptable method for cardiac fibrosis measurement. In clinical practice, the Colocalization software should provide similar precision and accuracy as the Genie tool, because cardiac fibrosis values are rarely exceeding 40 %, and a slight overestimation in the lower range may be acceptable. The Colocalization software is less complex, simpler to use and calibrate, and less expensive. Furthermore it can be run fully automated from image scanning to the final results and it is very time efficient. The Colocalization algorithm is less dependent on human investigator input at any point of the process (except initial settings for color deconvolution), making it more transparent and manageable for users.

### *6.4. Summary*

Finalizing data presented in this study we can state that therapeutic strategies for myocarditis and dilated cardiomyopathy should be obtained through analysis of the acute, subacute and chronic phases. Most of recent studies are concentrated on the investigation of acute myocardial injuries, whereas chronic myocardial injuries are less symptomatic but more complicated for investigation and treatment. In this study we investigated molecular mechanisms dominating in chronic viral cardiomyopathy due to viral persistence in dilated heart without ongoing inflammation and chronic inflammatory DCM. The aetiology of inflammatory DCM was assumed to be either infectious, toxic or autoimmune. The most important thing is that both types of investigated chronic DCM, showed different molecular mechanism suggesting more options for further DCM treatment.

However, chronic DCM patients are usually treated according to general guidelines regardless of their etiology. Our data suggest that conventional therapeutic agents for chronic DCM and heart failure such as β-blockers, angiotensin-converting enzyme (ACE) inhibitors, angio-tensin receptor blockers and other can be combined with others such as collagen I metabolism regulating (in case of chronic viral DCM) and anti-apoptotics (in case of idiopathic chronic inflammatory DCM). The foreseeable future therapeutic approach for various types of chronic dilated

cardiomyopathy should be directed to control and regulation of molecular mechanisms progressing DCM. The technological and scientific progress is also improving a quality of estimation of myocardial functioning regulating processes such as fibrosis. We sincere hope that our data contribute not only to a better understanding of molecular mechanism progressing chronic DCM but also to new therapeutic options.

# 7. CONCLUSIONS

1. Chronic viral DCM did not induce inflammation, fibrosis and cardiomyocyte death. Destruction of myocardial contraction, particularly trough changed collagen I turnover, dominates in virus-positive DCM.
2. The improper functioning of collagen I in virus-positive biopsies is affected by reduction of TGF-β1 (3.55 fold) and activation of MMP1 and MMP2 (2.19 and 2.43 fold). Decreased serum levels of galectin-3, adiponectin and BNP (1.16, 1.88 and 1.97 fold) indirectly down-regulated collagen I turnover and diminished general resistance of viral myocardium.
3. Chronic inflammatory-positive myocardium compared to inflammatory-negative showed the highest level of infiltrated T-lymphocytes(14.6 cell/$mm^2$) that significantly correlated with 3.50 fold augmented secretion of inflammatory cytokines, particularly IL-6.
4. Chronic myocardial inflammation significantly increased the release of caspase-9,-8, and-3 (6.24, 3.10, 3.62 folds, ($p < 0.05$)) into serum. Significant increase of Hsp60 (8.97 fold) in serum showed the impairment of mitochondria and also the importance of intrinsic apoptotic pathway in inflammatory DCM.
5. The extrinsic apoptotic pathway (FasR, FasL and caspase-8) was a supporter, but not a main leader in the progression of chronic inflammatory DCM. The pro-apoptotic Bax is an important intersection point for the extrinsic-and intrinsic apoptotic pathways.
6. Slight increase of MMP9/TIMP1 ratios both in biopsies (from 0.19 to 0.48, $p<0.05$) and in serums (from 0.23 to 0.25) and two fold increased release of hsTnT into serum attenuated fibrosis in chronically-inflamed myocardium.
7. The Genie algorithm proved to be the method of choice with the single drawback of a slight underestimation bias that can be acceptable for clinical and research demands to quantify the extent of fibrosis in myocardial biopsies.

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# 9. LIST OF PUBLICATIONS ON THE TOPIC OF THE DISSERTATION

## *9.1. Publications*


1. Daunoravičius D, Grabauskienė V, Bogomolovas J, Jablonskienė V, Vitkus D. Galectin-3: a novel marker of fibrosis and heart failure. *Laboratorinė medicina*. 2011; 4 (52): 205–211.
2. Daunoravičius D, Grabauskienė V, Jasulaitis A. Inflammatory dilated cardiomyopathy: new perspective in diagnostics and treatment. *Medicinos teorija ir praktika* 2012; 3: 39–44.
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4. Daunoravičius D, Laurinavičius A, Žurauskas E, Laurinavičienė A, Grabauskienė V. Quantitative evaluation of cardiac fibrosis by digital image analysis versus pathologist visual scoring. *Proceedings of International Conference Biomedical Engineering* 2012, p. 91–95.
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6. Daunoravičius D, Besusparis J, Žurauskas E, Laurinavičienė A, Bironaitė D, Pankuweit S, Plancoulaine B, Herlin P, Bogomolovas J, Grabauskienė V, Laurinavičius A. Quantification of myocardial fibrosis by digital image analysis and interactive stereology. *Diagnostic Pathology* 2014; 9: 114.
7. Bogomolovas J, Brohm K, Čelutkienė J, Balčiūnaitė G, Bironaitė D, Bukelskienė V, Daunoravičus D, Witt C, Fielitz J, Grabauskienė V, Labeit S. Induction of Ankrd1 in dilated cardiomyopathy correlates with the heart failure progression. *BioMed Research International* 2014.

### *9.2. Oral presentations*

1. Daunoravičius D, Grabauskienė V. The diagnostics and ethiopathogenetic treatment possibilities for inflammatory cardiomyopathy – first experience in Lithuania. 5th National Scientific Conference „Science for the human health", Kaunas, Lithuania. 2011 April 7, 2011.
2. Daunoravicius D, Jakubauskas A, Griskevicius L, Cibiras S, Zurauskas E, Maneikiene V, Celutkiene J, Rucinskas K, Jasulaitis A, Grabauskiene V. Inflammatory cardiomyopathy: aetiopathogenetic markers for diagnosis of disease subentities and for aetiology directed treatment. Heart failure 2012 Congress, Berlgrade, Serbia. May 19–22, 2012.
3. Daunoravičius D, Grabauskienė V. Dilated cardiomyopathy: evolution of aetiopathogenetic diagnosis and new possibilities of treatment. International Conference „Evolutionary Medicine: New Solutions for the Old Problems", Vilnius, Lithuania. June 12–15, 2012.
4. Daunoravicius D, Besusparis J, Zurauskas E, Laurinaviciene A, Bironaite D, Grabauskiene V, Laurinavicius A. Quantification of myocardial fibrosis by digital image analysis methods and pathologist visual scoring versus digital stereology. Heart failure 2014 Congress, Athens, Greece. May 17–20, 2014.
5. Bironaite D, Daunoravicius D, Bogomolovas J, Jakubauskas A, Vitkus D, Zurauskas E, Zasytyte I, Rucinskas K, Brunk U, Venalis A, Grabauskiene V. The role of collagen in virus-positive dilated cardiomyopathy. World Congress of Cardiology Scientific Sessions 2014**,** Melbourne, Australia. May 4–7, 2014. Supplement to Global Heart, Vol 9, Issue 1C, 2014, PM051, e71. doi:10.1016/j.gheart.2014.03.1461
6. Bironaite D, Daunoravicius D, Bogomolovas J, Jakubauskas A, Vitkus D, Zurauskas E, Zasytyte I, Rucinskas K, Brunk U, Venalis A, Grybauskiene V. Apoptotic markers in diagnosinginflammatory dilated cardiomyopathy. World Congress of Cardiology Scientific Sessions 2014**,** Melbourne, Australia. May 4–7, 2014. Supplement to Global Heart, Vol 9, Issue 1C, 2014, PM050, e71 doi:10.1016/j.gheart.2014.03.1460
7. Bironaite D, Daunoravicius D, Bogomolovas J, Cibiras S, Vitkus D, Zurauskas E, Zasytyte I, Labeit S, Venalis A, Grabauskiene V. Mechanism

of fibrosis in inflammatory dilated cardiomyopathy. The 18th Annual Scientific Meeting of the HFSA, Las Vegas, Nevada. September 14–17, 2014. Journal of Cardiac Failure August 2014: Volume 20, Issue 8, Supplement, S81. doi: http://dx.doi.org/10.1016/j.cardfail.2014.06.229.

# 10. ANNEX

**VILNIAUS UNIVERSITETO MEDICINOS FAKULTETAS**

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**VILNIAUS REGIONINIS BIOMEDICININIŲ TYRIMŲ ETIKOS KOMITETAS**
M.K.Čiurlionio 21/27, LT-03101,Vilnius Tel.(85) 2686998, el.p.: rbtek@mf.vu.lt

# LEIDIMAS ATLIKTI BIOMEDICININĮ TYRIMĄ

2011-09-06 Nr.158200-09-382-103

Tyrimo pavadinimas:

**Imunohistologinių lėtinio intramiokardinio uždegimo žymenų analizė ir reikšmė diferencijuojant dilatacinės kardiomiopatijos priežastis ir parenkant gydymo taktiką**

Protokolo Nr.: 2011/07
Versija: 1
Data: 2011.07.06
Asmens informavimo forma ir Informuoto asmens sutikimo forma (lietuvių kalba):
Versija: 2
Data: 2011-09-05

Asmens informavimo forma ir Informuoto asmens sutikimo forma kontrolinei grupei (lietuvių kalba):
Versija: 1
Data: 2011-09-05

Pagrindiniai tyrėjai: V.Grabauskienė (D.Daunoravičius)

Biomedicininio tyrimo vieta:
Įstaigos pavadinimas: Vilniaus universitetinė ligoninė „Santariškių klinikos“
Įstaigos adresas: Santariškių g. 2, LT-08661 Vilnius

Leidimas išduotas Vilniaus regioninio biomedicininių tyrimų etikos komiteto posėdžio (protokolas Nr. 158200-2011/09), vykusio 2011 m. rugsėjo 06 d., sprendimu.

| Vilniaus regioninio biomedicininių tyrimų etikos komiteto ekspertų grupės nariai | | | |
|---|---|---|---|
| Nr. | Vardas, pavardė | veiklos sritis | dalyvavo posėdyje |
| 1 | doc. Dr.Laimutė Jakavonytė | filosofija | taip |
| 2 | doc. Dr. Kęstutis Žagminas | epidemiologija | taip |
| 3 | dr. Indrė Isokaitė | teisė | ne |
| 4 | dr. Marija Veniūtė | visuomenės sveikata | ne |
| 5 | doc.dr. Jolanta Gulbinovič | medicina | ne |
| 6 | prof.dr. Vytautė Pečiulienė | medicina, odontologija | taip |
| 7 | Laura Malinauskienė | medicina | taip |
| 8 | dr. Gražina Pastavkaitė | klinikinė psichologija | ne |
| 9 | Ugnė Šakūnienė | pacientų teisės | taip |

Pirmininkė Vytautė Pečiulienė

VILNIAUS UNIVERSITETAS * Vilniaus regioninis biomedicininių tyrimų etikos komitetas * MEDICINOS FAKULTETAS

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## 12. DISCLOSURE

There is no conflict of interest.

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