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https://orcid.org/0000-0002-0619-3881

VILNIUS UNIVERSITY

Ieva Kažukauskienė

Prognostic Utility of Myocardial, Serum and Echocardiographic Biomarkers in Patients with Non-Ischemic Dilated Cardiomyopathy

DOCTORAL DISSERTATION

Medical and Health Sciences,
Medicine (M 001)

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Academic supervisor – Prof. Dr. Virginija Grabauskienė (Vilnius University, Medicine and Health Sciences, Medicine M 001).

This doctoral dissertation will be defended in a public meeting of the Dissertation Defence Panel:

Chairman – **Prof. Dr. Jolanta Dadonienė** (Vilnius University, Medical and Health Sciences, Medicine – M 001).

Members:

Prof. Dr. Sigita Glaveckaitė (Vilnius University, Medical and Health Sciences, Medicine – M 001),

Dr. Algirdas Grevys (Oslo University, Natural Sciences, Biochemistry – N 004),

Dr. Vilius Janušauskas (Vilnius University, Medical and Health Sciences, Medicine – M 001),

Prof. Dr. Diana Zakarkaitė (Vilnius University, Medical and Health Sciences, Medicine – M 001).

The dissertation shall be defended at a public meeting of the Dissertation Defence Panel at 10 a.m. on 27th June 2022 in the Great Auditorium of Vilnius University Faculty of Medicine.
Address: Čiurlionio str. 21/27, Vilnius, Lithuania.

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VILNIAUS UNIVERSITETAS

Ieva Kažukauskienė

Neišeminės dilatacinės kardiomiopatijos seruminių, miokardinių ir echokardiografinių žymenų prognostinės vertės tyrimai

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Mokslinė vadovė:
prof. dr. Virginija Grabauskienė (Vilniaus universitetas, medicinos ir sveikatos mokslai, medicina – M 001).

Gynimo taryba:
Pirmininkė – **prof. dr. Jolanta Dadonienė** (Vilniaus universitetas, medicinos ir sveikatos mokslai, medicina – M 001).

Nariai:
Prof. dr. Sigita Glaveckaitė (Vilniaus universitetas, medicinos ir sveikatos mokslai, medicina – M 001),

Dr. Algirdas Grevys (Oslo universitetas, gamtos mokslai, biochemija – N 004),

Dr. Vilius Janušauskas (Vilniaus universitetas, medicinos ir sveikatos mokslai, medicina – M 001),

Prof. dr. Diana Zakarkaitė (Vilniaus universitetas, medicinos ir sveikatos mokslai, medicina – M 001).

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ABBREVIATIONS

ACE-I	angiotensin-converting enzyme inhibitor
ARB	angiotensin II receptor blocker
AUC	area under the curve
BMI	body mass index
BNP	B-type natriuretic peptide
BP	blood pressure
B19V	parvovirus B19
CI	confidence interval
DT	deceleration time
E/A	ratio of early-diastolic LV inflow velocity (E) to atrial-systolic velocity (A)
EBV	Epstein-Barr virus
E/e´	ratio of early-diastolic LV inflow velocity (E) to early-diastolic mitral annular velocity (e´)
eGFR	estimated glomerular filtration rate
ESC	European Society of Cardiology
EV	enterovirus
GLS	global longitudinal strain
HCV	hepatitis C virus
HFrEF	heart failure with reduced ejection fraction
HHV-6	human herpes virus 6
HR	hazard ratio
hs-CRP	high-sensitivity C-reactive protein
iDCM	inflammatory dilated cardiomyopathy
IL-6	interleukin 6
KRAS	Kirsten rat sarcoma viral oncogene homolog
LAVi	left atrial indexed volume
LBBB	left bundle branch block
LV	left ventricle
LVEDD	left ventricular end-diastolic diameter
LVEF	left ventricular ejection fraction
mPAP	mean pulmonary arterial pressure
MRA	mineralocorticoid receptor antagonist
NYHA	New York Heart Association

NI-DCM	non-ischemic dilated cardiomyopathy
non-iDCM	non-inflammatory dilated cardiomyopathy
PCWP	pulmonary capillary wedge pressure
PVR	pulmonary vascular resistance
RAP	right atrial pressure
ROC	receiver operating characteristic
RV	right ventricle
suPAR	soluble urokinase-type plasminogen activator receptor
TAPSE	tricuspid annular plane systolic excursion
TNF-α	tumor necrosis factor α
TR	tricuspid regurgitation
UBC	ubiquitin C
uPAR	urokinase-type plasminogen activator receptor
VZV	varicella zoster virus
WBC	white blood cell
WHF	World Heart Federation

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LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. **Kažukauskienė I,** Baltrūnienė V, Jakubauskas A, Žurauskas E, Maneikienė V.V, Daunoravičius D, Čelutkienė J, Ručinskas K, Grabauskienė V. *Prevalence and prognostic relevance of myocardial inflammation and cardiotropic viruses in non-ischemic dilated cardiomyopathy.* Cardiology journal. Gdansk: Via Medica. ISSN 1897-5593. eISSN 1898-018X. 2020, first on line, p. [1–24].

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II. **Kažukauskienė I,** Baltrūnienė V, Rinkūnaitė I, Žurauskas E, Vitkus D, Maneikienė V.V, Ručinskas K, Grabauskienė V. *Inflammation-related biomarkers are associated with heart failure severity and poor clinical outcomes in patients with non-ischemic dilated cardiomyopathy.* Life 2021, 11 (10), 1006; https://doi.org/10.3390/life11101006

III. Baltrūnienė V, Bironaitė D, **Kažukauskienė I,** Bogomolovas J, Vitkus D, Ručinskas K, Žurauskas E, Augulis R, Grabauskienė V. *The role of serum adiponectin for outcome prediction in patients with dilated cardiomyopathy and advanced heart failure.* BioMed research international: Hindawi. ISSN 2314-6133. eISSN 2314-6141. 2017, Vol. 2017, Article ID 3818292, [p. 1–13].

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IV. **Kažukauskienė I,** Balčiūnaitė G, Baltrūnienė V, Čelutkienė, J, Maneikienė V.V, Čibiras S, Ručinskas K, Grabauskienė V. *Left ventricular global longitudinal strain predicts elevated cardiac pressures and poor clinical outcomes in patients with non-ischemic dilated cardiomyopathy*. Cardiovasc Ultrasound London: BioMed Central. eISSN 1476-7120. 2021, vol. 19, no. 1, art. no. 21, p. [1–13].
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1. INTRODUCTION

1.1. The Research Problem and Relevance of the study

Heart failure is a heterogeneous, multi-etiologic and complex clinical syndrome resulting from impaired ventricular filling (diastolic dysfunction) or impaired ejection of blood (systolic dysfunction) that is associated with symptoms such as breathlessness, fatigue, and pulmonary and/or peripheral edema. Heart failure affects up to 2% of the adult population in developed countries [1], and more than 10 % of the population over 70 years of age [1–3]. The syndrome leads to considerable morbidity and mortality: the 5-year survival after the diagnosis is 50%. Although pharmacological and device therapies have significantly improved quality of life and decreased mortality of heart failure patients in the past two decades, heart failure remains the leading cause of death in developed countries [4].

Heart failure is commonly classified according to the evaluation of the central measure–left ventricular ejection fraction (LVEF)–which is expressed as a percentage of how much blood the left ventricle ejects during each contraction. If LVEF is less than 40%, the heart failure is classified as heart failure with reduced ejection fraction (HFrEF) (an equivalent for systolic dysfunction). HFrEF is a multi-etiologic syndrome arising from different cardiac lesions, including the myocardium, pericardium, vasculature, cardiac valves, or heart conduction system, and comprises of approximately 50-60 % of heart failure patients. The most common cause of HFrEF is ischemic heart disease, especially in the elderly population. However, in younger patients, non-ischemic dilated cardiomyopathy (NI-DCM) is one of the leading causes of HFrEF: NI-DCM is the underlying cause of HFrEF in approximately one-third of the cases [5,6].

NI-DCM is a chronic heart disease that presents with left ventricle (LV) dilatation and impaired ventricle function (left or both ventricles) that is not caused by coronary artery disease or abnormal loading conditions [1]. Decades of research have revealed a wide etiologic spectrum for NI-DCM, including infectious agents, genetic abnormalities, neuromuscular or endocrine disorders, auto-immune mechanisms, drugs, toxins, nutritional deficiency, or electrolyte disturbance [7,8]. This wide diversity of etiologic factors requires a wide array of expensive diagnostic tools to identify an exact cause of the

disease. Unfortunately, the availability of diagnostic tools differs significantly worldwide; therefore, many cases of NI-DCM that could in theory be identified are [mistakenly instead] classified as idiopathic dilated cardiomyopathy [9–12].

Endomyocardial biopsy, analyzed by immunohistochemistry and viral polymerase chain reaction (PCR), became an essential procedure for diagnosing the cause of NI-DCM [13–15]. Consequently, cardiotropic viruses are now recognized as a crucial etiologic factor of heart failure and are found in the myocardium of up to two-thirds of NI-DCM patients [16,17]. However, the data concerning the impact of cardiotropic viruses on clinical significance and prognosis remains debated [18,19].

Myocardial inflammation, confirmed by endomyocardial biopsy, is also known to be a significant causal factor and is responsible for the progression of LV dilatation [20–23]. During the past decades, diagnostic criteria for inflammation in the myocardium have been updated several times [13,24–26]. The latest definition of myocardial inflammation was endorsed by the European Society of Cardiology (ESC) Working Group on Myocardial and Pericardial Diseases [13]. However, the prevalence of myocardial inflammation diagnosed by this criterion has not yet been estimated. At this time, immunohistochemistry and viral PCR are used to characterize etiopathogenetic subsets of NI-DCM patients, but prospective data are lacking for these subsets.

Due to its etiopathogenetic diversity, the progression of NI-DCM varies among individuals. Despite guideline-directed treatment, a significant proportion of NI-DCM patients deteriorate (progressively) either until death or until they receive a heart transplant. NI-DCM is the most common indication for heart transplantation worldwide, accounting for more than 50% of all heart transplants in the age group of 18–59 [27]. However, there are no prognostic strategies for risk stratification in patients who have developed NI-DCM. Furthermore, Dziewiecka et al. recently showed that most risk assessment models, which have been created for heterogeneous heart failure populations, have suboptimal accuracy for NI-DCM patients [28]. Therefore, there is a need for reliable markers for identifying high-risk patients because they require close follow-up and timely decisions regarding advanced treatments, as well as to facilitate the search for specific therapeutic targets.

This thesis is based on four published research articles. Its overall aim is to examine the correlations between various biomarkers and heart failure severity

and clinical outcomes in the cohort of patients with pure NI-DCM. Paper I analyzes the prevalence and prognostic relevance of cardiotropic viruses and myocardial inflammation. Papers II and III investigate the association between inflammation-related biomarkers and the severity of the disease, as well as the prognostic value of these biomarkers. Finally, paper IV investigates global longitudinal strain (GLS) association with disease severity and poor clinical outcomes.

1.2. Aim of the Study

Hypothesis: Myocardial, serum and echocardiographic biomarkers indicate heart failure severity and predict poor clinical outcomes in patients with NI-DCM.

The aim of the thesis: To analyze the relationship of myocardial, serum, echocardiographic biomarkers and heart failure severity parameters and to assess the prognostic value of these biomarkers in a cohort of well-defined pure NI-DCM patients.

1.3. Objectives of the Study

1. To evaluate the prevalence of myocardial inflammation and cardiotropic viruses in NI-DCM patients by immunohistochemistry and PCR (Paper I).
2. To assess the prognostic value of myocardial inflammation and cardiotropic viruses in a cohort of patients with non-ischemic dilated cardiomyopathy (Paper I).
3. To assess the association of serum white blood cell count (WBC), high-sensitivity C-reactive protein (hs-CRP), adiponectin, interleukin 6 (IL-6), tumor necrosis factor α (TNF-α), soluble urokinase Plasminogen Activator Receptor (suPAR)) and myocardial inflammation-related (CD3+, CD4+, CD68+, CD45ro+, HLA-DR+, ICAM-1+) biomarkers with heart failure severity parameters and adverse cardiac events in cohort of pure NI-DCM patients (Papers II, III).
4. To assess the prognostic value of global longitudinal strain (GLS) in a prospective well-defined pure NI-DCM cohort (Paper IV).

1.4. Scientific Novelty of the Study

This doctoral research, which resulted in a total of five published papers, evaluated the prognostic value of various myocardial, serum and echocardiographic biomarkers in a cohort of well-defined pure NI-DCM patients over five years of follow-up. Previously, the prognostic value of the significant amount of different biomarkers was estimated in heterogeneous chronic heart failure cohorts but not in the pure NI-DCM cohort.

To the best of our knowledge, the prognostic value of myocardial inflammation, diagnosed by ESC criterion 2013 [13], was evaluated for the first time in the cohort of NI-DCM patients by our research. Moreover, this research evaluated the prognostic value of some biomarkers (e.g., CD3+, CD45ro+, hs-CRP, adiponectin) in NI-DCM patients over a longer follow-up duration than in previously published studies.This research is also the first to evaluate the prognostic value of IL-6, TNF-α, suPAR in a cohort of NI-DCM patients, as well as the first to evaluate the prognostic impact of the combination of all three inflammation-related cytokines (adiponectin, IL-6, TNF-α). Finally, our research identified specific thresholds of GLS, IL-6, TNF-α and adiponectin, which help identify high-risk patients.

1.5. The Practical Value of the Study

Patients with NI-DCM have a high mortality rate even under current heart failure treatment. For this reason, it is important to research etiopathogenetic factors (e.g., cardiotropic viruses, myocardial inflammation) for better risk stratification of NI-DCM patients and for target-specific treatment strategies.

This research demonstrated that endomyocardial biopsy evaluated by immunohistochemistry provides not only diagnostic but also prognostic information in patients with NI-DCM. Moreover, assessment of inflammation-related cytokines and GLS measurement might aid in patients' risk stratification and identification of high-risk patients. Recognition of high-risk patients would affect the intensity of follow-up, aid treatment decision making, and help prioritize patients on the heart transplantation list.

1.6. Defended Statements of the Thesis

1. Cardiotropic viruses in the myocardium and myocardial inflammation are prevalent among NI-DCM patients.
2. Detection of cardiotropic viruses in the myocardium and diagnosis of inflammatory NI-DCM is not associated with poor clinical outcomes.
3. Distinct types of inflammatory cells in the myocardium, such as CD3+ and CD45ro+, are associated with adverse cardiac events in NI-DCM patients.
4. Inflammation-related cytokines (IL-6, TNF-α and adiponectin) are associated with poor clinical outcomes and can identify high-risk NI-DCM patients.
5. GLS is associated with heart failure severity and can predict adverse cardiac events in NI-DCM patients.

2. LITERATURE REVIEW

2.1. Etiopathogenesis of NI-DCM

NI-DCM is a multi-etiologic disease. However, the most common causes of the disease are genetics and inflammation.

Genetic etiology is determined in approximately one-third of NI-DCM cases [29] and various studies have identified more than 250 genes related to NI-DCM. Of these genes, approximately 60 are included in genetic testing panels. However, recently, an international panel of individual experts in NI-DCM genetics performed a systematic review and curation of published evidence supporting monogenic relationships of genes with NI-DCM [30]. Of the 267 genes known to be associated with NI-DCM, 51 were enrolled in the curation. The panel found that out of 51 genes, just 19 were evidence-genes (definitively/strongly or moderately related with NI-DCM) and should be used in clinical practice. Moreover, these genes explained only a minority of NI-DCM cases (20–35%). In addition, the panel's investigation demonstrated that genetic architecture is complex and diverse, while genes related to NI-DCM span 10 ontologies such as ion channels, sarcomere proteins, sarcolemma, cytoskeleton, nuclear envelope, intercellular junction molecules; therefore, genetic diagnostics is challenging [30]. Furthermore, evidence shows that genetic background plays an important role in the pathogenesis of NI-DCM when it is induced by other (not genetic) etiologic factors [29].

Inflammation plays a crucial role in NI-DCM initiation and progression. Myocardial damage, independently of etiologic factors (genetic or environmental), triggers an inflammatory response leading to the recruitment of inflammatory cells to the heart, a process called acute myocarditis. The primary goal of this inflammatory response is to adapt cardiomyocytes to physiologic stress and repair damaged tissue. However, in many cases, due to unresolved tissue damage or dysregulation of an inflammatory response, the inflammatory response switches from acute to chronic state–in other words, switching from host defense to host injury–in a process called inflammatory dilated cardiomyopathy. Although inflammatory dilated cardiomyopathy is a multi-etiologic disease, the most common causes are infection and autoimmunity. Therefore, this thesis will focus on inflammatory dilated cardiomyopathy caused by a viral infection and autoimmune response associated with a viral infection.

2.1.1. Inflammatory Dilated Cardiomyopathy. Diagnostic Criteria of Inflammatory Dilated Cardiomyopathy

Over the past few decades, the definition of inflammatory dilated cardiomyopathy has been developed [8,13,24,25]. Inflammatory dilated cardiomyopathy is a subgroup of NI-DCM and is defined as myocardial inflammation combined with cardiac dysfunction and ventricular remodeling [13,24]. As stated by the ESC working group on myocardial and pericardial diseases, the gold standard for diagnosing inflammatory dilated cardiomyopathy is endomyocardial biopsy with polymerase chain reaction (PCR) and immunohistochemical evaluation [13]. PCR analysis became an essential procedure for identifying infectious agents associated with inflammatory dilated cardiomyopathy. Consequently, studies have demonstrated that viruses are a predominant cause of viral inflammatory dilated cardiomyopathy [31]. Therefore, immunohistochemical evaluation of biopsy samples is necessary to diagnose or exclude myocardial inflammation.

Diagnostic criteria for inflammation in the myocardium have been updated several times [13,24–26]. In 1985, histological Dallas criteria were proposed to diagnose myocardial inflammation: "histological evidence of inflammatory infiltrates within the myocardium associated with myocyte degeneration and necrosis of non-ischemic origin" [26]. However, after a couple of decades, the criteria were criticized as subjective (diagnosis of inflammation significantly varied among pathologists) and not sensitive enough to diagnose myocardial inflammation [32]. Moreover, it was proved that the rate of inflammation detection by immunohistochemistry was significantly higher than detection by histological Dallas criteria [18,33]. Therefore, Dallas criteria were changed to more sensitive immunohistochemical criteria.

Diagnostic immunohistochemical criteria of myocardial inflammation were introduced at the end of the last century. A task force of the World Heart Federation's (WHF) Council on Cardiomyopathies defined myocarditis (myocardial inflammation) as being when in endomyocardial biopsy detected $\geq$14 infiltrating leukocytes/mm^2 (T lymphocytes (CD3) and macrophages) [25]. Later, in 2013, the European Society of Cardiology (ESC) working group in myocardial and pericardial diseases revised and updated diagnostic immunohistochemical criteria [13]. Based on this document, myocardial inflammation was diagnosed when tests of the endomyocardial sample detected "$\geq$14 leucocytes/mm^2 including up to 4 monocytes/mm^2 with the presence of CD3 positive T-lymphocytes $\geq$7 cells/mm^2" [13].

Regardless of proposed diagnostic criteria by WHF or ESC, criteria for diagnosing myocardial inflammation vary markedly within different studies. For example, a meta-analysis by Katzmann et al. [33] enrolled 61 publications in which patients (n=10491) underwent endomyocardial biopsy with immunohistochemical evaluation. The studies used 13 different criteria, and therefore, the epidemiology of inflammatory dilated cardiomyopathy differed significantly across the studies. In addition, few studies evaluated the prevalence of inflammatory dilated cardiomyopathy by using the latest ESC criteria for diagnosis.

2.1.2. Etiology of Inflammatory Dilated Cardiomyopathy: The Role of Viruses

Infectious agents–predominantly viruses–are one of the leading causes of acute myocarditis [31,34] and account for ~30% of the etiology of NI-DCM [35–41]. Acute myocarditis is an acute inflammatory cardiac disorder [31, 34] that is suspected by clinical presentation and confirmed by histological, immunological and immunohistochemical evaluation of endomyocardial biopsy samples.

Several phases of viral-induced myocarditis can be distinguished (Figure 1). Firstly, the active phase (lasting 1–7 days) is caused by the virus entering the host cell and activating the innate immune response leading to the damage of the cardiomyocyte [42–44]. Activation of the innate immune response causes cytokine release and recruitment of immune cells to the heart [45]. The acute phase then switches to a subacute phase in which the adaptive immune response is activated (lasting 1–4 weeks) [13,42], leading to the increase of pro-inflammatory cytokines and the recruitment of immune cells in the myocardium. After the acute and/or subacute phase, there are several possible options for the disease course: (1) the virus is successfully cleared and the tissue damage is repaired; (2) the virus is not cleared and persists in the myocardium with or without inflammation; (3) a virus–induced autoimmune inflammatory response (possibly due to molecular mimicry) persists even if the virus is cleared from the myocardium [46,47]. In the case of viral persistence and/or persistent myocardial damage, the proteins of viruses and/or injured cells are constantly released. These proteins, along with a dysregulated immune system response, cause persistent low-grade inflammation leading to chronic immune-mediated tissue injury [48]. Approximately half of acute viral myocarditis cases progress to chronic inflammatory state (inflammatory dilated cardiomyopathy) [49].

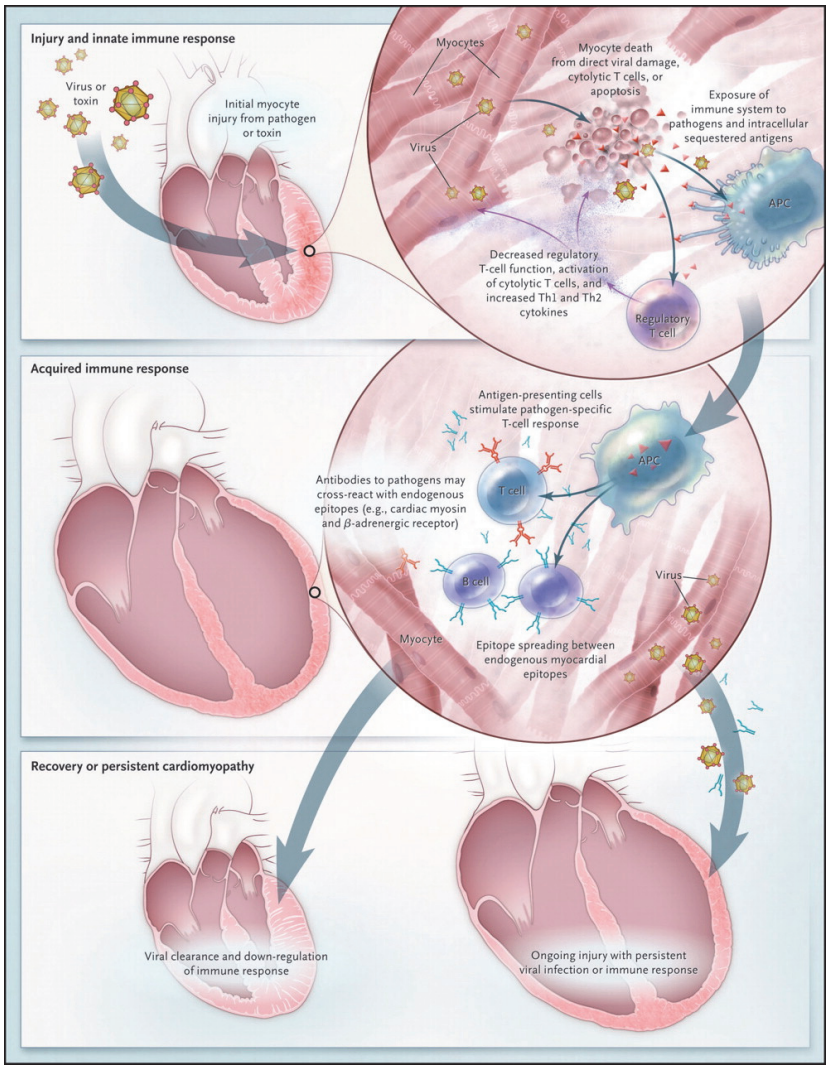

Figure 1. Pathogenesis of myocarditis. APC denotes antigen-presenting cell. Reproduced with permission from Cooper (2009) [20], Copyright Massachusetts Medical Society.

Although different viruses vary considerably in their ability to induce the disease and affect its course, previous studies showed the association between viral persistence in the myocardium and progressive remodeling as well as impairment of left ventricular function [50]. In addition, eliminating the virus from the myocardium (e.g., by antiviral treatment) improves left ventricular

function [51,52]. Despite this evidence, discussion continues among researchers about the role of viruses in the progression of the disease, while viruses are also found in healthy hearts [53], demonstrating the relevance of host immune response.

Numerous viruses can damage the myocardium and induce an inflammatory response. Based on viruses' action on the heart, they could be classified into three groups: direct infiltration (cardiotropic and vasculotropic viruses such as adenoviruses, enteroviruses, parvovirus B19 (PVB19)), indirect infiltration (lymphotropic viruses such as cytomegalovirus (CMV), Epstein-Barr virus (EBV), human herpesvirus 6 (HHV-6)), and viruses that do not infiltrate the heart but trigger an inflammatory response by causing a cytokine storm (e.g., SARS-CoV-2) or cellular immune response against the heart, induced possibly by molecular mimicry [50].

The prevalence of these different viruses has varied over time. Three decades ago, the most frequently detected viruses were adenoviruses and enteroviruses. In the past two decades, herpesviruses (HHV–6, EBV and CMV) and PB19 have been the most frequently found in the myocardium. In addition, various studies have found co-infection of several different viruses (double or triple viral infection). Co-infection accounts for up to 30% of virus-positive NI-DCM patients [54,55].

Adenoviruses and enteroviruses are cytolytic viruses that induce direct myocardial injury and trigger an immune response even after viral clearance [56]. However, about half of the patients with adenovirus- or enterovirus-induced myocarditis recover without residual injury [52].

The prevalence of myocarditis induced by herpesviruses is not high, but when they are detected, the most common strains are HHV-6, EBV and VZV. These viruses induce myocarditis by direct cytopathic effect and secondary endothelial dysfunction or immune-mediated damage. These viruses can have lifelong persistence in various cells in the body [48].

PVB19 can enter endothelial cells, induce apoptosis of the cardiomyocyte [57] and trigger an immune response [58]. PVB19 can cause acute myocarditis and can also persist with reactivation episodes [31]. In addition, autopsy studies have found PVB19 in healthy hearts with no signs of cardiac inflammation or cardiomyopathy [59,60].

In summary, the data concerning the impact of the presence of cardiotropic viruses on clinical significance and prognosis remains a topic of debate and discussion [18,19].

2.1.3. Etiology of Inflammatory Dilated Cardiomyopathy: The Role of Autoimmunity

Myocardium damage by viruses can secondarily induce an autoimmune response, possibly due to molecular mimicry leading to activation of virus-specific T lymphocytes. Activated T-cells attack the myocardium, and activated inflammatory cells secrete proinflammatory cytokines ((e.g., tumor necrosis factor (TNF), interferon- (IFN-)γ, interleukin (IL)-1a, IL-1b and IL-2), which further exacerbate damage of the myocardium. Furthermore, activated B cells produce autoantibodies against specific autoantigens, which aggravates the myocardial damage and worsens cardiac function.

Heart-specific antibodies are detected in more than half of NI-DCM patients [61]. Autoantibodies can be against different parts of contractile apparatus, like matrix proteins [37–40]. These autoantibodies can directly affect the function of cardiomyocytes and stimulate the progression of the disease [62–64]. Previous studies have found a vast array of different autoantibodies in NI-DCM patients, including β1-adrenergic and M2 muscarinic receptors, cardiac myosin and troponin I, adenine nucleotide translocator, L-type Ca^{2+} channel and the Na+/K+ ATPase [64–66]. However, the most frequently detected autoantigens are against cardiac α-myosin and β-myosin heavy-chain isoforms [67]. Furthermore, HLA molecules can also be autoantigens leading to autoimmune response [68]. In addition, genetic predisposition might be a key factor influencing the progression of autoimmune myocarditis to inflammatory dilated cardiomyopathy [29,69]. Taken together, pathologic autoimmune response augments myocardial damage, leading to chronic inflammation and cardiac remodeling [70–72].

2.2. Pathogenesis of Inflammatory Dilated Cardiomyopathy

In recent years, the concept of chronic heart failure pathogenesis has changed dramatically. It has become clear that chronic heart failure is not simply a hemodynamic failure and not a problem of impaired neuroendocrine activation; it is a far more complex process, a systemic disorder involving immune activation, metabolic alterations, and pathologic processes skeletal muscle [73].

2.2.1. Pathogenesis: Role of Chronic Inflammation.

Chronic inflammation underlies the pathogenesis in a wide array of cardio-vascular diseases, including atherosclerosis, hypertension, myocarditis, myo-

cardial infarction and chronic heart failure [74]. Mounting evidence shows a significant role of inflammation in the pathogenesis of various heart failure types [75–77]. However, whether inflammation is a cause or a consequence of chronic heart failure is still and ongoing discussion. While chronic heart failure and chronic inflammation are mutually related [78], heart failure leads to malfunctioning and/or death of the cells that trigger an inflammatory response. On the other hand, chronic inflammation stimulates cardiac fibroblasts that induce an inflammatory response by expressing chemokines. Chemokines induce activation of endothelial adhesion molecules and recruitment of immune cells in the myocardium [79,80]. These processes maintain the vicious circle of chronic inflammation and worsening heart failure [80].

Acute damage of the myocardium (by environmental or genetic factors) induces an immune response causing the recruitment of inflammatory cells to the myocardium as well as pro-inflammatory cytokines synthesis [45]. This process is essential for repairing damaged tissue, healing and/or clearing an infectious agent [45]. However, in up to half of cases, an immune response is dysregulated and leads to an exaggerated and/or chronic inflammatory process causing cardiac remodeling and dysfunction [81,82]. This induced process (myocardial damage and ongoing inflammation) sets off a vicious circle of myocardial damage, the persistence of chronic inflammation, and cardiac remodeling [83]. Additionally, previous studies have shown that myocardial inflammation, confirmed by endomyocardial biopsy, is a factor responsible for the progression of LV dilatation [20–22,84]. However, it is still unclear how the immune response shifts from host defense to host injury.

In the literature, long-lasting chronic inflammation is also referred to as a low-grade inflammation, in other words, an intermediate condition between inflammation and basal (non-inflammatory) state [85]. Chronic inflammation is a complex and cumbersome process consisting of multiple factors, and various factors are strongly related to each other and act in multiple ways. Even so, for clarity, inflammation types can be usefully divided into two main groups: myocardial inflammation and systemic inflammation.

2.2.2. Pathogenesis: The Role of Myocardial Inflammation

The importance of immune cells in the pathogenesis of inflammatory cardiomyopathy has been demonstrated in various studies. However, results are inconsistent, and while each type of immune cell has multiple actions, just some of these actions have been identified.

T-cells (CD3+) are known critical mediators of cardiac damage [86–89]. Activation of T-lymphocytes seems to be a crucial component of autoimmune inflammatory cardiomyopathy [90]. However, different subtypes of T-cells might have opposite effects on the pathogenesis of the diseases. For example, CD4+ T-cells are associated with left ventricular remodeling [91], while TH17 cells are associated with the progression of NI-DCM [92], and regulatory T-cells attenuate myocardial inflammation and progression of the disease [87,93].

B-cells are a link between an innate and adaptive immune response. Although their infiltration in the myocardium is not increased in patients with inflammatory cardiomyopathy, they contribute by maintaining chronic inflammation by secreting autoantibodies [94]. Autoantibodies are directed against numerous cardiac proteins leading to damage of the myocardium and cardiac dysfunction [65].

Macrophages (CD68+) participate in the innate immune response. These cells have profibrotic properties and contribute to the fibrosis process in the necrotic tissue [95]. Furthermore, M2 macrophages are associated with collagen formation leading to left ventricular remodeling [96–99]. In addition, another crucial role of macrophages in sustaining chronic inflammation is the secretion of pro-inflammatory cytokines [100,101].

The expression of cell adhesion molecules (e.g., intercellular cell adhesion (ICAM)-1, human leukocyte antigen (HLA)) on endothelial cells are increased during inflammation. These cells mediate the migration of immune cells (T-cells, macrophages) to the myocardium [102]. Thus the increase of T-lymphocytes is associated with increased expression of cell adhesion molecules. A study on mice by Salvador et al. [103] demonstrated that T-cell recruitment in the myocardium mediated by ICAM-1 is strongly associated with pathological cardiac remodeling.

Various immune cells promote cardiac remodeling, collagen deposition, and fibrosis [41,104,105]. In addition, immune cells produce various cytokines, which induce not only a local (in the myocardium) immune response but also systemic inflammation.

2.2.3. Pathogenesis: The Role of Systemic Inflammation

Systemic inflammation plays a crucial role in the pathogenesis of chronic heart failure (including NI-DCM) [106]. Systemic inflammation is reflected by increased levels of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) and decreased levels of anti-inflammatory cytokines (IL-10, TGF-β) that are

secreted by infiltrating immune cells and resident cells in the myocardium, including cardiomyocytes, cardiac fibroblasts, and endothelial cells [107]. Increased levels of cytokines can affect the heart via different mechanisms, including cardiomyocyte apoptosis, extracellular matrix degradation, and fibrosis. TNF-α can induce cardiomyocyte apoptosis [108,109]. TNF-α and IL-1beta can change Ca^{2+} homeostasis in cardiomyocytes, leading to impaired inotropy of the ventricle [110–112] and inducing hypertrophy [109]. IL-6 reduces titin phosphorylation, causing increased stiffness of the ventricle [113]. All these mechanisms lead to the progression of cardiac fibrosis and remodeling. In addition, systemic inflammation in heart failure patients also affects other organs leading to anemia, endothelial dysfunction, atherogenesis and cachexia (Figure 2) [114], further exacerbating heart failure.

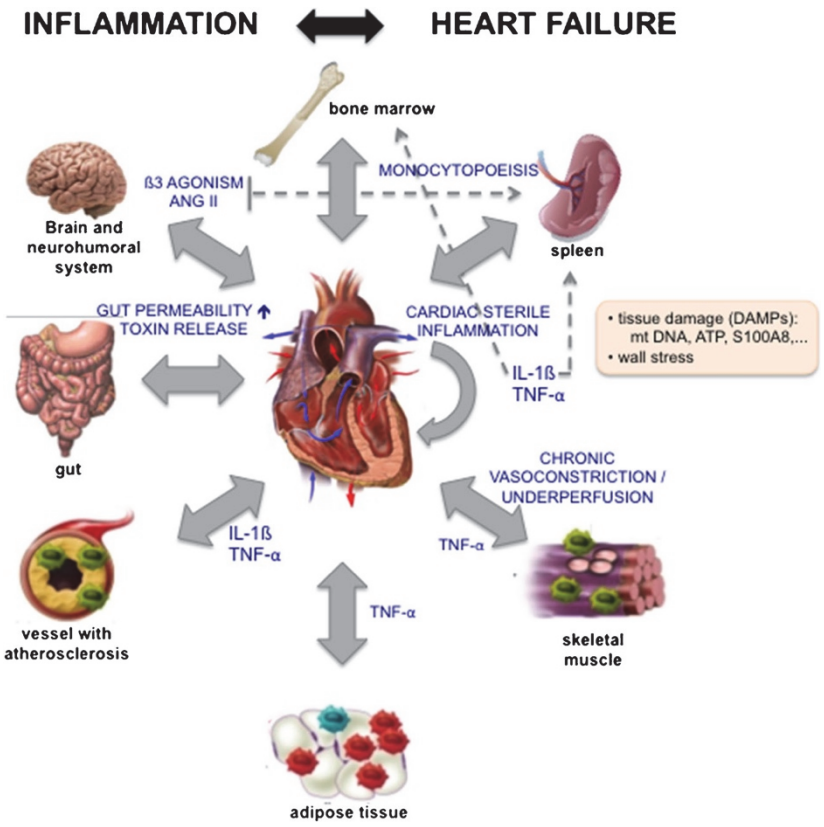

Figure 2. Inflammation and heart failure interconnect with each other. Induced systemic inflammation by cardiac damage affects multiple organs causing multiple effects: cell death or growth, fibrosis, remodeling, metabolism disturbances, and inflammation. Figure reproduced from Van Linthout and Tschöpe (2017) [78] (CC BY 4.0).

Consistent with deleterious effects of the most studied cytokines (IL-6 and TNF-α), it was suggested that they are potential markers for heart failure [101,115–117]. It was demonstrated that increased levels of cytokines are associated with the severity of the disease [118] and poor clinical outcomes in heart failure patients [118–120]. Furthermore, these cytokines were used as targets for treatment. Unfortunately, the results were disappointing: patients treated with immunomodulatory drugs had the same outcomes as the control group [121]. Therefore, profound characterization of chronic inflammatory response might be important not only for understanding the pathogenesis but also for searching for new therapeutic targets.

2.3. Prognosis of NI-DCM

Owing to the pathogenic diversity of NI-DCM, the disease progression differs significantly among patients. Since drugs reducing activation of the neurohumoral system were introduced into daily clinical practice, the survival of heart failure patients has increased remarkably [122]. However, regardless of that, a significant proportion of NI-DCM patients deteriorate (progressively) either until death or until they receive a heart transplant. Therefore, the prognostication in this patients group is crucial.

Identifying high-risk heart failure patients is essential for decision making, patient treatment, the frequency of follow-ups and prioritization for heart transplantation. A wide array of parameters (e.g., clinical characteristics, circulating biomarkers, measurements of various diagnostic tests, treatment strategies) are associated with heart failure severity and poor clinical outcomes. Some of the parameters have good accuracy for identifying high-risk heart failure patients and are involved in various prognostic scores, which are also used in clinical practice. Although these scores have good accuracy for predicting poor clinical outcomes in heart failure or HFrEF population, the prognostication of adverse cardiac events in a subpopulation of NI-DCM patients is moderate. Two aspects might account for this: development of the scores in heterogeneous HFrEF populations and differences between the two main HFrEF subpopulations (i.e., ischemic heart disease and NI-DCM).

Prognostic scores have been developed, and their prognostic value was verified in large patient cohorts with chronic heart failure [123–130]. However, this population consists of multi-etiologic diseases, primarily NI-DCM and ischemic heart disease. Ischemic heart disease patients stand out

not just because of a poorer prognosis than patients with non-ischemic heart failure [131,132], but also in having different values (sometimes worse) of some biomarkers. Thus, the prognostic role of various biomarkers in chronic heart failure studies might be primarily determined by patients with ischemic etiology. On the other hand, there is a lack of data on the predictive value of various biomarkers in NI-DCM patients, whereas the developed prognostic scores for the NI-DCM population have only moderate accuracy [28]. Therefore, the clinical applicability of these scores is limited and the precise risk stratification remains challenging in NI-DCM patients. Thus there is a need for novel biomarkers in order to better aid the risk stratification of patients.

2.3.1. Prognostic Value of Cardiotropic Viruses in NI-DCM Patients

Although viruses are a key component in the pathogenesis of inflammatory cardiomyopathy, their prognostic role is ambiguous. Kuehl et al. [51] demonstrated an association between viral infection in the myocardium and deterioration of left ventricular function, while other studies [18,133] found no association between viruses in the myocardium and poor clinical outcomes. In addition, an autopsy study found PVB19 genomes in up to 44% of healthy hearts [134]. Recently, a study of retrospective analysis of endomyocardial biopsies revealed that persistence of PVB19 with transcriptional activity but not sole genomes are associated with adverse clinical outcomes [135].

2.3.2. Prognostic Value of the Myocardial Inflammation in NI-DCM patients

Although myocardial inflammation is a key process in the pathogenesis of inflammatory cardiomyopathy, the prognostic value of myocardial inflammation has rarely been assessed. Instead, most studies focus on the pathophysiologic processes and their associations with left ventricular function or treatment strategies.

Several studies analyzed the predictive value of myocardial inflammation or distinct types of inflammatory cells in patients with acute myocarditis or NI-DCM. Kindermann et al. [18] diagnosed myocardial inflammation for half of the 181 patients with acute myocarditis based on the immunohistochemical criteria >14 leukocytes/mm^2 (CD3+ T lymphocytes and/or CD68+ macrophages) in the myocardium, in addition to enhanced expression of HLA class II molecules. They showed that myocardial inflammation is associated with a

higher risk of adverse cardiac events (death or heart transplantation). Another study by Zuern et al. [136] evaluated the prognostic value of myocardial inflammation (CD3+, CD68+ and MHCII cells) in a cohort of 227 chronic heart failure patients (including chronic myocarditis 110 and DCM 71 patients) over 3 years of follow-up. No association was found between inflammatory markers and poor clinical outcomes. These findings are supported by another study done by Greulich et al. [133], which enrolled 108 patients who underwent endomyocardial biopsy due to suspected myocarditis or NI-DCM. Myocardial inflammation was diagnosed in 62% of the patients by detecting ">14 leukocytes per mm^2 (CD3+ T lymphocytes and/or CD68+ macrophages) in the myocardium, in addition to enhanced expression of HLA class molecules" in the biopsy samples. However, myocardial inflammation was not associated with poor clinical outcomes. On the other hand, a study by Nakayahma et al. [96] identified separate inflammatory cells in the myocardium as predictors of 6.9 ± 2.4 years of poor clinical outcomes in a cohort of 182 NI-DCM patients. They found that patients with a higher count of CD3+ (T lymphocytes ≥14 cells/mm^2), CD68+ (macrophages ≥32 cells/mm^2) and CD163+ (M2 macrophages ≥10 cells/mm^2) had worse clinical outcomes.

In sum, the prognostic role of myocardial inflammation on clinical outcomes varies in different studies. One of the reasons might be diverse diagnostic criteria [18,33,96,137,138] . In addition, to our knowledge, none of the previous studies estimated the prognostic value of the latest criteria proposed by ESC for diagnosing myocardial inflammation.

2.3.3. Prognostic Value of C-Reactive Protein

C-reactive protein (CRP) is a non-specific inflammatory protein released by hepatocytes. CRP is associated with an inflammatory response in various chronic diseases such as hypertension, atherosclerosis, diabetes mellitus, chronic kidney disease [139,140], and chronic heart failure [141–143]. Higher CRP levels are associated with a higher hospitalization and mortality rate in heart failure patients [144,145]. Few studies have evaluated CRP prognostic value in NI-DCM patients, but the ones that have, have shown an association between higher CRP levels and poor clinical outcomes over 2.6 and 3.5 years of follow-up [146,147]. On the other hand, a study by Lamblin et al. [144] showed CRP association with adverse cardiac events in a heterogeneous HFrEF cohort and ischemic heart failure patients over 2.7 years of follow-up, but not in NI-DCM patients. To our best knowledge, none of these studies

estimated the prognostic value of CRB in NI-DCM patients for long-term (5 years) outcomes.

2.3.4. Prognostic Value of Adiponectin

Adiponectin is an adipocyte-derived cytokine (adipokine) that is also synthesized in cardiac muscle cells and connective tissue cells within the heart [148]. Adiponectin has a critical signaling function in the heart which is particularly important in patients with heart failure. Adiponectin has anti-apoptotic, fibrosis reducing, and oxidative stress diminishing properties in the myocardium [149,150]. Lower serum levels of adiponectin is an independent cardiovascular risk factor in coronary artery disease [151–154]. Low serum levels of adiponectin also increase cardiovascular risk and inflammation in hypertension, coronary artery disease, obesity, and insulin resistance [151,155,156]. On the other hand, high adiponectin levels are associated with increased risk of recurrent cardiovascular events [157] and mortality in patients with acute myocardial infarction [158] and heart failure [159,160]. In HFrEF patients, adiponectin levels are increased and correlate with mortality, disease severity, and heart failure symptoms [161,162].

Although it seems that adiponectin has an additive role in predicting the course of chronic heart failure [163–165], it has not been officially recognized as a biomarker in HF with reduced ejection fraction [166]. Tamura et al. [165] showed that adiponectin could be an independent predictor of mortality in patients with ischemic heart failure. However, they did not find a significant impact of high serum adiponectin levels on the mortality of patients with non-ischemic heart failure.

2.3.5. Prognostic Value of Soluble Urokinase-Type Plasminogen Activator Receptor (suPAR)

Soluble urokinase-type plasminogen activator receptor (suPAR) is a soluble form of the urokinase-type plasminogen activator receptor (uPAR), which is a membrane-bound receptor with a glycosyl-phosphatidylinositol anchor. When the urokinase plasminogen activator attaches to uPAR, the receptor cleaves from the anchor and is released as a soluble form, namely suPAR [167]. suPAR originates in various cells, e.g., activated T-cells, monocytes, macrophages, endothelial cells, and fibroblasts. suPAR is exerted in various physiologic processes such as inflammation, plasminogen activation, cell adhesion, modulation, and proliferation [167].

suPAR is an inflammatory marker that reflects activation of the immune system. In addition, it was found to be a good prognostic biomarker in numerous diseases: infectious diseases (e.g., tuberculosis, malaria, HIV), cancer, autoimmune diseases, chronic kidney disease, and cardiovascular diseases such as atherosclerosis [168–170]. Recently, it was demonstrated that suPAR could be a useful prognostic biomarker in chronic heart failure patients [171]. However, to the best of our knowledge, its predictive value in NI-DCM patients has not been estimated.

2.3.6. Prognostic Value of Interleukin 6 (IL-6)

IL-6 is a cytokine with a pleiotropic effect on immune response (especially in the acute phase), inflammation, and hematopoiesis [172]. IL-6 is secreted by most tissues (including the myocardium) and immune cells. The cytokine plays an important role in transitioning from an innate to adaptive immune response [173]. The primary role of IL-6 is to stimulate the immune response and maintain tissue integrity. However, dysregulated immune response might cause constant IL-6 elevation, leading to sustain chronic inflammation and fibrosis induction [174].

IL-6 has a protective effect (e.g., antioxidative, anti-apoptotic) on the heart [175–177]. In order to prevent myocardial damage in acute viral myocarditis, IL-6 limits viral replication [178]. Moreover, in cases of ischemia-reperfusion injury or myocardial infarction, IL-6 has a beneficial effect while playing a role in ischemic preconditioning by causing a cascade of biochemical events in the cardiomyocyte caused by brief ischemia, protecting the myocardium from subsequent ischemia [179]. On the other hand, persistent elevation of IL-6, e.g., after myocardial infarction or in heart failure, is associated with progressive left ventricular remodeling [180,181 and poor clinical outcomes [176,181]. To sum up, in acute cases of myocardial damage, IL-6 has protective effects, while in chronic myocardial injury, IL-6 action becomes deleterious and contributes to chronic heart failure.

Previous studies have found increased IL-6 concentrations in chronic heart failure [182,183] and demonstrated that increased serum levels of IL-6 are associated with heart failure severity [183–185]. In addition, IL-6 was found to be a predictor of mortality in patients with acute heart failure and acute coronary syndromes (n=75) [186] as well as chronic heart failure [182,183].

It is known that IL-6 concentrations differ between heart failure phenotypes, heart failure severity, and even between etiologic groups of heart failure.

Previous studies have shown that NI-DCM patients have lower concentrations of IL-6 than patients with ischemic heart failure [187,188]. However, the prognostic value of IL-6 was assessed in heterogenous cohorts of chronic heart failure patients but not separately in NI-DCM patients.

2.3.7. Prognostic Value of Tumor Necrosis Factor α (TNF-α)

TNF-α is a cytokine with pleiotropic effects secreted by activated monocytes, macrophages, natural killers and T-lymphocytes, in addition to other types of cells, including cardiomyocytes [189,190]. TNF-α plays an important role in immune response in order to heal and repair damaged tissue. The cytokine is essential for inducing an innate immune response by activation of transcriptional factor (NF-kB), leading to activation of immune cell recruitment, increased secretion of pro-inflammatory cytokines, and promoting apoptosis via activation of metalloproteinase [190–192].

TNF-α plays a crucial role in the pathogenesis of various diseases (e.g., infection, autoimmune diseases, obesity, diabetes mellitus, cancer) [193–199]. In addition (and like IL-6), TNF-α has a protective effect on the heart by controlling innate immune response and tissue injury. On the other hand, persistent TNF-α elevation has deleterious effects on the heart, leading to the remodeling of the ventricle: it impairs myocyte contractility, promotes cardiomyocyte hypertrophy, and induces cardiomyocyte apoptosis and fibrosis of the myocardium [200–205].

Previous studies have found increased TNF-α levels in numerous cardiovascular diseases, like acute viral myocarditis [206], atherosclerosis [200,201,207,208]. Moreover, it was demonstrated that serum TNF-α concentration positively correlates with the level of cardiac dysfunction [201,206] or severity of heart failure [118,185,209]. Furthermore, TNF-α is associated with increased mortality and poor clinical outcomes in chronic heart failure patients [208,210,211]. However, attempts to use this cytokine as a treatment target in HFrEF patients were unsuccessful [121,212,213].

Elevated TNF-α levels were also found in patients with NI-DCM [187,188]. However, compared with ischemic heart failure patients, TNF-α expression and the serum concentration were significantly lower in patients with NI-DCM [187,188]. Although TNF-α was found to be a prognostic marker in a heterogeneous chronic heart failure population, no studies on the prognostic value in NI-DCM patients has been found.

2.3.8. Prognostic Value of Global Longitudinal Strain (GLS)

Left ventricular global longitudinal strain (GLS) is a well-validated, easily-performed echocardiographic parameter for evaluating myocardial deformation. GLS predicts poor prognosis in various cardiac diseases, including ischemic heart disease [214,215] and heart failure with reduced ejection fraction (HFrEF) [216–218]. However, studies investigating mixed HFrEF populations have also included patients with ischemic heart disease (usually half of the cohort), who have a worse prognosis than patients with non-ischemic heart failure [131,132]. Therefore, the prognostic significance of GLS in the HFrEF population has been confounded by the inclusion of ischemic heart failure patients. Moreover, a recent study by Zuo et al. [219] demonstrated that GLS patterns are different between ischemic heart disease and NI-DCM, which might have an affect on the GLS prognostic value. Furthermore, the predictive value of GLS has not been evaluated in patients with only NI-DCM.

3. RESEARCH DESIGN AND METHODS

3.1. Ethical Approval

The study was conducted in accordance with the Declaration of Helsinki. All patients provided written informed consent. Ethical approval was obtained from the local Lithuanian Bioethics Committee (license numbers 158200-09-382-l03; 158200-382-PP1-23; and 158200-17-891-413).

3.2. Study Population. Inclusion and Exclusion Criteria.

A prospective cohort study with retrospective analysis was done in the Vilnius University Hospital Santaros Klinikos. We enrolled 57 patients with suspected NI-DCM that were admitted to the hospital for diagnostic evaluation between January 2010 and December 2013. The median duration of heart failure symptoms before enrollment was 12 (5–60) months. The inclusion criteria were symptoms and signs of heart failure, with echocardiographic evidence of left ventricular dilation and reduced LVEF (≤45%).

Exclusion criteria were:

- significant coronary artery disease, defined as at least 50 % proximal stenosis of a coronary artery or a history of myocardial infarction;
- known causes of heart failure, such as primary valvular or heart muscle disease, hypertensive heart disease, endocrine disease, advanced chronic kidney disease, drug or alcohol abuse;
- acute myocarditis (new-onset symptoms during the past three months) or acute myocardial infarction suspected by clinical presentation or diagnostic tests.

Patients underwent transthoracic echocardiography on the same day or day before interventional procedures: coronary angiography and right heart catheterization.

All patients were treated according to the ESC guidelines [220,221]. At the time of inclusion, none of the patients were treated with inotropic agents. Specific etiology-directed treatment was not administered.

3.3. Medical Examinations

Study patients underwent a detailed medical interview, physical examination and routine laboratory tests, including complete blood count, high sensi-

tivity C-reactive protein (hs-CRP), creatinine (CKD-EPI creatinine equation was used to estimate glomerular filtration rate (GFR)). Additionally, high-sensitivity troponin T (hs-TnT) was measured in serum using an Elecsys 2010 analyzer (Roche Diagnostics, Indianapolis, Indiana) and B-type natriuretic peptide (BNP) using ARCHITECT i analyzer (Abbott, Illinois, USA). Laboratory tests were performed in the laboratory of our university hospital, which is accredited according to the international standard EN/ISO-IEC 17,025.

Mandatory investigations included coronary angiography to exclude coronary artery disease, right heart catheterization for hemodynamic evaluation. During the same procedures, right ventricle endomyocardial biopsy was performed for the immunohistochemical evaluation and the detection of viruses by PCR.

3.4. Biochemical Assays of Serologic Inflammation-Related Markers

Plasma samples were stored at −80 °C until analysis. The proinflammatory serum cytokines TNF-α and IL-6 were measured by solid-phase, chemiluminescent immunometric assays using IMMULITE/Immulite 1000 systems (Immulite, Siemens) according to the manufacturer's instructions: TNF-α (Catalog number LKNFZ (50 tests) and LKNF1 (100 tests)), IL-6 (Catalog number LK6PZ (50 tests) and LK6P1 (100 tests)) and expressed as pg/mL. Adiponectin was measured using the Millipore Adiponectin assay according to the manufacturer's recommendations (Millipore, Burlington, MA, USA) and expressed as mg/mL. Levels of human soluble urokinase-type plasminogen activator receptor (suPAR) were estimated by ELISA assay according to the manufacturer's recommendations (Abbexa). Absorbance was measured at 450 nm with a spectrophotometer (Varioskan®Flash, Thermo Fisher Scientific, Vantaa, Finland). Final concentrations of suPAR are expressed as ng/mg protein.

3.5. Echocardiography

Echocardiographic evaluation was carried out using commercially available ultrasound machines (GE Vivid 7 or 9) with a 2.5-MHz probe. Images were digitally stored and analyzed offline using EchoPAC version PCBT08. We used a routine protocol of our laboratory for conventional M-mode, two-dimensional, Doppler, and tissue Doppler echocardiographic measurements [222]. LV end-diastolic (LVEDD) diameter was measured from the parasternal long-axis view and indexed to the body surface area. LV end-systolic and end-

diastolic volumes were measured, and LVEF was calculated by the Simpson biplane method. Left atrium volume was measured by a biplane area-length method from the apical four- and two-chamber views and indexed to the body surface area. Mitral E and A peak velocity and deceleration time were measured, and the ratio of early-diastolic LV inflow velocity to atrial-systolic velocity (E/A) calculated. The average tissue Doppler-derived early diastolic mitral annular velocity (e') was obtained from the mitral annulus' septal and lateral sides. The average ratio of early-diastolic LV inflow velocity to early-diastolic mitral annular velocity (E/e´) was calculated. Left ventricular diastolic dysfunction was graded according to the ESC guidelines [223]. Mitral regurgitation and tricuspid regurgitation (TR) severity, as well as right ventricular function, were assessed visually.

3.6. Myocardial Deformation Analysis by Two-Dimensional Speckle Tracking Echocardiography

Echocardiographic images were acquired at 50–70 frames/s (with individual adjustment) for LV GLS analysis. A digital loop was acquired from three apical views (four-, two- and three-chamber views). After the manual cardiac cycle selection, the LV endocardial border was manually traced at the end-systolic frame (aortic valve closure was used for the end-systole timing). The investigator visually assessed the detected region of interest (ROI) and, if necessary, manually modified the ROI to ensure accurate tracking of the speckles. In the case of inaccurate speckle tracking, ROI was readjusted. We calculated the GLS by averaging the mean values of all valid segments.

Forty-one patients had echocardiographic images of sufficient quality for further two-dimensional myocardial deformation analysis. These patients comprise the cohort analyzed in the Paper IV.

3.7. Right Heart Catheterization

A Swan-Ganz catheter was inserted using a femoral approach in a supine position. The zero reference level of fluid-filled transducers was set at the mid-axillary line. Right atrium pressure, mean pulmonary artery pressure (mPAP), and pulmonary capillary wedge pressure (PCWP) were obtained. Wedge position in the PCWP measurement was confirmed by fluoroscopy, waveform changes, and arterial saturation$\geq$95%. Cardiac output (CO) was measured using Fick's method and calculated by an equation: CO (l/min)=

oxygen consumption (ml/min) / ((aorta SaO_2 – pulmonary artery SvO^2) x hemoglobin × 1.34). Only oxygen consumption (VO_2) was estimated indirectly using VO2 nomograms based on age, weight and sex. Mixed venous blood was sampled for oximetry from the pulmonary artery, arterial blood – from the aorta. We calculated cardiac index (CO indexed to the body surface area), and pulmonary vascular resistance (PVR) ((mean PA pressure − PA wedge pressure)/CO) [224]. Each pressure measurement was recorded over a brief breath-hold at the end of expiration and was averaged over three consecutive cardiac cycles via computerized analysis [225].

3.8. Endomyocardial Biopsy

Right ventricular endomyocardial biopsy was obtained using a flexible bioptome *(Westmed)* via the right femoral vein [226]. Biopsies were drawn from the right interventricular septum. At least 3 endomyocardial biopsies were subjected to conventional histologic and immunohistochemical evaluation and 2 endomyocardial biopsies were stored at −70 C in the biobank as retained biosamples. Endomyocardial biopsies were immediately placed on ice and investigated within 24 hours.

Three endomyocardial biopsy procedures were discontinued because of arrhythmias or right ventricular perforation, and as a result, immunohistochemical analysis was not performed for two of these patients and PCR analysis – for one patient due to a lack of biopsy material. Therefore, out of 57 patients, immunohistochemical evaluation was performed for 55 patients and PCR – for 56 patients.

3.9. Histological and Immunohistochemical Assessment of Endomyocardial Biopsies

Endomyocardial biopsy 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 Hematoxylin and Eosin (H&E) according to the standard protocol for the routine histological evaluation. Histological diagnosis was based on the Dallas criteria [26,227]. The experienced pathologist evaluated endocardium (thickness, subendocardial fat, fibrosis, and inflammation); myocardium (muscle fiber number, size, and damage); interstitium (fibrosis, fat, edema, and inflammation); and intramural vessels (size, signs of

inflammation, damage, and luminal stenosis). Immunohistological assessment of endomyocardial biopsies was carried out as described elsewhere [227].

We detected infiltrative inflammatory cells in the myocardium using the following antibodies: T-lymphocyte CD3 (Agilent DAKO, Hamburg, Germany), active-memory T-lymphocyte CD45ro (Agilent DAKO, Hamburg, Germany), macrophage CD68 (Agilent DAKO, Hamburg, Germany), T-helper cell CD4 (Agilent DAKO, Hamburg, Germany), intracellular adhesion molecule 1 (ICAM-1) CD54 (Leica Biosystems, Newcastle, United Kingdom) and MHC class II cell surface receptor HLA-DR (Agilent DAKO, Hamburg, Germany). Positive cells were registered by an experienced pathologist and expressed as the number of cells per mm^2. Myocardial inflammation was diagnosed according to the criterion established by the ESC Working Group on Myocardial and Pericardial Diseases. This criterion is immunohistochemical detection of significant focal or diffuse cellular infiltration in the endomyocardial biopsy ($\geq$ 14 leucocytes/mm^2, including up to 4 monocytes/mm^2 with the presence of CD3 positive T-lymphocytes $\geq$ 7 cells/mm^2) [13]. Inflammatory endothelial activation was diagnosed if immunohistochemical analysis revealed $\geq$ 3 cells expressing adhesion molecules, i.e., ICAM-1 (CD54) and/or HLA-DR [227].

3.10. Detection of Viral Genomes

Genomic DNA and total RNA were extracted simultaneously using the ZR-Duet DNA/RNA Miniprep kit (Zymo Research, Irvine, CA, USA). RNA (1 µg) was reverse transcribed in 20 µl reaction volumes using random hexamers and the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Vilnius, Lithuania) according to the vendor's recommendations and diluted up to 100 µl with deionized water after the reaction.

Nested PCR primers for the detection of adenovirus [228], herpes simplex viruses 1 and 2, varicella-zoster virus (VZV), cytomegalovirus (CMV), parvovirus B19 (B19V), Epstein-Barr virus (EBV), hepatitis C virus (HCV), enterovirus (EV), and rubella virus [229] are described elsewhere. Primer sequences for the nested PCR of human herpes virus 6 (HHV-6, GenBank accession no. NC001664.2 and NC000898.1); Kirsten rat sarcoma viral oncogene homolog (KRAS, GenBank accession no. NM033360); and ubiquitin C (UBC, GenBank accession no. NM021009) are presented in Table 1. Forward primers for the second PCR step (N2Fw) were labeled with 6-carboxyfluorescein at the 5′ end. All primers were synthesized by Metabion Company (Martinsried, Germany).

Table 1. Primers for detection of human herpes virus 6 (HHV6), Kirsten rat sarcoma viral oncogene homolog (KRAS) and ubiquitin C (UBC).

Primer	Sequence (5′ – 3′)
HHV6-N1 Forward	ACCCGAGAGATGATTTTGCGTG
HHV6-N1 Reverse	GCAGAAGACAGCAGCGAGATAG
HHV6-N2 Forward	CATAGCAACCTTTTCTAGCTTTGAC
HHV6-N2 Reverse	TCTATAACATAAATGACCCCTGGGA
UBC-N1 Forward	TTCTTTCCAGAGAGCCGAAC
UBC-N1 Reverse	CCCATCTTCCAGCTGTTTTC
UBC-N2 Forward	TGGGTCGCAGTTCTTGTTTG
UBC-N2 Reverse	CCTTCCTTATCTTGGATCTTTGCC
KRAS-N1 Forward	CTTTGGAGCAGGAACAATGTCT
KRAS-N2 Forward	AATCCAGACTGTGTTTCTCCCT
KRAS-N1/N2 Reverse	TACACAAAGAAAGCCCTCCCC

All PCRs were run on a TProfessional Standard thermocycler (Biometra, Göttingen, Germany), as described by Allard et al.[228]. KRAS and UBC detection was used to validate the extraction of nucleic acids and was performed in parallel according to the conditions for viral DNA and RNR, respectively. Final PCR products were diluted 10-fold and sized by capillary electrophoresis on a Genetic Analyzer 3130xl, using GeneScan 600 LIZ™ Size Standard and Gene Mapper Software v4.1 (Applied Biosystems, Foster City, CA, USA). For positive results, the genomic DNA or RNR specimens from peripheral blood samples were also tested to exclude the contamination.

3.11. Biochemical Assays of Serologic Inflammation-Related Markers

Plasma samples were stored at −80°C. The proinflammatory serum cytokines TNF-α and IL-6 were measured by solid-phase, chemiluminescent immunometric assays using IMMULITE/Immulite 1000 systems (Immulite, Siemens) according to manufactures instructions: TNF-α (Catalog number LKNFZ (50 tests) and LKNF1 (100 tests)), IL-6 (Catalog number LK6PZ (50 tests) and LK6P1 (100 tests)) and expressed as pg/mL. Adiponectin was measured by Millipore Adiponectin assay according to manufacturers' recommendations (Millipore, USA) and expressed as mg/mL. Levels of Human Soluble Urokinase-Type Plasminogen Activator Receptor (suPAR) were estimated by ELISA assay according to the manufacturer's recommendations

(Abbexa). Absorbance was measured at 450 nm with a spectrophotometer (Varioskan®Flash, Thermo Scientific). Final concentration of suPAR is expressed as ng/mg of protein.

3.12. Follow-up

Patients were followed up for five years after enrollment in the study. The clinical outcome measure was defined retrospectively and was a composite endpoint of left ventricle assist device implantation, heart transplantation, or cardiovascular death. The time of the first event was included in the analysis. Adverse cardiac events were confirmed by medical records, national death registry records, or telephone interviews with the patients' families. The clinical relevance of choosing these outcomes as a composite endpoint was based on the idea that all these outcomes are clinically relevant and reflect the same clinical and probably pathophysiological state of advanced heart failure when there are no more viable alternatives of treatment.

3.13. Statistical Analysis

Data analyses (Papers I-IV) included the whole cohort – 57 patients. However, 41 patients comprise the cohort of Paper IV, intended for GLS predictive value, because forty-one patients had echocardiographic images of sufficient quality for further two-dimensional myocardial deformation analysis.

Data analysis was performed using the R studio package (1.0.143, 3.5.1 and 4.0.3 versions) and SPSS package (version 23.0 for Windows; IBM.SPSS statistics). A p-value of < 0.05 was considered statistically significant. The Shapiro–Wilk statistic tested continuous variables for normal distribution. Normally distributed continuous variables were expressed as the mean ± standard deviation. Other continuous variables were expressed as the median (25^{th} percentile, 75^{th} percentile), and categorical data as counts and percentages. Continuous variables were compared by Student's independent t-test when normally distributed or by the Mann–Whitney-U test when non-normally distributed. Comparisons of categorical variables between the groups were made using the chi-square test or Fisher's exact test if expected values were < 5. The association between echocardiographic and hemodynamic parameters was assessed using Spearman correlation.

The receiver operating characteristic (ROC) curve was used to identify the optimal cut-off value for the outcome prediction of each inflammation-

related biomarker and echocardiographic parameters, and to estimate their accuracy for predicting composite outcome measures. Differences between areas under the curve (AUC) were tested using the bootstrap method. Kaplan–Meier analysis was used to compare the cumulative survival rates between the groups of NI-DCM patients stratified by cut-off values of each inflammation-related biomarker, the GLS or LVEF, or by the presence of cardiotropic virus or inflammation in the myocardium. The log-rank statistic was used to evaluate the statistical significance of differences between the curves.

Cox proportional hazards regression analysis was performed to evaluate which baseline parameters were associated with poor composite outcomes. We performed univariate Cox regression analysis for all baseline variables. All variables with a p-value < 0.1 in the univariate analysis were included in multivariate Cox regression analysis, which was performed using the stepwise backward elimination.

It is important to note that inflammation-related biomarkers were not enrolled in Paper IV, GLS – in Paper II. Therefore, multivariate Cox regression analysis in Paper II and IV did not evaluate the prognostic impact of GLS and inflammation-related biomarkers, respectively.

4. RESULTS

4.1. Baseline patients' characteristics – Papers I–IV

Fifty-seven NI-DCM patients (mean age 47.3 ± 10.9 years; 45 (79 %) males) with chronic heart failure participated in the study. The average LVEDD was 6.8 ± 0.9 cm, average LVEF - 26.08 ± 9.5 %, and average pulmonary artery wedge pressure - 21.8 ± 8.9 mm Hg. Of these patients, 41 (72 %) were NYHA III class and 10 (17%) were NYHA IV class. At the moment of enrollment to the study, the patients were normotensive to hypotensive: the average of systolic blood pressure was 116 ± 20 mm Hg and average of diastolic pressure was 80 ± 10 mm Hg. Patients also had an impaired GLS (-8.1 ± 3.7%). The median (25^{th} percentile, 75^{th} percentile) heart failure duration was 12 (5, 60) months. Baseline characteristics are shown in Table 2.

Table 2. Baseline characteristics for the study population.

Variable	All patients (n=57)
Clinical characteristics	
Age, years	47.3 ± 10.9
Male gender, n (%)	45 (79%)
Body mass index, kg/m^2	26.84 (23.4-31.6)
Systolic blood pressure, mm Hg	116 ± 20
Diastolic blood pressure, mm Hg	80 ± 10
Heart rate, beats/min	77 (66-93)
Atrial fibrillation, n (%)	11 (19%)
LBBB, n (%)	14 (25%)
NYHA III-IV class, n (%)	51 (90%)
Duration of heart failure symptoms, months	12 (5-60)
Laboratory findings	
Hemoglobin, g/l	142 ± 16
eGFR, ml/min/1.73 m^2	111 ± 30
BNP, ng/l	728 (90-1887)
WBC x 10^9/L	7.92 (5.92-10.06)
hs-CRP, mg/L (n=56)	4.6 (1.5-15.7)
IL-6, pg/mL (n=55)	2.5 (4.7)
TNF-α, pg/mL (n=55)	8.6 (6.6-10)
Adiponectin, µg/mL (n=55)	14.2 (7.97-28.85)
suPAR, ng/mg protein (n=45)	1.79 (0.84-2.65)

Variable	All patients (n=57)
Echocardiographic parameters	
LVEF, %	26.08 ± 9.5
LVEDD, cm	6.8 ± 0.9
GLS, % (n=41)	-8.1 ± 3.72
LV diastolic dysfunction grade III (n=54)	23 (43 %)
Functional MR ≥ moderate, n (%)*	32 (56%)
RV end-diastolic diameter, cm	3.3 ± 0.6
Severe RV systolic dysfunction, n (%)*	16 (28%)
Functional tricuspid regurgitation ≥moderate, n (%)*	20 (35%)
Hemodynamic measurements (n=54)	
PCWP, mmHg	21.8 ± 8.9
Mean RAP, mmHg	11 (6.8-13.3)
mPAP, mmHg	29 ± 18
PVR, Wood units	2 (1.2-3.2)
Cardiac index, L/min/m^2	2.2 (1.6-2.8)
Concomitant cardiac medication	
ACE-I/ARB, n (%)	41 (72%)
Beta-blocker, n (%)	54 (95%)
MRA, n (%)	51 (90%)
Diuretics, n (%)	53 (93%)
Virus-positive endomyocardial biopsies	
Total, n (%)	29 (52%)
B19V, n (%)	25 (45%)
EBV, n (%)	1 (2%)
EV, n (%)	1 (2%)
HHV-6, n (%)	5 (9%)
VZV, n (%)	1 (2%)
HCV, n (%)	1 (2%)
Infiltrative inflammatory cells (n=55)	
CD3+, cells/mm^2	10 (7-16)
CD45ro+, cells/mm^2	7 (5-10)
CD68+, cells/mm^2	4 (3-5)
CD4+, cells/mm^2	4 (2-6)
ICAM-1/CD54+, cells/mm^2	0 (0-1)
HLA DR, cells/mm^2	5 (4-6)

Values are expressed as: median (25^{th} percentile, 75^{th} percentile), mean ± SD or n (%).*assessed visually.

ACE-I – Angiotensin-converting enzyme inhibitor; ARB – Angiotensin II receptor blocker; BNP – B type natriuretic peptide; B19V – parvovirus B19; EBV – Epstein-Barr virus; eGFR – Estimated glomerular filtration rate; EV – enterovirus; GLS – global longitudinal strain; HCV – hepatitis C virus; HHV – 6 – human herpes virus 6; hs-CRP – high-sensitivity C reactive protein; IL-6 – interleukin 6; LBBB – left bundle branch block; LV – left ventricle; LVEDD – left ventricular end-diastolic diameter; LVEF – left ventricular ejection fraction; mPAP – pulmonary arterial pressure; MR – mitral regurgitation; MRA - mineralocorticoid receptor antagonist; NYHA – New York Heart Association; PCWP – pulmonary capillary wedge pressure; PVR – pulmonary vascular resistance; RAP – right atrial pressure; RV – right ventricle; suPAR – soluble urokinase-type plasminogen activator receptor; TAPSE – tricuspid annular plane systolic excursion; TNF-α –tumor necrosis factor α; VZV – varicella zoster virus; WBC – white blood cells;

4.2. The Prevalence of Cardiac Inflammation and Cardiotropic Viruses in the Myocardium (Paper I)

4.2.1. The Prevalence of the Myocardial Inflammation (Paper I)

Immunohistochemical analysis was performed on 55 endomyocardial biopsies. Myocardial inflammation was detected in 31 of the 55 (54%) NI-DCM patients. Patients were divided into two groups: inflammatory DCM (iDCM) and non-inflammatory DCM (non-iDCM). No difference was observed in baseline characteristics of the two groups ($p > 0.05$; Table 3), except for lower systolic blood pressure and higher level of B-type natriuretic peptide (BNP) in the iDCM group ($p \leq 0.05$).

Inflammatory endothelial activation (increased expression of HLA-DR and ICAM [$\geq$ 3 cells/mm^2]) was detected by immunohistochemistry in 50 (91%) patients. The expression did not, however, differ between the iDCM and non-iDCM groups ($p > 0.05$). Nonetheless, it should be interpreted with caution, while these proteins are not only markers for endothelial activation, but are also found on the surface of immune cells.

Table 3. A comparison of baseline characteristics of non-iDCM and iDCM patients, and patients with and without viral genome.

Variable	non-iDCM (n=24)	iDCM (n=31)	p	Virus-negative (n=27)	Virus-positive (n=29)	p
Clinical characteristics						
Age, years	48.3 ± 13	46.6 ± 9,6	0.58	48.44 ± 12.68	46.07 ± 9.28	0.43
Male gender, n (%)	17 (71%)	26 (82%)	0.25	23 (85%)	21 (72%)	0.23
Body mass index, kg/m^2	27.3 (24.3-32.5)	25.7 (23.1-31)	0.45	28.1 (24.2-31.4)	25.2 (21.8-30.5)	0.24
Systolic blood pressure, mm Hg	123 ± 20	110 ± 17	**0.01**	115 ± 20	118 ± 22	0.63
Diastolic blood pressure, mm Hg	78 (70-83)	80 (69-80)	0.43	80 (70-80)	80 (70-80)	0.76
Heart rate, beats/min	73 (66-87)	86 (66-98)	0.18	79 (68-91)	76 (66-100)	0.83
Atrial fibrillation, n (%)	3 (13%)	8 (26%)	0.31	6 (22%)	5 (17%)	0.64
LBBB, n (%)	6 (25%)	8 (26%)	0.99	10 (37%)	4 (14%)	0.15
NYHA III-IV class, n (%)	20 (83%)	29 (94%)	0.64	24 (89%)	26 (90%)	1
Laboratory findings						
Hemoglobin, g/l	137.7 ± 13.7	144.7 ± 14.7	0.08	142.7 ±16.6	141.3 ±14.6	0.73
eGFR, ml/min/1.73 m^2	104 ± 31	116 ± 30	0.17	109 ± 28	110 ± 31	0.87
BNP, ng/l	214 (57-1501)	1017 (317-2750)	**0.05**	916 (601-3171)	228 (75-1404)	**0.04**
C-reactive protein, mg/l	5.2 (6.6)	4.5 (15.3)	0.74	6.6 (13.5)	2.4 (10.3)	0.12
IL-6, pg/ml	2.2 (3.1)	2.9 (7.4)	0.21	4.62 (6.3)	2.01 (2.6)	**0.04**
Echocardiographic parameters						
LVEF, %	25.6 ±11.8	26.7 ±7.1	0.69	26.9 ± 9.2	26.7 ± 9.9	0.94
LVEDD, cm	6.8 ± 0.9	6.9 ± 0.9	0.57	7.0 ± 0.9	6.7 ± 0.8	0.12
LV diastolic dysfunction grade III, n (%) (n=54)	10 (44%)	13 (43%)	0.99	13 (48%)	10 (37%)	0.41
Functional MR ≥ moderate, n (%)*	13 (54%)	19 (61%)	0.6	17 (63%)	14 (48%)	0.27
RV end-diastolic diameter, cm	3.2 ± 0.8	3.4 ± 0.5	0.3	3.4 ± 0.6	3.3 ± 0.6	0.63
Severe RV systolic dysfunction, n (%)*	8 (33%)	8 (26%)	0.54	10 (37%)	5 (17%)	0.09
Functional tricuspid regurgitation ≥moderate, n (%)*	9 (38%)	11 (36%)	0.88	11 (41%)	9 (31%)	0.45
Hemodynamic measurements (n=54)						
PCWP, mmHg	21.5 ± 9.6	22.6 ± 8.8	0.65	25 ± 9	19 ± 8	**0.02**
Mean RAP, mmHg	10 (7-13)	11 (5-15)	0.96	12 (8-16)	8 (5-13)	0.09
mPAP, mmHg	31 ± 12	32 ± 11	0.67	35 ± 11	28 ± 9	**0.02**
PVR, Wood units	1.5 (1.0-2.6)	2.3 (1.7-3.3)	0.08	2.4 (1.6-4.3)	1.6 (1.0-2.4)	**0.04**

Variable	non-iDCM (n=24)	iDCM (n=31)	p	Virus-negative (n=27)	Virus-positive (n=29)	p
Concomitant cardiac medication						
ACE-I/ARB, n (%)	18 (75%)	18 (58%)	0.19	15 (56%)	22 (76%)	0.11
Beta-blocker, n (%)	24 (100%)	28 (90%)	0.25	24 (89%)	29 (100%)	0.11
MRA, n (%)	20 (83%)	30 (97%)	0.16	26 (96%)	24 (83%)	0.2
Diuretics, n (%)	21 (88%)	30 (97%)	0.31	25 (93%)	27 (93%)	1
Virus-positive endomyocardial biopsies						
Total, n (%)	15 (63%)	14 (47%)	0.25	0	29 (52%)	
B19V, n (%)	13 (54%)	12 (40%)	0.3	0	25 (45%)	
EBV, n (%)	0 (0%)	1 (3%)	1	0	1 (2%)	
EV, n (%)	0 (0%)	1 (3%)	1	0	1 (2%)	
HHV-6, n (%)	2 (8%)	3 (10%)	1	0	5 (9%)	
VZV, n (%)	1 (4%)	0 (0%)	0.44	0	1 (2%)	
HCV, n (%)	0 (0%)	1 (3%)	1	0	1 (2%)	
Infiltrative inflammatory cells (n=55)						
CD3+, cells/mm^2	7 (6-8)	15 (12-20)	**<0.001**	10 (7-16)	9 (7-12)	0.66
CD45ro+, cells/mm^2	5 (4-6)	10 (6-12)	**<0.001**	7 (5-10)	6 (5-8)	0.42
CD68+, cells/mm^2	3 (2-4)	5 (3-7)	**<0.001**	5 (3-6)	3 (3-5)	**0.01**
CD4+, cells/mm^2	4 (2-5)	4 (2-8)	0.52	3 (2-6)	4 (2-6)	0.82
ICAM-1/CD54+, cells/mm^2	0 (0-1)	0 (0-1.5)	0.45	0 (0-2)	0 (0-1)	0.26
HLA DR, cells/mm^2	4.5 (4-6)	5.0 (5-7)	0.1	5 (5-6)	5 (4-6)	0.23

Values are expressed as: median (25th percentile-75th percentile), mean ± SD or n (%). The p values in bold indicate a value <0.05. * assessed visually.

3.2.2. ACE-I – Angiotensin-converting enzyme inhibitor; ARB – Angiotensin II receptor blocker; BNP – B type natriuretic peptide; B19V – parvovirus B19; EBV – Epstein-Barr virus; eGFR – Estimated glomerular filtration rate; EV – enterovirus; GLS – global longitudinal strain; HCV – hepatitis C virus; HHV – 6 – human herpes virus 6; iDCM – inflammatory dilated cardiomyopathy; IL-6 – interleukin 6; LBBB – left bundle branch block; LV – left ventricle; LVEDD – left ventricular end-diastolic diameter; LVEF – left ventricular ejection fraction; mPAP – pulmonary arterial pressure; MR – mitral regurgitation; MRA - mineralocorticoid receptor antagonist; NYHA – New York Heart Association; non-iDCM – non-inflammatory dilated cardiomyopathy; PCWP – pulmonary capillary wedge pressure; PVR – pulmonary vascular resistance; RAP – right atrial pressure; RV – right ventricle; TAPSE – tricuspid annular plane systolic excursion; VZV – varicella zoster virus.

4.2.2. Prevalence of Cardiotropic Viruses (Paper I)

Polymerase chain reaction analysis was performed on 56 endomyocardial biopsies. Viral genomes were detected in the myocardium of 29 (52%) of the 56 NI-DCM patients. Of these 29 patients, 25 (86%) had the B19V genome, and other 5 (17%) had HHV6 genome. Other viruses (VZV, CMV, EBV, HCV, EV), were detected in single cases (n = 1 [3%] of each type). Three (10%) of virus-positive patients had a double infection and one of them (3%) a triple infection. Co-detection of B19V and HHV6 prevailed (n = 3 [10%]). The remaining 27 (48%) patients were virus-negative. There were no differences in most baseline parameters between the virus-positive and virus-negative groups ($p > 0.05$), except for higher BNP and IL-6 levels, worse hemodynamic parameters (Table 3), and a higher number of infiltrative CD68+ cells in the virus-negative group (Figure 3).

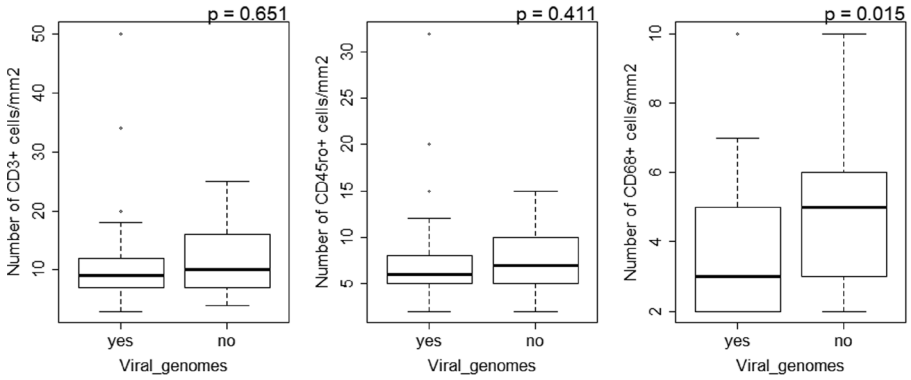

Figure 3. Comparison of inflammatory cell (CD3+, CD45ro+, and CD68+) counts in the virus-negative and virus-positive biopsies.

4.2.3. Sub-Groups of Idiopathic NI-DCM (Paper I)

Both PCR analysis and immunohistochemical evaluation were performed on 54 NI-DCM patients. Based on the detection of viral genome - in combination with positive or negative immunohistochemistry - four specific sub-groups of patients were distinguished:

- Virus-positive (chronic) myocarditis (15 (28 %) patients): both cardiotropic virus and myocardial inflammation is present.
- Autoreactive inflammatory dilated cardiomyopathy (16 (30%) patients): no cardiotropic virus was detected but myocardial inflammation was present.

- Viral dilated cardiomyopathy (14 (26 %) patients): viral genome was detected but no signs of myocardial inflammation.
- Non-inflammatory dilated cardiomyopathy (9 (17 %) patients): neither viral genome nor inflammation was detected.

4.3. Association of Inflammation-Related Biomarkers and GLS with Heart Failure Severity (Papers II-IV)

4.3.1. Association Between Inflammation-Related Biomarkers and Heart Failure Severity (Papers II-III)

We evaluated the correlations between inflammation-related biomarkers and parameters that reflect heart failure severity (Table 4). WBC, suPAR, infiltrative CD68+, CD4+, CD54+, and HLA-DR+ cells did not correlate with any of the heart failure severity parameters. A weak but significant positive correlation was found between infiltrative CD3+ and CD45ro+ cells and BNP levels, while hs-CRP significantly but weakly correlated with LVEF and BNP. Furthermore, levels of IL-6, TNF-α and adiponectin increased according to NYHA class (Figure 4) and correlated with all heart failure severity parameters (LVEF, mPAP, PCWP, and BNP), except TNF-α, which did not correlate with PCWP. Finally, IL-6 and adiponectin levels correlated most strongly with parameters of heart failure severity, particularly BNP.

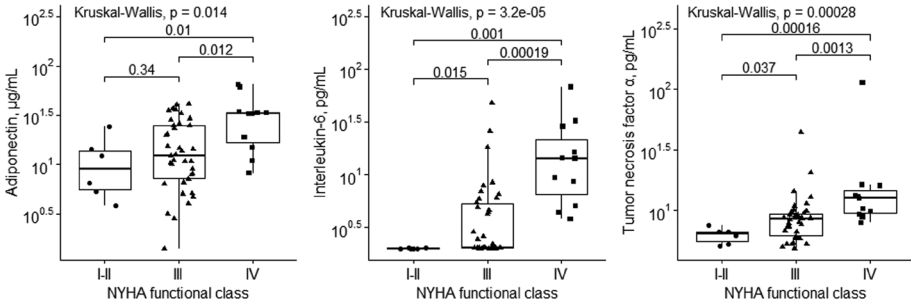

Figure 4. Serum cytokine levels based on New York Heart Association (NYHA) functional class.

Then we evaluated the correlations between inflammatory cells in the myocardium and inflammation-related biomarkers in serum (Table 5). Inflammatory cells did not correlate with biomarkers of systemic inflammation, except moderate correlation between CD68+ cells/mm^2 and IL-6.

Table 4. Correlations between inflammatory-related biomarkers and parameters of heart failure severity.

	LVEF, %		mPAP, mmHg		PCWP, mmHg		BNP, ng/L	
	r	**p**	**r**	**p**	**r**	**p**	**r**	**p**
WBC x 10^9/L	-0.09	0.5	0.23	0.12	0.05	0.7	0.01	0.97
hs-CRP, mg/L	**-0.3**	**0.03**	0.19	0.19	0.17	0.2	0.33	0.02
IL-6, pg/mL	**-0.56**	**<0.0001**	**0.48**	**<0.001**	**0.42**	**<0.01**	**0.66**	**<0.0001**
TNF-α, pg/mL	**-0.29**	**0.03**	**0.39**	**<0.01**	0.25	0.08	**0.5**	**<0.01**
Adiponectin, µg/mL	**-0.37**	**<0.01**	**0.43**	**<0.01**	**0.39**	**<0.01**	**0.65**	**<0.0001**
suPAR, ng/mg protein	-0.07	0.66	0.2	0.21	0.11	0.48	0.25	0.11
CD3+, cells/mm^2	-0.01	0.96	0.12	0.41	0.12	0.4	**0.31**	**0.02**
CD45ro+, cells/mm^2	-0.04	0.78	0.06	0.67	0.03	0.84	**0.28**	**0.04**
CD68+, cells/mm^2	-0.16	0.23	0.09	0.51	0.06	0.69	0.22	0.11
CD4+, cells/mm^2	0.18	0.18	-0.03	0.81	0.02	0.9	-0.01	0.98
CD54+, cells/mm^2	-0.1	0.7	0.1	0.57	0.12	0.39	0.15	0.28
HLA-DR+, cells/mm^2	-0.19	0.16	-0.02	0.88	0.04	0.76	0.12	0.38

Bold indicates p-value <0.05. BNP – B type natriuretic peptide, hs-CRP – high sensitivity C-reactive protein, IL-6 – interleukin 6, LVEF – left ventricle ejection fraction, mPAP – mean pulmonary arterial pressure, PCWP – pulmonary capillary wedge pressure, suPAR – soluble urokinase-type plasminogen activator receptor, TNF-α – tumor necrosis factor α, WBC – white blood cell.

Table 5. Correlations between inflammatory cells in the myocardium and inflammatory-related biomarkers in serum.

	CD3+, cells/mm^2		CD45ro+, cells/mm^2		CD68+, cells/mm^2		CD4+, cells/mm^2		CD54+, cells/mm^2		HLA_DR+, cells/mm^2	
	r	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**
WBC x 10^9/L	-0.12	0.42	**0.1**	0.52	**0.12**	0.23	**-0.06**	0.67	0.1	0.48	-0.14	0.35
hs-CRP, mg/L	0.19	0.18	0.23	0.12	0.26	0.07	0.003	0.99	0.01	0.97	0.12	0.39
IL-6, pg/mL	0.21	0.12	0.09	0.51	**0.35**	**0.009**	-0.22	0.11	-0.03	0.89	0.09	0.53
TNF-α, pg/mL	0.21	0.14	0.01	0.94	0.2	0.15	-0.07	0.64	-0.05	0.72	0.1	0.49
Adiponectin, µg/mL	0.14	0.3	0.11	0.42	0.05	0.72	-0.2	0.87	0.02	0.91	0.13	0.37
suPAR, ng/mg protein	0.08	0.62	0.16	0.3	0.13	0.42	0.19	0.23	0.1	0.52	0.09	0.55

Bold indicates p-value <0.05. hs-CRP – high sensitivity C-reactive protein, IL-6 – interleukin 6, suPAR – soluble urokinase-type plasminogen activator receptor, TNF-α – tumor necrosis factor α, WBC – white blood cell.

4.3.2. Association Between GLS and Heart Failure Severity Parameters (Paper IV)

We evaluated the association between GLS and other conventional echocardiographic parameters with invasive cardiac pressures and cardiac index. GLS as well as average E/e', TR velocity, left atrial indexed volume (LAVi) and LVEF significantly correlated with cardiac pressures. (Table 6). Correlations between myocardial deformation parameter (GLS) and invasive hemodynamic measurements are also plotted in Figure 5. Additionally, we evaluated correlations between GLS and serum biomarkers. GLS strongly correlated with BNP, but not with troponin T (Figure 5).

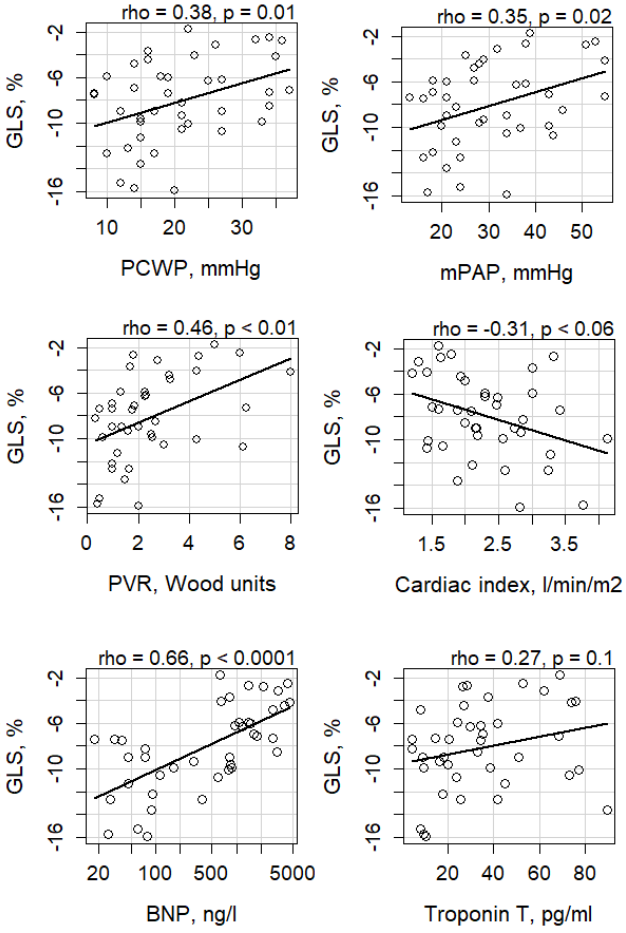

Figure 5. Correlations between myocardial deformation parameters and invasive hemodynamic measures, and serum biomarkers. BNP – B type natriuretic peptide; GLS – global longitudinal strain; mPAP – mean pulmonary arterial pressure; PCWP – pulmonary capillary wedge pressure; PVR – pulmonary vascular resistance.

Table 6. Correlations between echocardiographic parameters and invasive hemodynamic ones.

	PCWP, mmHg		**mPAP, mmHg**		**PVR, Wood units**		**Cardiac index, l/min/m^2**	
	r	p	r	P	r	p	r	p
DT, ms	-0,13	0.42	-0.17	0.28	**-0.4**	0.01	0.21	0.21
E/A	0.22	0.21	**0.38**	0.03	**0.62**	<0.0001	**-0.55**	<0.01
Average E/e’	**0.37**	0.02	**0.36**	0.02	**0.31**	0.048	-0.02	0.92
TR velocity, m/s	**0.54**	<0.001	**0.59**	<0.0001	**0.5**	<0.001	-0.19	0.24
LAVi, ml/m^2	**0.40**	<0.01	**0.47**	<0.01	**0.6**	<0.0001	**-0.45**	<0.01
LVEF, %	**-0.38**	0.01	**-0.41**	<0.01	**-0.45**	<0.01	0.26	0.11
GLS, %	**0.38**	0.01	**0.35**	0.02	**0.46**	<0.01	-0.31	0.06

The bold font character means statistically significance ($p < 0.05$).

DT – deceleration time; E/A – ratio of early-diastolic LV inflow velocity (E) to atrial-systolic velocity (A); E/e’ – ratio of early-diastolic LV inflow velocity (E) to early-diastolic mitral annular velocity (e´); GLS – global longitudinal strain; LAVi – left atrium indexed volume; LVEF – left ventricular ejection fraction; PCWP – pulmonary capillary wedge pressure; mPAP – mean pulmonary arterial pressure; PVR – pulmonary vascular resistance; TR – tricuspid regurgitation.

4.4. Poor Clinical Outcomes (Papers I, II, IV)

Of the 57 patients, 27 (47%) reached the composite outcome measure during the 5-year follow-up period:

- 10 (18%) patients died;
- 9 (16%) underwent heart transplantation (the urgency status according to heart allocation policy [230]: 6 patients had status 1-3 and 3 patients – status 4);
- 8 (14 %) had an LVAD implantation (7 patients had 1-3 INTERMACS profile [231], 1 patient – 4 profile).

The other patients remained on conventional medical heart failure therapy. The 5-year cumulative survival rate was 53%.

Based on these outcomes, we divided the cohort into two groups: event (n=27) and event-free (n=30) groups. Patients in the event group tended to have a longer duration of symptoms before enrollment than patients in the event-free group, but the difference was not statistically significant. Patients who experienced adverse outcomes had significantly lower systolic blood pressure, more impaired LV function, GLS and enlarged right ventricle (Table 7). In addition, there were higher levels of BNP and inflammation-related markers (IL-6, TNF-α and adiponectin) in the event group compared to patients that remained event-free (Table 8).

Table 7. Baseline characteristics for the study population, stratified by outcome.

Variable	Event-Free Group (n = 30)	Event Group (n = 27)	p
Clinical characteristics			
Age, years	48 (47-53)	46 (39-54)	0.19
Male gender, n (%)	23 (79)	22 (79)	0.95
NYHA III-IV class, n (%)	24 (83)	27 (96)	0.19
Body mass index, kg/m^2	27.8 (22.7-32.4)	26.6 (23.6-30.4)	0.8
Systolic blood pressure, mmHg	123 (110-130)	106 (94-116)	**<0.01**
Diastolic blood pressure, mmHg	80 (70-80)	70 (69-80)	0.11
Atrial fibrillation, n (%)	6 (20)	5 (19)	0.89
Dyslipidemia, n (%)	6 (20)	4 (15)	0.73
Rheumatologic disease, n (%)	1 (3)	3 (11)	0.34
Duration of heart failure symptoms, months	10 (4-48)	48 (10-72)	0.08

Variable	Event-Free Group (n = 30)	Event Group (n = 27)	p
Laboratory findings			
Hemoglobin, g/L	146 (137-154)	143 (126-150)	0.41
eGFR, mL/min/1.73 m^2	83 (73-100)	93 (73-107)	0.27
BNP, ng/L	248 (46-893)	1280 (343-2681)	**<0.01**
hs-troponin T, pg/mL	25.8 (16.2-45.1)	31 (22.7-59.7)	0.34
Concomitant cardiac medication			
ACE-I/ARB, n (%)	21 (73)	19 (68)	0.71
Beta-blocker, n (%)	28 (97)	26 (93)	0.61
MRA, n (%)	24 (83)	27 (96)	0.19
Loop diuretics, n (%)	26 (90)	27 (96)	0.61
Echocardiographic parameters			
LVEF, %	30 (21-35)	22 (18.8-26)	**0.03**
LV end-diastolic diameter, cm	6.7 (6.2-7.1)	6.9 (6.5-7.5)	0.17
GLS, % (n=41)*	-9.9 ± 2.8	-6.2 ± 3.7	**<0.001**
LAVi, mL/m^2 (n=41)*	55 (53-76)	73 (46-81)	0.63
Mitral DT, ms (n=41)*	145 (111-187)	142 (98-187)	0.29
Mitral E/A (n=41)*	2 (0.8-2.7)	2.4 (1.2-3.3)	0.44
Average E/e' (n=41)*	13.7 (10.9-15.1)	15.0 (13.1-17.4)	0.13
Grade III LV diastolic dysfunction, n (%)	7 (24)	16 (57)	**0.01**
Severe RV systolic dysfunction, n (%)	5 (17)	11 (39)	0.06
RV end-diastolic diameter, cm	3.1 (2.6-3.4)	3.6 (3.1-3.9)	**<0.01**
TR systolic jet velocity (m/sec) (n=41)*	2.5 (2.4-2.7)	2.8 (2.5-3.0)	0.16
Hemodynamic measurements (n=54)			
PCWP, mmHg	18 (14.8-24.8)	23 (16-34)	0.11
mPAP, mmHg	25 (21-37.3)	34 (27-43)	0.06
Cardiac index, L/min/m^2	2.27 (1.93-2.81)	2.0 (1.5-2.49)	0.14

Values are expressed as: median (25th percentile-75th percentile) or n (%). Bold indicates a p-value < 0.05. *Parameters were compared between smaller subgroups (Paper IV): event-free group – 21 patients, event groups – 20 patients.

ACE-I – Angiotensin-converting enzyme inhibitor, ARB – Angiotensin II receptor blocker, BNP – B type natriuretic peptide, DT – deceleration time; E/A – ratio of early-diastolic LV inflow velocity (E) to atrial-systolic velocity (A); E/e' – ratio of early-diastolic LV inflow velocity (E) to early-diastolic mitral annular velocity (e´); eGFR – estimated glomerular filtration rate, LV – left ventricle, LVEF – left ventricle ejection fraction, mPAP – mean pulmonary arterial pressure, MRA – mineralocorticoid receptor antagonist, NYHA – New York Heart Association, PCWP – pulmonary capillary wedge pressure, RV – right ventricle, TR – tricuspid regurgitation.

Table 8. Inflammation-related biomarkers of the study population, stratified by outcome.

Variable	Event-Free Group (n = 30)	Event Group (n = 27)	p
Inflammation-related serum biomarkers			
WBC x 10^9/L (n=57)	8.33 (6.19-10.75)	7.83 (5.7-9.13)	0.48
hs-CRP, mg/L (n=56)	2.4 (1.3-15)	6.6 (2.7-16.1)	0.27
IL-6, pg/mL (n=55)	2.0 (2.0-3.8)	5.24 (2.0-13.7)	**0.002**
TNF-α, pg/mL (n=55)	7.5 (6.1-9.3)	9.0 (7.9-12.9)	**0.03**
Adiponectin, μg/mL (n=55)	10.9 (5.63-18.96)	23.4 (11.1-32.9)	**0.01**
suPAR, ng/mg protein (n=45)	1.77 (0.92-2.84)	1.79 (0.79-2.65)	0.92
Inflammatory cells in myocardium (n=55)			
CD3+, cells/mm^2	10 (7-13)	10 (7-50)	0.39
CD45ro+, cells/mm^2	7 (5-9)	6.5 (5-10.5)	0.71
CD68+, cells/mm^2	4 (3-5)	3.5 (3-5)	0.96
CD4+, cells/mm^2	4 (2-6)	3 (2-8)	0.71
CD54+, cells/mm^2	0 (0-2)	0 (0-1)	0.29
HLA-DR+, cells/mm^2	5 (4-6)	5 (4-7)	0.71

Values are expressed as: median (25th percentile, 75th percentile). Bold indicates a p-value <0.05.
hs-CRP – high-sensitivity C-reactive protein, IL-6 – interleukin 6, suPAR – soluble urokinase-type plasminogen activator receptor, TNF-α – tumor necrosis factor α, WBC – white blood cell.

4.4.1. Prognostic Utility of Cardiotropic Viruses and Myocardial Inflammation (Paper I)

Kaplan-Meier survival curves demonstrated no difference in survival rates of patients in the virus-positive and virus-negative groups ($p>0.05$; Figure 6) as well as no difference between the survival curves of the patients with inflammatory dilated cardiomyopathy (iDCM) and non-inflammatory dilated cardiomyopathy (non-iDCM) ($p > 0.05$; Figure 6).

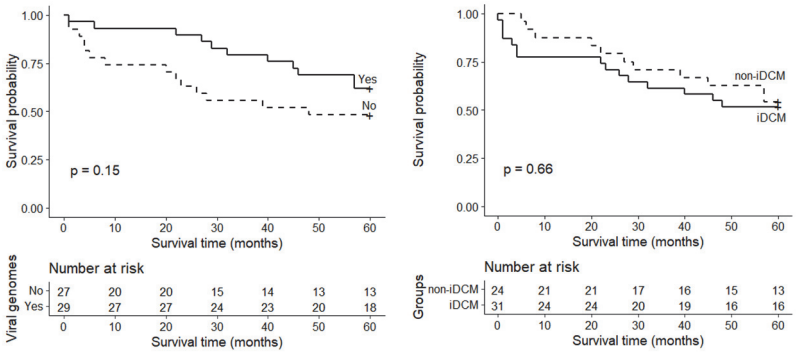

Figure 6. The Kaplan-Meier analysis of virus-positive and virus-negative, and of the inflammatory dilated cardiomyopathy (iDCM) and non-inflammatory dilated cardiomyopathy (non-iDCM) groups.

4.4.2. Inflammation-Related Biomarkers and Risk Prediction (Paper II)

We performed ROC analysis to identify the best cut-off value for each inflammation-related biomarker for predicting outcomes. Cut-off values are presented in Table 9. IL-6, TNF-α and adiponectin predicted adverse cardiac events with the highest accuracy (AUC 0.77, 0.65 and 0.70, respectively).

Table 9. Cut-off values identified by ROC analysis of inflammation-related biomarkers for predicting adverse cardiac events.

	Cut-off	Specificity, %	Sensitivity, %	AUC (95% CI)
WBC x 109/L	10.66	32	88	0.56 (0.39-0.72)
hs-CRP, mg/L	4.62	65	65	0.61 (0.44-0.77)
IL-6, pg/mL	4.53	83	64	**0.77 (0.64-0.89)**
TNF-α, pg/mL	7.81	53	80	**0.65 (0.50-0.79)**
Adiponectin, μg/mL	17.14	73	60	**0.70 (0.55-0.73)**
suPAR, ng/mg of protein	0.85	80	35	0.51 (0.34-0.69)
CD3+, cells/mm^2	13	75	41	0.59 (0.43-0.74)
CD45ro+, cells/mm^2	11.5	89	26	0.53 (0.37-0.68)
CD68+, cells/mm^2	9	96	11	0.51 (0.36-0.66)
CD54+, cells/mm^2	2.5	93	7	0.43 (0.29-0.56)
CD4+, cells/mm^2	3.5	61	56	0.53 (0.37-0.69)
HLA-DR+, cells/mm^2	5.5	68	41	0.53 (0.38-0.68)

Bold indicates p-value <0.05. AUC – area under the curve, hs-CRP – high-sensitivity C-reactive protein, CI – confidence interval, IL-6 – interleukin 6, suPAR – soluble urokinase-type plasminogen activator receptor, TNF-α – tumor necrosis factor α, WBC – white blood cell.

Kaplan-Meier survival analyses with log-rank tests were subsequently performed to reveal alterations in survival probability among patient groups based on the obtained cut-off values (Figure 7). Higher levels of inflammation-related markers (hs-CRP > 4.6 µg/mL, IL-6 > 4.53 pg/mL, TNF-α > 7.81 pg/mL, adiponectin >17.14 µg/mL) and higher numbers of infiltrative inflammatory cells (CD3+ >13 cells/mm^2 and CD45ro+ >11.5 cells/mm^2) were associated with lower survival rates (Figure 7). No significant differences in survival probability were obtained for the other inflammation-related biomarkers.

Univariate Cox regression analysis showed that systolic and diastolic blood pressure, echocardiographic parameters (LVEF, RV end-diastolic diameter, severe RV dysfunction), right heart catheterization measurements (mPAP, PCWP), inflammation-related serum biomarkers (hs-CRP > 4.62 µg/mL, IL-6 > 4.53 pg/mL, TNF-α > 7.81 pg/mL and adiponectin > 17.14 µg/mL) as well as inflammatory CD3+ > 13 cells/mm^2 and CD45ro+ > 11.5 cells/mm^2 in the myocardium predicted adverse clinical events. Parameters with a p-value < 0.1 in univariate Cox regression analysis are shown in Table 10. Multivariate Cox analysis revealed that IL-6 > 4.53 pg/mL, adiponectin > 17.14 µg/mL, hs-troponin T, CD45ro+ > 11.5 cells/mm^2 and diastolic blood pressure could independently predict adverse cardiac events.

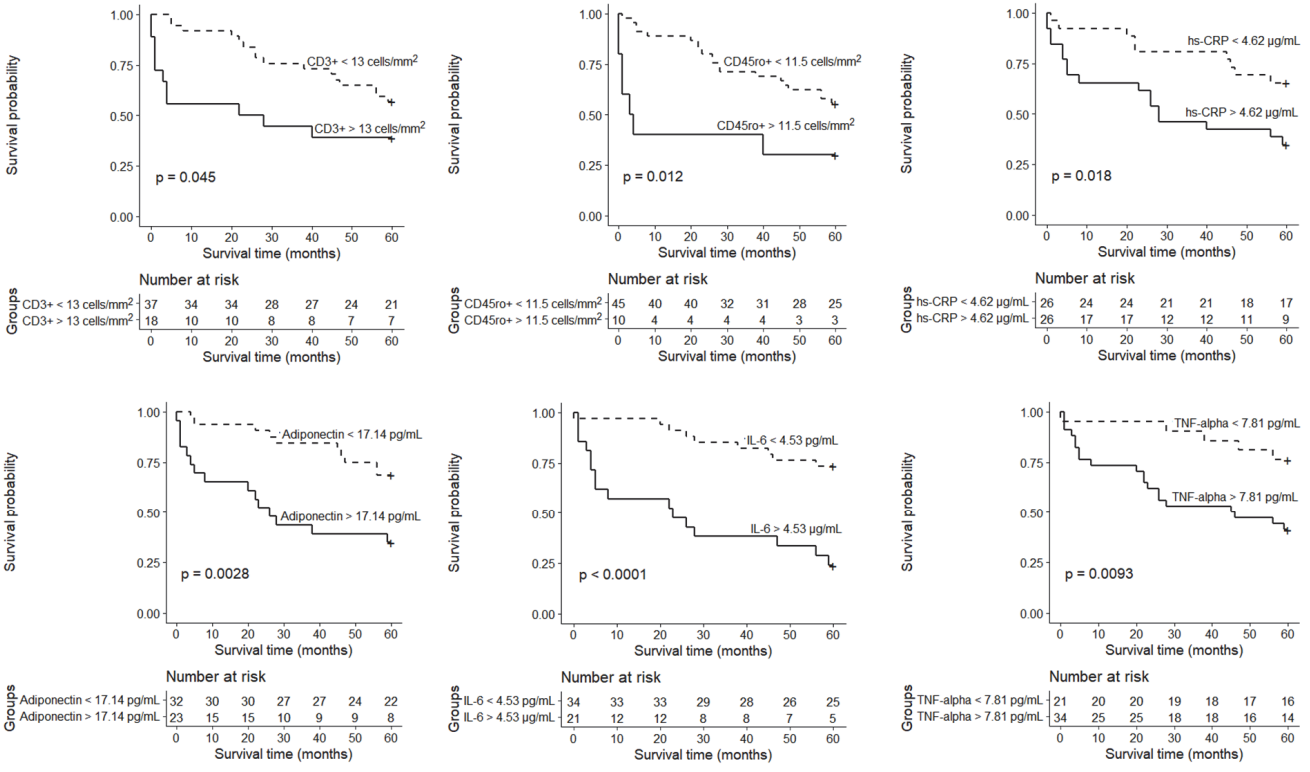

Figure 7. Survival curves stratified by inflammation-related biomarkers during the five-year follow-up. hs-CRP – high-sensitivity C reactive protein, TNF-alpha – tumor necrosis factor α.

Table 10. Results of Cox regression analysis for predictors of adverse cardiac events.

	Univariate		Multivariate	
Variable	**HR (95% CI)**	**p**	**HR (95% CI)**	**p**
Systolic BP, mmHg	0.97 (0.95-0.995)	0.02		
Diastolic BP, mmHg	0.96 (0.93-1.0)	0.05	0.95 (0.91-0.99)	**0.01**
LVEF, %	0.93 (0.88-0.97)	0.003		
Severe RV systolic dysfunction	2.81 (1.29-6.11)	0.009		
LV diastolic dysfunction grade III	3.15 (1.45-6.84)	0.003		
RV end-diastolic diameter, cm	2.65 (1.4-5.0)	0.003		
PCWP, mmHg	1.06 (1.01-1.11)	0.01		
mPAP, mmHg	1.05 (1.01-1.08)	0.01		
log BNP, ng/L	1.47 (1.16-1.86)	0.002		
hs-troponin T, pg/mL	1.004 (1-1.01)	0.068	1.005 (1.0-1.01)	**0.04**
IL-6 > 4.53 pg/mL	4.78 (2.09-10.89)	0.0002	6.26 (2.52-15.58)	**<0.0001**
hs-CRP > 4.62 µg/mL	2.57 (1.14-5.79)	0.02		
TNF-α > 7.81 pg/mL	3.42 (1.28-9.15)	0.01		
Adiponectin >17.14 µg/mL	3.2 (1.43-7.16)	0.005	5.43 (1.01-10.33)	**<0.001**
CD3+ > 13 cells/mm^2	2.18 (1.01-4.7)	0.048		
CD45ro+ >11.5 cells/mm^2	2.89 (1.22-6.87)	0.012	3.23 (1.01-10.33)	**0.048**

BNP – B type natriuretic peptide, BP – blood pressure, CI – confidence interval, HR – hazard ratio, hs – high-sensitivity, hs-CRP – high-sensitivity C reactive protein, IL-6 – interleukin 6, LVEF – left ventricle ejection fraction, mPAP – mean pulmonary arterial pressure, PCWP – pulmonary capillary wedge pressure, RV – right ventricle, TNF-α – tumor necrosis factor α.

4.4.2.1. Combined assessment of Inflammation-Related Cytokines (Paper II)

Since all three cytokines that were measured predicted adverse cardiac events with the highest accuracy (Table 9), they were subjected to further analysis. Figure 8 shows a risk stratification based on the three cytokines. All patients (n=12) with IL-6 < 4.53 pg/mL, adiponectin < 17.14 µg/mL and TNF-α < 7.81 pg/mL had a 100% five-year event-free survival, whereas all patients (n=10) with concentrations of these three cytokines above the cut-off values experienced adverse cardiac events. Patients with one or two cytokines above the cut-off value had a similar five-year survival: 50% and 40% event-free survival rate, respectively.

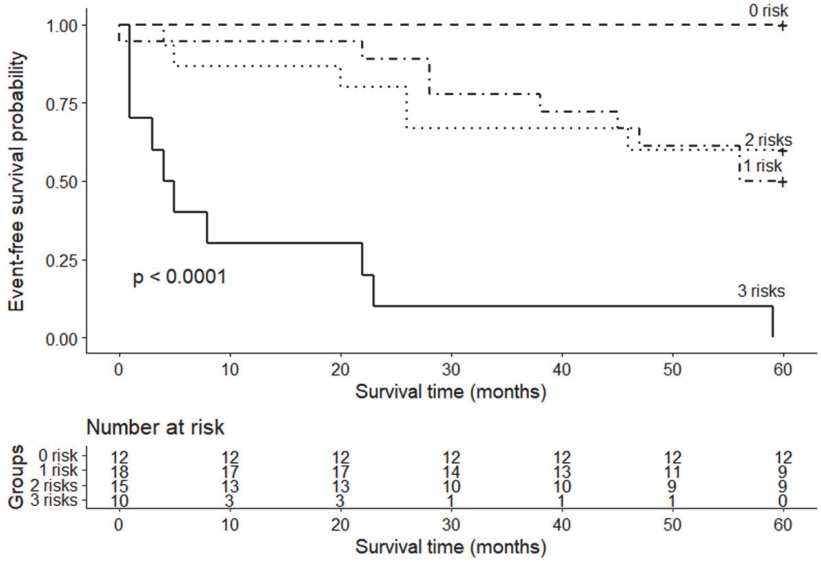

Figure 8. Event-free survival from adverse cardiac events according to a triple inflammation-related cytokine risk stratification model using adiponectin > 17.14 µg/mL, IL-6 > 4.53 pg/mL and TNF-α > 7.81 pg/mL as unfavorable risk factors.

4.5. Prognostic Utility of GLS (Paper IV)

We then used GLS and LVEF to identify high-risk patients in terms of adverse cardiac events. For comparison to GLS, we chose LVEF as the most widely used prognostic echocardiographic parameter in clinical practice. ROC analyses showed, that GLS and LVEF predicted poor clinical outcomes during

the long-term follow-up (Figure 9). The differences between the curves were not significant. Additionally, the analysis identified optimal cut-off values for the composite outcome measure: -5.96% (60% sensitivity, 100% specificity) for GLS and 24.5% for LVEF (80% sensitivity, 81% specificity).

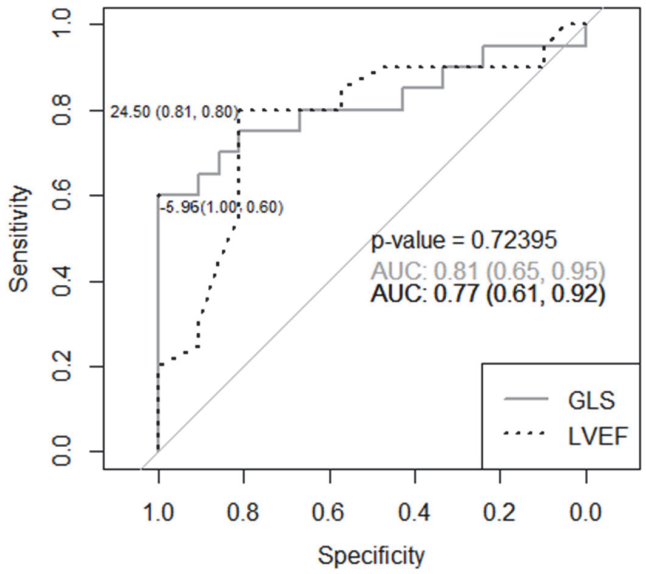

Figure 9. ROC analysis of GLS and LVEF identified high-risk patients for an adverse cardiac event. GLS – global longitudinal strain, LVEF – left ventricular ejection fraction.

To obtain additional information for risk stratification, we performed survival analysis with GLS and LVEF. The survival curve estimations demonstrated that patients with GLS values above the cut-off and LVEF below it had significantly lower event-free survival rates during five-year follow-up ($p<0.001$) (Figure 10). The graph shows that more than half of the patients with GLS above the cut-off value experienced adverse cardiac events during the first year of follow-up. In addition, univariate Cox analysis demonstrated that GLS values above cut-off indicated 12-fold (HR 12.47; 95% CI 4.6-33.82, <0.0001) higher risk of poor clinical outcomes at five years.

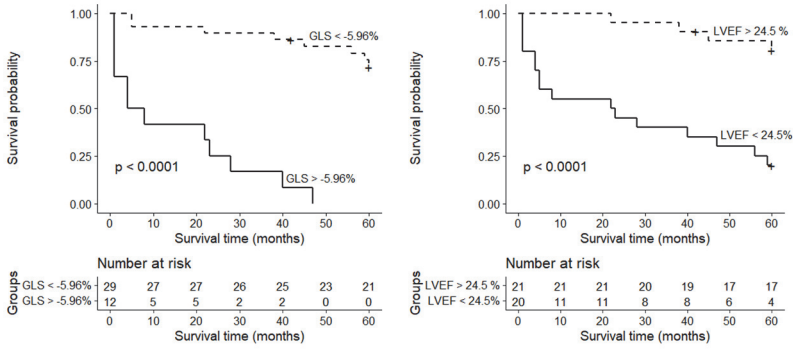

Figure 10. Survival curves stratified by GLS and LVEF during long-term follow-up. GLS – global longitudinal strain; LVEF – left ventricle ejection fraction.

Given that LVEF is a well-established prognostic marker, a subgroup survival analysis was performed to evaluate whether GLS has an additional predictive value in patients stratified by LVEF. In patients with LVEF values above the cut-off, GLS did not stratify the risk, while all patients had GLS values below the cut-off. However, for patients with LVEF below the cut-off value, the risk was further stratified by GLS (Figure 11). Patients with GLS above the cut-off value (>-5.96%) had significantly increased events rates within the severely reduced LVEF group.

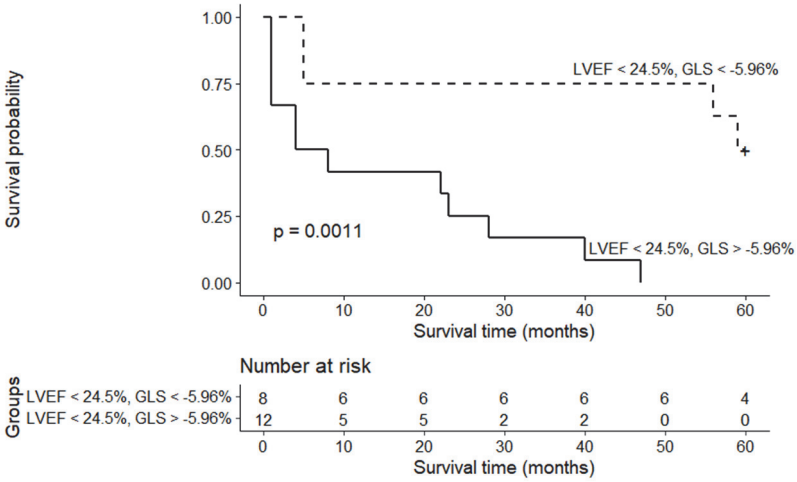

Figure 11. Composite adverse cardiac events probability according to GLS within severely reduced LVEF patients. GLS – global longitudinal strain; LVEF – left ventricle ejection fraction.

We then performed univariate Cox proportional-hazards model analysis to evaluate the prognostic significance of GLS as continuous variable. All baseline variables from were enrolled in univariate Cox regression analysis. The analysis showed that GLS, LVEF, BNP, Troponin T cardiac pressures and right ventricle enlargement were associated with adverse cardiac events during five-year follow-up (Table 11). GLS increase by 1% was associated with 41% higher risk of adverse cardiac events during long-term follow-up.

Table 11. Results of Cox regression analysis for predictors of adverse cardiac events during long-term follow-up.

	Univariate		**Multivariate**	
	HR (95% CI)	**p**	**HR (95% CI)**	**P**
BNP, ng/l	1.001 (1-1.001)	<0.0001	1.001 (1.0 -1.001)	0.02
Troponin T, pg/ml	1.004 (1.001-1.008)	0.02	1.004 (1.0-1.009)	0.04
mPAP, mmHg	1.1 (1.04-1.16)	0.002		
PCWP, mmHg	1.18 (1.05-1.13)	0.007		
PVR, Wood units	1.28 (1.03-1.59)	0.03		
RV end-diastolic diameter, cm	2.45 (1.24-4.87)	0.01		
LVEF, %	0.89 (0.83-0.96)	<0.01		
LV GLS, %	1.41 (1.18-1.68)	<0.0001	1.25 (1.01-1.55)	0.04

BNP – B type natriuretic peptide; CI – confidence interval; GLS – global longitudinal strain; HR – hazard ratio; LVEF – left ventricular ejection fraction; mPAP – mean pulmonary arterial pressure; PCWP – pulmonary capillary wedge pressure; PVR – pulmonary vascular resistance; RV – right ventricle.

The significant univariate predictors were enrolled in multivariate Cox regression analysis, which was performed using stepwise backward elimination method. GLS showed significant association with the occurrence of adverse cardiac events during long-term follow-up (adjusted HR 1.25 (95% CI 1.01-1.55); p = 0.04), even after adjusting for univariate outcome predictors (Table 11).

5. DISCUSSION

5.1. Prevalence of Myocardial Inflammation and Cardiotropic Viruses and Their Prognostic Value in NI-DCM Patients (Paper I)

This prospective study summarizes our experience identifying etiopathogenetic markers of idiopathic NI-DCM for diagnosis of distinct disease sub-entities, and evaluates their prognostic value. In this study, we used the criterion defined by the ESC Working Group on Myocardial and Pericardial Diseases [13] for diagnosing inflammatory dilated cardiomyopathy. Inflammatory dilated cardiomyopathy was diagnosed in 54% of the patients by immunohistochemistry. The rate of the inflammatory dilated cardiomyopathy was similar compared with the study by Palecek et al [232]. The prognostic value of myocardial inflammation and different inflammatory cells varies in different studies, possibly due to the diversity of diagnostic protocols [33]. Though, we did not find any study which evaluated the prognostic value of inflammatory dilated cardiomyopathy diagnosed by the ESC criterion. In our cohort, inflammatory dilated cardiomyopathy had no impact on clinical outcomes. However, a higher count of CD3+ and CD45ro+ cells was associated with a poor clinical outcome.

Our study found a high prevalence of cardiotropic viruses (52% of the patients), of which the most frequently detected were B19V and HHV6. Three patients (10%) were co-infected with B19V and HHV6. These findings support previous studies, in which B19V and HHV6 were the most frequent viruses [16,39,233]. Furthermore, the high prevalence of B19V suggests that chronic DCM might have developed from the previous B19V–associated myocarditis [234]. However, there is conflicting evidence about the viral genome's impact on the long-term prognosis. Several studies say that viral genomes were associated with worsening LV function, the need for heart transplantation, and death [16,51]. While other studies report that the existence of viral genomes per se is not associated with poor clinical outcome [18,60,235,236]. In our cohort, the detection of a virus had no impact on clinical outcome.

However, half of our virus-positive patients had no myocardial inflammation. This finding is also reported by Kuehl et al., in whose study NI-DCM patients had symptoms of heart failure and viral genome, but no evidence of inflammation [16,51]. Kindermann et al. [18] have also reported that the frequency of inflammation is independent of any evidence of the virus

genome. Several studies have detected B19V in healthy people's hearts with no evidence of inflammation [59,60,237].

In contrast, half of our inflammatory dilated cardiomyopathy patients had no viral genome, and the cause of inflammation remains unknown. This finding supports the idea that myocardial inflammation could be maintained by an autoimmune process leading to the deterioration of LV function [38]. Contrarily, this high prevalence of cardiac inflammation might be due to the advanced NI-DCM phenotype. The question remains if this inflammation is a consequence of this advanced stadium or if it acts as a causal factor.

Interestingly, the macrophage count was higher in the virus-negative group. It could be hypothesized that CD68+ macrophages have an impact on NI-DCM pathogenesis because of their pro-inflammatory activity. As known from previous studies, macrophages can cause or maintain persistent LV systolic dysfunction and LV remodeling [238]. While recent studies have noted the importance of macrophage profiles and their function in heart diseases, much is still unknown about their impact on NI-DCM pathogenesis [239].

Virus-negative patients had worse hemodynamic parameters and higher B-type natriuretic peptide levels than the virus-positive patients, although the echocardiographic parameters did not differ between the two groups. Worse hemodynamic status might be explained by chronic immune activation and myocardial inflammation, given that higher numbers of macrophages and higher levels of IL-6 were detected in this virus-negative group. Macrophages secrete IL-6 [240], which might increase the severity of pulmonary hypertension [241].

Although heart failure treatment has become more effective, there are still many refractory NI-DCM patients who do not respond to any available treatment. Therefore, developing alternative therapies is essential. We distinguished four etiopathogenetic groups, for whom could be suitable specific therapeutic strategy selected [8,13,242] or novel treatment options established [19]. Treatment strategies based on the etiopathogenetic approach of the disease might improve LV function, prevent the progression of heart failure, and, in some cases, exclude patients from the heart transplant list.

5.2. Association of Inflammation-Related Biomarkers with Heart Failure Severity and Their Prognostic Role in NI-DCM Patients (Papers II-III)

This study evaluates the association of inflammation-related biomarkers with heart failure severity, as well as their prognostic value, in a cohort of NI-DCM patients. The main findings are that:

- hs-CRP is associated with certain heart failure severity parameters (LVEF, BNP) and adverse cardiac events;
- suPAR is not associated with heart failure severity or poor clinical outcomes in NI-DCM patients;
- Higher counts of CD3+ T lymphocytes and CD45ro+ memory T cells correspond to a poorer clinical outcome.
- Higher levels of inflammation-related cytokines (IL-6, TNF-α, adiponectin) are associated with heart failure severity and predict poor clinical outcomes.
- An increase in serum levels of all three cytokines (IL-6 > 4.53 pg/mL, TNF-α > 7.81 pg/mL and adiponectin > 17.14 mg/mL) can identify high-risk patients.

hs-CRP is an acute-phase protein produced mainly by hepatocytes under the influence of cytokines. Elevated hs-CRP levels are detected and associated with poor clinical outcomes in chronic heart failure patients [243,244]. However, the predictive value of hs-CRP in NI-DCM patients has not been resolved. Lamblin et al. [144] evaluated hs-CRP predictive value in 546 patients with heart failure with reduced ejection fraction (non-ischemic etiology ~60%). hs-CRP was a predictor of mortality in the whole cohort and ischemic heart failure subgroup during the median 2.7 years' follow-up but not in non-ischemic patients. Li et al. [146] found that hs-CRP was an independent predictor of mortality during an average of 2.6 years of follow-up in 622 NI-DCM patients. Ishikawa et al. [147] also demonstrated that hs-CRP was an independent predictor of poor clinical outcome in a cohort of 84 NI-DCM patients during 42 months of follow-up. In our study, which employed a longer follow-up period than previous studies, we found that hs-CRP was associated with long-term adverse cardiac events, although it did not remain a significant predictor following multivariate analysis.

suPAR is a proinflammatory marker originating from proteolytic cleavage and releasing of the membrane-bound urokinase-type plasminogen activator receptor from vascular endothelial and immune cells [167]. It is associated with

infectious diseases, systemic inflammation, malignancies [169,245,246] and cardiovascular diseases, predominantly ischemic heart disease [247]. Several studies have analyzed suPAR in chronic heart failure patients, but the results have been inconsistent. Lichtenauer et al. [248] found no difference in suPAR concentrations between NI-DCM (n=65) and ischemic heart failure (n=59) patients. However, they observed an increase in suPAR according to NYHA stage, which reached a plateau at NYHA stage III. In another study, Koller et al. [171] found that suPAR was associated with adverse cardiac events in 319 chronic heart failure patients (55% NYHA class II, 43%class III). van den Berg et al. [249] found suPAR was associated with adverse cardiac events in 263 mixed-etiology chronic heart failure patients (26% had NYHA III-IV class) during a median of 2.2 years of follow-up. In contrast to earlier findings, we did not detect any association between suPAR and heart failure severity parameters or adverse cardiac events. A possible explanation for this might be that most patients in our cohort were classified as NYHA III-IV when suPAR levels would be expected to reach a plateau [248]; consequently, this marker possibly loses its prognostic value in our more advanced NI-DCM patient cohort.

The presence of infiltrative inflammatory cells in the myocardium can be assessed to diagnose inflammation [13]. However, only a few studies have estimated the prognostic value of distinct inflammatory cells, with inconsistent results [96,136]. Zuern et al. [136] found no association between CD3+ and CD68+ cells and clinical outcome in 227 patients with congestive heart failure (79% chronic myocarditis or non-inflammatory DCM) during three-years of follow-up. Nakayama et al. [96] evaluated CD3+ and CD68+ cells in the myocardium in 182 DCM patients. During an average of 6.9 years' follow-up, both inflammatory cells types were associated with poor clinical outcomes but did not remain significant in a multivariate approach. Our results demonstrate that CD3+ and CD45ro+ cells, but not CD68+, CD4+, CD54+ or HLA-DR+ cells, were associated with poor clinical outcomes during the five-year follow-up period. Higher counts of CD45ro+ cells remained an independent predictor of adverse cardiac events in the multivariate analysis. These divergent results might be influenced by differences in statistical analysis and follow-up duration, unequal proportions of different etiopathogenetic phenotypes in the NI-DCM cohorts [250], as well as biopsy collection occurring at different time points over the course of the disease [251].

Adiponectin is an adipocyte-derived cytokine, which acts in obesity-linked diseases [252], renal failure, and various cardiovascular diseases [253]. However, its actions are multifaceted and controversial [254]. Adiponectin has cardioprotective [255], anti-atherogenic [256] and anti-inflammatory effects [257,258] in the context of atherosclerotic cardiovascular diseases. Despite these beneficial effects, adiponectin levels increase with the clinical worsening of chronic heart failure [259,260], and are associated with higher mortality [89,261]. Our study results support previous findings demonstrating an association of adiponectin with heart failure severity parameters and adverse clinical outcomes in NI-DCM patients. Previously, Wojciechowska et al. [262] found that elevated adiponectin levels were associated with poor clinical outcomes in 129 NI-DCM patients during three-years of follow-up. Our results are consistent with these findings and show that adiponectin remains a significant predictor in NI-DCM patients during the five-year follow-up. However, in agreement with other authors, role of APN as a predictive marker in chronic heart failure is highly dependable on various clinical characteristics (age, sex, BMI, NYHA class, treatment received, renal function, type of heart failure, etc.) [263–266]. For this reason, clinical interpretation of APN level in patient's plasma is not straightforward and not so easily applicable in clinical practice.

IL-6 and TNF-α are proinflammatory cytokines produced mostly by activated monocytes and macrophages. The proinflammatory role of these cytokines in systemic inflammation is well established in various pathologies, including cardiovascular diseases such as atherosclerotic cardiovascular disease and chronic heart failure [267,268]. In addition, the prognostic role of IL-6 and TNF-α in chronic heart failure patients has been demonstrated in previous studies [269,270]. However, approximately half of these cohorts were composed of ischemic heart failure patients, which might have had a significant impact on the predictive value of these cytokines, considering that serum concentrations of IL-6 and TNF-α are significantly higher in ischemic heart failure patients than in patients with NI-DCM [187,188]. To the best of our knowledge, the prognostic value of IL-6 and TNF-α has not previously been estimated in a NI-DCM patient population. We found an association between the cytokines, IL-6 and TNF-α, and adverse cardiac events during the five-year follow-up period. In addition, IL-6 > 4.53 pg/mL was an independent predictor of long-term poor clinical outcomes.

The present study also revealed that inflammation-related cytokines (IL-6, TNF-α and adiponectin) were associated with heart failure severity. These results are consistent with those of previous studies, which showed an association between circulating cytokines and heart failure progression (i.e. NYHA functional class and/or BNP levels) [163,187,260]. Additionally, our results supplement and consolidate previous findings, in which, besides having an association with NYHA and BNP, cytokines also correlated with other heart failure severity parameters, such as LVEF, PCWP (except TNF-α), and mPAP. Moreover, these cytokines showed the highest accuracy in predicting clinical outcomes out of all the inflammation-related biomarkers that we investigated. Our most important clinically relevant finding was that the combination of all three cytokines could help identify high-risk patients. Patients who had low concentrations of all three biomarkers did not experience adverse cardiac events. In contrast, all patients with higher concentrations than the cut-off values of all three cytokines experienced adverse cardiac events during follow-up, mainly within the first two years. Therefore, the use of inflammation-related cytokines might aid in identifying high-risk NI-DCM patients, which could lead to changes in follow-up intensity, the timing for device therapy, or heart transplantation.

Overall, this research evaluated both biomarkers of myocardial and systemic inflammation. A significant finding to emerge from this study is that inflammatory cells in the myocardium did not correlate with biomarkers of systemic inflammation, except CD68+ cells with IL-6. Inflammatory cells are mainly studied in patients with acute myocarditis, but not NI-DCM; therefore, there is a lack of consistent data in an advanced disease phase [50]. Therefore, we hypothesize that the absence of the association may partly be explained by the existence of different etiopathogenetic sub-groups in this cohort with different myocardial inflammation statuses [250]. The other possible explanation for this might be that pathological processes in the myocardium, including inflammation, cause malfunction and remodeling of the heart, which triggers a systemic inflammatory response that proceeds to a low-grade inflammation (para-inflammation) state leading to further disease progression [85]. Another significant finding from this study might support this hypothesis: systemic inflammation-related biomarkers (cytokines) predicted clinical outcomes more significantly and with higher accuracy than infiltrative inflammatory cells. This finding also reflects the importance of chronic low-grade systemic inflammation in the disease progression.

5.3. The Relation Between GLS and Heart Failure Severity and Outcomes in Patients with NI-DCM (Paper IV)

This study evaluates the association between myocardial deformation parameter GLS and invasively assessed cardiac pressures and PVR as well as the prognostic value of GLS for predicting clinical outcomes in NI-DCM patients. The main findings are that:

- GLS correlates with heart failure severity parameters (PCWP, mPAP, PVR, BNP);
- GLS is significantly associated with poor clinical outcome in a well-defined NI-DCM cohort;
- GLS >-5.96% predicts adverse clinical events during five-year follow-up. In the subgroup of patients with severely reduced LVEF, GLS values above the cut-off have an additional predictive value.

Prior studies have demonstrated that GLS has a significant predictive value in heart failure patients [216–218,271], in various cardiovascular pathologies [272–279], and even in the general population [280]. To our knowledge, this is the first study to investigate the predictive potential of GLS in patients with chronic heart failure due to NI-DCM. Our study showed that GLS is predictor of poor long-term outcomes in a well-defined cohort of patients with NI-DCM. Furthermore, GLS remained a significant long-term predictor when added to a model with other prognostic parameters.

Guidelines define a GLS≤–20% as a normal value in healthy subjects [222]. A recent meta-analysis reports that a GLS>-16% indicates significant myocardial dysfunction [281]. There are no defined GLS cut-off values for risk estimation in different cardiac pathologies, although these would be useful for prognostication, management, and future studies. Motoki et al. [216] included 194 patients with various etiology chronic heart failure. They identified a GLS cut-off value of -6.95% to be a predictor of poor clinical outcomes during a five-year follow-up. We identified the cut-off value of GLS -5.96% of adverse cardiac events during a five-year follow-up. The worse GLS cut-off value in our study compared to theirs might be due to the severity of heart failure in our cohort (NYHA III-IV functional class 88% versus 39%) and a higher event rate (49% versus 40%). Sengelov et al. [217] included 1065 patients with various origin HFrEF and found a mortality rate of 16.7% during the median follow-up of 40 months. They identified a GLS cut-off value of -5.9% to be a useful predictor of increased mortality in patients with

severely reduced LV systolic function (LVEF < 22%). Our study's identified cut-off value is in line with their study, despite differences in heart failure etiology, follow-up duration, and event rate. It is important to note that our cut-off value had 100% specificity, supported by Kaplan–Meier analysis: all patients with GLS > -5.96% experienced adverse cardiac events before the end of follow-up, with more than half of the patients experiencing cardiac events during the first year of follow-up. This cut-off might be useful for identifying patients with advanced heart failure.

6. LIMITATIONS

Our study's main limitation is the single-center small sample size, which prevents us from making generalizations about independent predictors of poor clinical outcomes or exact cut-off values for each biomarker in patients with NI-DCM. The small sample size also limited deeper subgroup analysis (e.g., urgent versus non-urgent heart transplantation or LVAD implantation or differentiation of the patients based on the type of infectious agent) and the ability to perform more extent survival analysis like competing risks regression models.

Secondly, the study had no control group for our laboratory investigations because of a shortage of donor hearts that would be healthy enough to be used in the lab but for some reason not suitable for transplantation. Thirdly, because of the financial expense, we neither investigated virus replication nor viral load for distinguishing active from incidental infection [282], nor performed autoantibody testing or genetic screening for pathogenic NI-DCM mutations. Fourth, due to limited experience in specific NI-DCM treatment and because of the treatment costs, none of our patients received etiology-directed treatment. Furthermore, we did not evaluate the dynamics of the biomarkers during the follow-up period, which could have helped us to understand the pathologic processes leading to heart failure progression more comprehensively. In addition, the study was held at a time when the right ventricle was a "forgotten" ventricle; therefore, it was not assessed thoroughly. Futhermore, the degree of valve regurgitation was evaluated visually.

This thesis was a continuation of research started in a previous study, which aimed to evaluate molecular mechanisms dominating in virus-positive and inflammatory dilated cardiomyopathy [283]. Therefore, *a priori* sample size calculation was performed for the study's primary aim, but unfortunately not for subsequent survival analysis presented in this thesis. In addition, *post hoc* was not also performed because analysis of observed results has been criticized as inappropriate [284,285], and sample size calculation is recommended only in the design stage.

Although we demonstrated the possibility for risk stratification by three inflammation-related cytokines and GLS, a larger study is needed to verify and build on the present research findings.

Despite its limitations, the study certainly provides a basis for a more extensive diagnostic and treatment study that is based on etiopathogenetic sub-entities and includes a control group. Furthermore, the use of GLS and inflammation-related cytokines to identify high-risk NI-DCM patients could lead to changes in follow-up intensity, the timing for device therapy, or prioritization on the heart transplantation list.

7. CONCLUSIONS

1. This research has shown that two markers, namely cardiotropic viruses and myocardial inflammation, are prevalent among NI-DCM patients. They are also helpful in identifying sub-groups of NI-DCM. (Paper I)

2. Cardiotropic viruses detected in myocardium and inflammatory NI-DCM, defined by ESC criteria, are not associated with adverse cardiac events. (Paper I)

3. An increased number of T-cells and memory T-cells in the myocardium are associated with poor long-term clinical outcomes. (Papers I, II)

4. Interleukin-6, adiponectin and memory T-cells are independent predictors of poor clinical outcomes. Interleukin-6, TNF-α and adiponectin are associated with disease severity, and a combination of them can identify high-risk NI-DCM patients. (Papers II, III)

5. GLS is associated with heart failure severity and predicts long-term adverse cardiac events in patients with NI-DCM. Worsening GLS is associated with incremental risk of long-term adverse cardiac events and might be used to identify high-risk patients. (Paper IV)

8. PRACTICAL RECOMMENDATIONS

1. Severely impaired GLS (> -5.96 %) and increased levels of three baseline inflammation-related cytokines (IL-6 < 4.53 pg/mL, adiponectin < 17.14 μg/mL and TNF < 7.81 pg/mL) can serve as additional biomarkers for identifying high-risk NI-DCM patients who need heart transplantation.

2. A further step would be a larger study to verify and build on the present research findings. A larger study is needed for deeper subgroups analyses: a trial comparing four different etiopathogenetic subgroups or a study analyzing NI-DCM patients with different disease severity (e.g., different NYHA classes) or different urgency for heart transplantation or mechanical circulatory support (e.g., different INTERMACS profiles or urgency statuses).

SUMMARY IN LITHUANIAN

SANTRUMPOS

AUC	plotas po kreive (angl. *area under the curve*)
BNP	B tipo natriuretinis peptidas
B19V	parvovirusas B19
CD3+	T limfocitų receptorius
CD4+	T pagalbininkų receptorius
CD45ro+	atminties T limfocitų receptorius
CD68+	makrofagų receptorius
CMV	citomegalovirusas
dj-CRB	didelio jautrumo C reaktyvusis baltymas
dj-troponinas T	didelio jautrumo troponinas T
DPS	dešiniojo prieširdžio spaudimas
DS	dešinysis skilvelis
DT	lėtėjimo greitis (angl. *decelaration time*)
e‘	ankstyvasis diastolinis mitralinio žiedo greitis
E/A	santykis kairiojo skilvelio ankstyvojo prisipildymo greičio (E) ir maksimalaus tėkmės per mitralinį vožtuvą greičio susitraukiant prieširdžiams (A)
EBV	Epšteino–Bar virusas
E/e’	kairiojo skilvelio ankstyvojo prisipildymo greičio (E) ir ankstyvojo mitralinio žiedo diastolinio greičio (e‘) santykis
EKD	Europos kardiologų draugija
EV	enterovirusas
GLS	bendra išilginė miokardo deformacija (angl. *global longitudinal strain*)
HCV	hepatito C virusas
HHV-6	žmogaus herpesvirusas 6 (angl. *human herpesvirus 6*)
HR	pavojaus rizika (angl. *Hazard Ratio*)
ICAM-1/CD54+	viduląstelinės adhezijos molekulės 1 (angl. *Intercellular Adhesion Molecule 1*)
IL-6	interleukinas 6
KPTi	kairiojo prieširdžio tūrio indeksas
KRAS	Kirsteno žiurkių sarkomos viruso onkogeno homologas
KS	kairysis skilvelis
KSGDD	kairiojo skilvelio galinis diastolinis dydis

KSIF	kairiojo skilvelio išstūmimo frakcija
KSPP	kairįjį skilvelį pavaduojantis prietaisas
MHC	didysis audinių suderinamumo kompleksas (angl. *major histocompatibility complex*)
MV	mitralinis vožtuvas
ne-uDKM	neuždegiminė dilatacinė kardiomiopatija
NI-DKM	neišeminė dilatacinė kardiomiopatija
NYHA	Niujorko širdies asociacija
PAS	plaučių arterijos spaudimas
PGR	polimerazės grandininė reakcija
PI	pasikliautinis intervalas
PKP	plaučių kraujagyslių pasipriešinimas
PKPS	plaučių kapiliarų pleištinis slėgis
ROC	kreivė, rodanti klasifikatoriaus jautrumo ir specifiškumo sąryšį (angl. *receiver operator curve*)
ROI	domėjimosi sritis (angl. *region of interest*)
SIFŠN	sumažėjusios išstūmimo frakcijos širdies nepakankamumas
suPAR	tirpus urokinazės plazminogeno aktyvavimo receptorius (angl. *soluble urokinase Plasminogen Activator Receptor*)
ŠI	širdies indeksas
ŠMT	širdies minutinis tūris
TNF-α	naviko nekrozės faktorius α
TV	triburis vožtuvas
UBC	ubikvitinas C
uDKM	uždegiminė dilatacinė kardiomiopatija
VZV	*Varicella zoster* virusas
ŽLA-DR	žmogaus leukocitų receptorius, DR izotipas

Ši daktaro disertacija parengta mokslinių publikacijų, kurios tolesniame tekste žymimos romėniškais skaitmenimis, rinkinio pagrindu:

I. **Kažukauskienė I,** Baltrūnienė V, Jakubauskas A, Žurauskas E, Maneikienė V.V, Daunoravičius D, Čelutkienė J, Ručinskas K, Grabauskienė V. *Prevalence and prognostic relevance of myocardial inflammation and cardiotropic viruses in non-ischemic dilated cardiomyopathy.* Cardiology journal. Gdansk: Via Medica. ISSN 1897-5593. eISSN 1898-018X. 2020, first on line, p. [1–24]. https://doi.org/10.5603/CJ.a2020.0088
II. **Kažukauskienė I,** Baltrūnienė V, Rinkūnaitė I, Žurauskas E, Vitkus D, Maneikienė V.V, Ručinskas K, Grabauskienė V. *Inflammation-related biomarkers are associated with heart failure severity and poor clinical outcomes in patients with non-ischemic dilated cardiomyopathy.* Life 2021, 11 (10), 1006; https://doi.org/10.3390/life11101006
III. Baltrūnienė V, Bironaitė D, **Kažukauskienė I,** Bogomolovas J, Vitkus D, Ručinskas K, Žurauskas E, Augulis R, Grabauskienė V. *The role of serum adiponectin for outcome prediction in patients with dilated cardiomyopathy and advanced heart failure.* BioMed research international: Hindawi. ISSN 2314-6133. eISSN 2314-6141. 2017, vol. 2017, Article ID 3818292, [p. 1–13].
https://doi.org/10.1155/2017/3818292
IV. **Kažukauskienė I,** Balčiūnaitė G, Baltrūnienė V, Čelutkienė J, Maneikienė V.V, Čibiras S, Ručinskas K, Grabauskienė V. *Left ventricular global longitudinal strain predicts elevated cardiac pressures and poor clinical outcomes in patients with non-ischemic dilated cardiomyopathy*. Cardiovasc Ultrasound London: BioMed Central. eISSN 1476-7120. 2021, vol. 19, no. 1, art. no. 21, p. [1–13].

1. ĮŽANGA

1.1.Tyrimo problema ir tyrimo aktualumas

Širdies nepakankamumas – tai heterogeniškas ir kompleksinis klinikinis sindromas, atsirandantis dėl sutrikusio skilvelių prisipildymo (diastolinė disfunkcija) arba kraujo išstūmimo (sistolinė disfunkcija), susijęs su tokiais širdies nepakankamumo simptomais kaip dusulys, nuovargis, plaučių ir (arba) periferinė edema. Išsivysčiusiose šalyse širdies nepakankamumu serga iki 2 % suaugusių gyventojų [1], o su amžiumi šis skaičius didėja – nuo jo kenčia

daugiau kaip 10 % vyresnių nei 70 metų gyventojų [1–3]. Šis sindromas lemia didelį sergamumą ir mirtingumą: penkerių metų išgyvenamumas po diagnozės nustatymo yra 50 %. Nors per pastaruosius du dešimtmečius farmakologinis gydymas ir medicinos prietaisų naudojimas gerokai pagerino šių pacientų gyvenimo kokybę ir sumažino mirtingumą, širdies nepakankamumas išlieka pagrindine mirties priežastimi išsivysčiusiose šalyse [4].

Širdies nepakankamumas paprastai klasifikuojamas pagal pagrindinio rodiklio – kairiojo skilvelio išstūmimo frakcijos (KSIF) – įvertinimą, kuris išreiškiamas procentais, kiek kraujo kairysis skilvelis išstumia per kiekvieną susitraukimą. Jei KSIF yra mažesnė nei 40 %, širdies nepakankamumas klasifikuojamas kaip sumažėjusios išstūmimo frakcijos širdies nepakankamumas (SIFŠN) (sistolinės disfunkcijos atitikmuo). SIFŠN sudaro apie 50–60 % širdies nepakankamumo atvejų. Tai didelės etiologinės įvairovės sindromas, atsirandantis dėl įvairių širdies pažeidimų, įskaitant miokardo, perikardo, kraujagyslių, širdies vožtuvų ar širdies laidumo sistemos pažeidimus. Dažniausia SIFŠN priežastis yra išeminė širdies liga, ypač vyresnio amžiaus žmonių populiacijoje. Tačiau jaunesnių pacientų viena iš pagrindinių SIFŠN priežasčių yra neišeminė dilatacinė kardiomiopatija (NI-DKM): NI-DKM sudaro apie trečdalį SIFŠN atvejų [5,6].

NI-DKM yra lėtinė širdies liga. Ji pasireiškia kairiojo skilvelio (KS) išsiplėtimu ir sutrikusia skilvelio (kairiojo arba abiejų skilvelių) funkcija, kurios nesukelia vainikinių arterijų liga ar neįprastos hemodinamikos sąlygos [1]. Dešimtmečius atliekami tyrimai atskleidė platų NI-DKM etiologinį spektrą, įskaitant infekcijų sukėlėjus, genetinius pakitimus, nervų ir raumenų ar endokrinines bei autoimunines ligas, vaistus, toksinus, mitybos nepakankamumą ar elektrolitų sutrikimus [7,8]. Tačiau didelė etiologinių veiksnių įvairovė lemia, kad tiksliai ligos priežasčiai nustatyti reikia daugybės brangių diagnostikos priemonių. Deja, diagnostikos priemonių prieinamumas visame pasaulyje labai skiriasi, todėl daugelis NI-DKM atvejų esant nenustatytiems etiologiniams veiksniams klasifikuojami kaip idiopatinė NI-DKM [9–12].

Endomiokardo biopsinės medžiagos analizė taikant imunohistochemiją ir polimerazės grandininę reakciją (PGR) tapo pagrindiniu tyrimu NI-DKM priežasčiai išsiaiškinti [13–15]. Todėl kardiotropiniai virusai yra pripažinti svarbiu širdies nepakankamumo etiologiniu veiksniu ir randami iki dviejų trečdalių pacientų, sergančių NI-DKM, miokarde [16,17]. Tačiau klinikinė kardiotropinių virusų reikšmė ir jų įtaką pacientų prognozei išlieka diskusijų objektu [18,19].

Miokardo uždegimas, patvirtintas endomiokardo biopsija, taip pat žinomas kaip svarbus priežastinis veiksnys, lemiantis KS dilatacijos progresavimą [20–23]. Per pastaruosius dešimtmečius miokardo uždegimo diagnostikos kriterijai buvo kelis kartus atnaujinti [13,24–26]. Naujausią miokardo uždegimo apibrėžimą patvirtino Europos kardiologų draugijos (EKD) miokardo ir perikardo ligų darbo grupė [13]. Tačiau pagal šį kriterijų diagnozuoto miokardo uždegimo paplitimas dar nebuvo tirtas. Be to, nors NI-DKM pacientų etiopatogenetiniai pogrupiai nustatomi naudojant imunohistochemijos ir virusinės PGR rezultatus, tačiau apie šiuos pogrupius trūksta perspektyviųjų duomenų.

Dėl etiopatogenetinės įvairovės NI-DKM progresuoja skirtingai. Nepaisant optimalaus, rekomendacijomis grįsto gydymo, nemažos dalies NI-DKM pacientų būklė blogėja (progresuoja) iki mirties arba iki tol, kol jiems persodinama širdis. NI-DKM yra visame pasaulyje dažniausia širdies transplantacijos indikacija, sudaranti daugiau kaip 50 % visų širdies transplantacijų 18–59 metų amžiaus grupėje [27]. Tačiau nėra tikslių prognostinių modelių NI-DKM pacientų rizikai stratifikuoti. Be to, Dziewiecka ir kt. [28] parodė, kad dauguma rizikos vertinimo modelių, sukurtų heterogeninėms širdies nepakankamumo populiacijoms, yra nepakankamo tikslumo NI-DKM sergantiems pacientams. Todėl reikia patikimų žymenų didelės rizikos NI-DKM pacientams nustatyti, nes juos reikia atidžiai stebėti ir laiku priimti sprendimus dėl sudėtingesnio gydymo. Patikimi žymenys taip pat galėtų palengvinti konkrečių terapinių taikinių paiešką.

Šis darbas parengtas keturių publikuotų mokslinių straipsnių pagrindu. Bendras jo tikslas – ištirti įvairių žymenų ir širdies nepakankamumo sunkumo bei klinikinių rodiklių sąsajas NI-DKM pacientų grupėje. Pirmame (I) straipsnyje analizuojamas kardiotropinių virusų ir miokardo uždegimo paplitimas bei prognostinė reikšmė. Antrame (II) ir trečiame (III) straipsniuose nagrinėtas ryšys tarp su uždegimu susijusių žymenų ir ligos sunkumo, taip pat šių žymenų prognostinė reikšmė. Galiausiai ketvirtame (IV) straipsnyje buvo tiriamas bendros miokardo išilginės deformacijos (angl. *global longitudinal strain* – GLS) ryšys su ligos sunkumu ir blogomis klinikinėmis baigtimis.

1.2. Tyrimo tikslas

Hipotezė: Miokardo, serumo ir echokardiografiniai žymenys rodo širdies nepakankamumo sunkumą ir prognozuoja blogas klinikines baigtis pacientams, sergantiems NI-DKM.

Disertacijos tikslas: Įvertinti ryšį tarp miokardinių, seruminių, miokardo deformacijos žymenų ir širdies nepakankamumo sunkumo rodiklių bei ištirti šių žymenų prognostinę vertę gerai charakterizuotoje, išgrynintoje NI-DKM pacientų kohortoje.

1.3. Tyrimo uždaviniai

1. Įvertinti kardiotropinių virusų ir miokardo uždegimo žymenų diagnostinę vertę NI-DKM pacientų kohortoje. (I straipsnis)
2. Nustatyti kardiotropinių virusų ir miokardo uždegimo žymenų prognostinę vertę NI-DKM pacientų kohortoje. (I straipsnis)
3. Nustatyti miokardinių (CD3+, CD4+, CD68+, CD45ro+, ŽLA-DR+, ICAM-1+) ir seruminių uždegimo žymenų (leukocitų, didelio jautrumo C reaktyviojo baltymo (dj-CRB), adiponektino, interleukino 6 (IL-6), naviko nekrozės faktoriaus α (TNF-α)) ryšį, tirpaus urokinazės plazminogeno aktyvavimo receptoriaus (angl. *soluble urokinase Plasminogen Activator Receptor* – suPAR) prognostinę vertę bei nustatyti minėtų žymenų koreliaciją su širdies nepakankamumo sunkumo rodikliais gerai charakterizuotoje, išgrynintoje NI-DKM pacientų kohortoje. (II ir III straipsniai)
4. Nustatyti bendros miokardo išilginės deformacijos prognostinę vertę gerai charakterizuotoje, išgrynintoje NI-DKM pacientų kohortoje (IV straipsnis)

1.4. Tyrimo mokslinis naujumas

Šiame doktorantūros tyrime, kurio rezultatai paskelbti penkiose mokslinėse publikacijose, įvertinta įvairių miokardo, serumo ir echokardiografinių žymenų prognostinė vertė gerai charakterizuotoje, išgrynintoje NI-DKM pacientų kohortoje per penkerius stebėjimo metus. Anksčiau įvairių skirtingų žymenų prognostinė vertė buvo tirta heterogeninėse lėtinio širdies nepakankamumo kohortose, bet išgrynintoje NI-DKM kohortoje vertinta nebuvo.

Mūsų turimomis žiniomis, miokardo uždegimo, diagnozuoto pagal EKD 2013 metų kriterijų [13], prognostinė vertė NI-DKM pacientų kohortoje pirmą kartą tirta ir vertinta mūsų tyrime. Be to, šiame tyrime kai kurių žymenų (pvz. CD3+, CD45ro+, dj-CRB, adiponektino) prognostinė vertė NI-DKM pacientų grupėje buvo įvertinta per ilgesnį stebėjimo laikotarpį nei anksčiau paskelbtuose tyrimuose. Negana to, tai pirmasis tyrimas, kuriame įvertinta

IL-6, TNF-α, suPAR prognostinė vertė NI-DKM pacientų grupėje. Taip pat tai pirmasis tyrimas, kuriame įvertinta visų trijų su uždegimu susijusių citokinų (adiponektino, IL-6, TNF-α) derinio prognostinė įtaka klinikinėms baigtims. Galiausiai mūsų tyrime nustatytos specifinės GLS, IL-6, TNF-α ir adiponektino ribos, kurios padeda nustatyti didelės rizikos pacientus.

1.5. Praktinė tyrimo vertė

Pacientų, sergančių NI-DKM, mirtingumas yra didelis net ir taikant dabartinį širdies nepakankamumo gydymą. Todėl etiopatogenetinių veiksnių (pvz. kardiotropinių virusų, miokardo uždegimo) tyrimai yra svarbūs siekiant geriau stratifikuoti NI-DKM pacientų riziką, taip pat išliekant tikslinių, specifinių gydymo strategijų paieškų poreikiui.

Šis tyrimas parodė, kad endomiokardo bioptatų ištyrimas taikant imunohistochemiją, suteikia ne tik diagnostinės, bet ir prognostinės informacijos pacientams, sergantiems NI-DKM. Be to, su uždegimu susijusių citokinų įvertinimas ir GLS matavimas gali padėti stratifikuoti pacientų riziką ir nustatyti didelės rizikos pacientus. Didelės rizikos pacientų atpažinimas turėtų įtakos tolesnio stebėjimo intensyvumui, padėtų priimti sprendimus dėl gydymo taktikos ir nustatyti pacientų prioritetus širdies transplantacijos sąraše.

1.6. Ginami disertacijos teiginiai

1. Kardiotropiniai virusai miokarde ir miokardo uždegimas yra paplitę tarp NI-DKM sergančių pacientų.
2. Kardiotropinių virusų radimas miokarde ir uždegiminio NI-DKM diagnozė nėra susiję su blogomis klinikinėmis baigtimis.
3. Skirtingų tipų uždegiminės ląstelės miokarde, pvz. CD3+ ir CD45ro+, yra susijusios su blogomis klinikinėmis NI-DKM pacientų baigtimis.
4. Uždegiminiai citokinai (IL-6, TNF-α ir adiponektinas) yra susiję su blogomis klinikinėmis baigtimis ir gali padėti nustatyti didelės rizikos NI-DKM pacientus.
5. Bendra išilginė miokardo deformacija (GLS) yra susijusi su širdies nepakankamumo sunkumu ir gali prognozuoti blogas klinikines NI-DKM pacientų baigtis.

2. TYRIMO PLANAS IR METODAI

2.1. Etinis patvirtinimas (I–IV straipsniai)

Tyrimas atliktas vadovaujantis Helsinkio deklaracija. Visi pacientai davė rašytinį informuoto asmens sutikimą. Bioetikos leidimas tyrimui gautas iš Lietuvos bioetikos komiteto (licencijos Nr. 158200-09-382-l03; 158200-382-PP1-23; 158200-17-891-413).

2.2. Tiriamoji populiacija. Įtraukimo ir neįtraukimo kriterijai (I–IV straipsniai)

Perspektyvusis kohortinis tyrimas su retrospektyviąja analize atliktas Vilniaus universiteto ligoninėje Santaros klinikose. Į tyrimą įtraukti 57 pacientai, kuriems buvo įtariama NI-DKM ir kurie nuo 2010 m. sausio mėn. iki 2013 m. gruodžio mėn. buvo paguldyti į ligoninę diagnozei patikslinti. Širdies nepakankamumo simptomų trukmės mediana iki įtraukiant į tyrimą buvo 12 (5–60) mėnesių.

Įtraukimo kriterijai buvo šie:

- širdies nepakankamumo simptomai ir požymiai;
- echokardiografiškai patvirtintas KS išsiplėtimas;
- sumažėjusi KSIF (≤45 %).

Neįtraukimo kriterijai buvo šie:

- reikšminga vainikinių arterijų liga, apibrėžiama kaip didesnė nei 50 % proksimalinės vainikinės arterijos stenozė arba anksčiau patirtas miokardo infarktas;
- žinomos širdies nepakankamumo priežastys, tokios kaip pirminė vožtuvų ar miokardo liga, hipertenzinė širdies liga, endokrininė liga, pažengusi lėtinė inkstų liga, piktnaudžiavimas narkotikais ar alkoholiu;
- ūminis miokarditas (naujai atsiradę simptomai per pastaruosius tris mėnesius) arba ūminis miokardo infarktas, įtariamas pagal klinikinį vaizdą arba diagnostinius tyrimus.

Pacientams tą pačią dieną arba dieną prieš intervencines procedūras (koronarografiją ir dešiniosios širdies kateterizaciją) atlikta transtorakalinė echokardiografija.

Visi pacientai buvo gydomi pagal EKD gaires [220,221]. Įtraukimo metu nė vienas pacientas nebuvo gydomas inotropiniais preparatais. Specifinis į etiologiją nukreiptas gydymas nebuvo taikomas.

2.3. Pacientų ištyrmas (I–III straipsniai)

Buvo surinkta išsami tyrimo dalyvių anamnezė, atlikta fizinė apžiūra ir įprastiniai laboratoriniai tyrimai, įskaitant bendrąjį kraujo tyrimą, dj-CRB, kreatininą (glomerulų filtracijos greičiui (GFR) įvertinti naudota CKD-EPI kreatinino lygtis). Taip pat serume buvo nustatomas didelio jautrumo troponinas T (dj-tropininas T) analizatoriumi Elecsys 2010 („Roche Diagnostics“, Indianapolis, Indianos valstija, JAV), o B tipo natriuretinis peptidas (BNP) – analizatoriumi ARCHITECT i („Abbott“, Ilinojus, JAV). Laboratoriniai tyrimai buvo atliekami mūsų universitetinės ligoninės laboratorijoje, akredituotoje pagal tarptautinį standartą EN/ISO-IEC 17 025.

Privalomieji tyrimai apėmė vainikinių arterijų angiografiją vainikinių arterijų ligai atmesti, dešiniosios širdies kateterizaciją hemodinamikai įvertinti. Tų pačių procedūrų metu iš dešiniojo skilvelio endomiokardo paimta biopsinės medžiagos imunohistocheminiam įvertinimui atlikti ir virusams nustatyti PGR metodu.

2.4. Su uždegimu susijusių serologinių žymenų biocheminiai tyrimai (I–III strapsniai)

Plazmos mėginiai iki analizės buvo laikomi –80 °C temperatūroje. Uždegiminiai citokinai TNF-α ir IL-6 serume buvo matuojami kietafaziais chemiliuminescenciniais imunometriniais metodais naudojant IMMULITE/Immulite 1000 sistemas („Immulite“, „Siemens“) pagal gamintojo instrukcijas: TNF-α (katalogo numeris KSNFZ (50 testų) ir KSNF1 (100 testų)), IL-6 (katalogo numeris KS6PZ (50 testų) ir KS6P1 (100 testų)), ir išreiškiami pg/ml. Adiponektinas buvo matuojamas naudojant „Millipore Adiponectin“ testą pagal gamintojo rekomendacijas (Millipore, Burlington, MA, JAV) ir išreiškiamas mg/ml. Žmogaus tirpaus urokinazės tipo plazminogeno aktyvatoriaus receptoriaus (suPAR) kiekis buvo įvertintas ELISA testu pagal gamintojo rekomendacijas (Abbexa). Absorbcija matuota 450 nm bangos ilgio spektrofotometru (Varioskan®Flash, Thermo Fisher Scientific, Vantaa, Suomija). Galutinė suPAR koncentracija išreikšta ng/mg baltymo.

2.5. Echokardiografija (I–IV straipsniai)

Echokardiografinis tyrimas atliktas naudojant komercinius ultragarso aparatus (GE Vivid 7 arba 9) su 2,5 MHz davikliu. Echokardiografiniai vaiz-

dai buvo surinkti ir skaitmeniniu būdu saugomi bei analizuojami vėliau, naudojant EchoPAC PCBT08 versiją. Naudojome įprastinį mūsų laboratorijos protokolą, skirtą įprastiniams M-režimo, dvimačiams, dopleriniams ir audinių dopleriniams echokardiografiniams vaizdams ir matavimams [198]. KS galinis diastolinis diametras (KSGDD) buvo išmatuotas parasternalinės ilgosios ašies vaizde ir indeksuotas pagal kūno paviršiaus plotą. Buvo išmatuoti KS galinis sistolinis ir galinis diastolinis tūriai, o KSIF apskaičiuotas Simpsono dviplaniu metodu. Kairiojo prieširdžio tūris buvo apskaičiuotas taikant dviplanį ploto ir ilgio metodą iš viršūninių keturių ir dviejų kamerų vaizdų. Indeksuojant kairiojo prieširdžio tūrį pagal kūno paviršiaus plotą, apskaičiuotas indeksuotas kairiojo prieširdžio tūris (KPTi). Buvo išmatuotas kairiojo skilvelio ankstyvojo prisipildymo greitis (E) ir maksimalus tėkmės per mitralinį vožtuvą greitis susitraukiant prieširdžiams (A) bei deceleracijos laikas (angl. *deceleration time* – DT) ir apskaičiuotas E/A. Ankstyvasis diastolinis mitralinio žiedo diastolinis greitis (e'), nustatytas audinių doplerio metodu, buvo gautas apskaičiavus vidurkį medialinio ir lateralinio mitralinio žiedo krašto diastolinių greičių viršūniniame keturių kamerų vaizde. Apskaičiuotas kairiojo skilvelio ankstyvojo prisipildymo greičio (E) ir ankstyvojo mitralinio žiedo diastolinio greičio (e') santykis (E/e'). Kairiojo skilvelio diastolinės disfunkcijos laipsnis buvo nustatytas pagal EKD rekomendacijas [223]. Vizualiai buvo įvertintas mitralinio ir triburio vožtuvo nesandarumo laipsnis ir dešiniojo skilvelio funkcija.

2.6. Miokardo deformacijos analizė naudojant dvimatę taškinio sekimo echokardiografiją (IV straipsnis)

Echokardiografiniai vaizdai KS GLS analizei buvo gaunami 50–70 kadrų per sekundę dažniu (su individualiu koregavimu). GLS analizei buvo išsaugoti trys skirtingi viršūniniai vaizdai (keturių, dviejų ir trijų kamerų vaizdai). Analizei tinkamas širdies ciklas buvo pasirenkamas individualiai rankiniu būdu. KS endokardo riba sistolės pabaigoje buvo pažymima rankiniu būdu (sistolės pabaigos laikui nustatyti naudotas aortos vožtuvo uždarymo momentas). Po to programa automatiškai pažymėdavo KS miokardą (angl. *region of interest* – ROI). Tyrėjas vizualiai įvertino pažymėtos ROI tinkamumą ir esant neatitikimui koregavo rankiniu būdu, kad būtų užtikrintas pažymėjimo tikslumas. Apskaičiavus vidurkį iš visų galiojančių segmentų vidutinių verčių buvo gaunamas GLS įvertis.

Keturiasdešimt vieno paciento echokardiografiniai vaizdai buvo pakankamos kokybės, kad būtų galima atlikti tolesnę dvimatę miokardo deformacijos analizę. Šie pacientai sudaro kohortą, analizuojamą IV straipsnyje.

2.7. Dešiniosios širdies kateterizavimas (IV straipsnis)

Dešiniosios širdies kateterizavimas buvo atliekamas Svano ir Ganzo (Swan–Ganz) kateteriu, kuris buvo įstumiamas gulinčiam pacientui punktuojant šlauninę veną. Skysčio pripildyto daviklio nulinis atskaitos lygis buvo nustatytas ties vidurine pažasties linija. Išmatuotas spaudimas dešiniajame prieširdyje, vidutinis plaučių arterijos spaudimas (PAS) ir plaučių kapiliarų pleištinis spaudimas (PPKS). Pleišto padėtis matuojant PPKS buvo patvirtinta vertinant fluoroskopijos vaizdus, hemodinamikos kreivę ir kraujo įsotinimą deguonimi (SaO_2 ≥95 %). Širdies minutinis tūris (ŠMT) išmatuotas Fiko metodu ir apskaičiuotas pagal lygtį: ŠMT (l/min) = deguonies suvartojimas (ml/min) / ((aortos SaO_2 – plaučių arterijos SvO_2) × hemoglobinas × 1,34). Tik deguonies suvartojimas (VO_2) buvo apskaičiuotas netiesiogiai, naudojant VO_2 nomogramas pagal amžių, svorį ir lytį. Mišraus veninio kraujo mėginiai oksimetrijai buvo imami iš plaučių arterijos, arterinio kraujo – iš aortos. Apskaičiuotas širdies indeksas (ŠI, indeksuotas pagal kūno paviršiaus plotą) ir plaučių kraujagyslių pasipriešinimas (PKP) ((vidutinis PAS – PPKS) / ŠMT) [224]. Kiekvienas spaudimo matavimas buvo atliktas paprašius pacientą trumpam sulaikyti kvėpavimą iškvėpimo pabaigoje. Atliekant kompiuterinę analizę buvo išvestas trijų iš eilės širdies ciklų vidurkis [225].

2.8. Endomiokardo biopsija (I–III straipsniai)

Dešiniojo skilvelio endomiokardo biopsija buvo atliekama lanksčiu biotomu („Westmed“), įstumtu per dešinę šlauninę veną [226]. Bioptatai buvo paimti iš dešiniosios tarpskilvelinės pertvaros. Endomiokardo bioptatai buvo nedelsiant dedami ant ledo ir ištirti per 24 valandas. Mažiausiai trys bioptatai buvo ištirti histologiškai ir imunohistochemiškai, o du bioptatai kaip biologiniai mėginiai buvo saugomi –70 °C temperatūroje biobanke.

Trys endomiokardo biopsijos procedūros buvo nutrauktos dėl aritmijos ar dešiniojo skilvelio perforacijos, todėl dviem iš šių pacientų nebuvo atlikta imunohistocheminė analizė, o vienam pacientui – PGR analizė dėl biopsinės medžiagos trūkumo.

2.9. Histologinis ir imunohistocheminis endomiokardo biopsijų įvertinimas (I–III straipsniai)

Histologinei analizei skirti endomiokardo biopsijos mėginiai buvo fiksuoti 10 % buferiniame formaline ir vėliau parafinuoti audinių apdorojimo aparate. Tyrimo metu buvo naudojami 3 μm storio pjūviai.

Bioptatų pjūviai buvo dažomi hematoksilinu ir eozinu (H&E) pagal standartinį įprastinio histologinio vertinimo protokolą. Histologinė diagnozė nustatyta remiantis Dalaso kriterijais [26,227]. Patyręs patologas įvertino endokardą (storį, subendokardinius riebalus, fibrozės ir uždegimo požymius); miokardą (raumeninių skaidulų skaičių, dydį ir pažeidimą); intersticiją (fibrozę, riebalus, edemą ir uždegimą); intramuralines kraujagysles (dydį, uždegimo požymius, pažeidimą ir liuminalinę stenozę). Imunohistologinis endomiokardo bioptatų vertinimas atliktas taip, kaip aprašyta kitur [227].

Infiltracinės uždegiminės ląstelės miokarde nustatytos naudojant šiuos antikūnus: T limfocitų CD3 (Agilent DAKO, Hamburgas, Vokietija), aktyvios atminties T limfocitų CD45ro (Agilent DAKO, Hamburgas, Vokietija), makrofagų CD68 (Agilent DAKO, Hamburgas, Vokietija), T pagalbininkų CD4 (Agilent DAKO, Leica Biosystems, Niukaslas, Jungtinė Karalystė), viduląstelinės adhezijos molekulės 1 (angl. *Intercellular Adhesion Molecule 1* – ICAM-1) CD54 (Leica Biosystems, Niukaslas, Jungtinė Karalystė) ir didžiojo audinių suderinamumo komplekso (angl. *major histocompatibility complex* – MHC) II klasės ląstelių paviršiaus receptoriaus žmogaus leukocitų antigeno DR izotipą (ŽLA-DR) (Agilent DAKO, Hamburgas, Vokietija). Uždegiminių ląstelių infiltraciją vertino patyręs patologas ir išreiškė ląstelių skaičiumi viename kvadratiniame milimetre. Miokardo uždegimas buvo diagnozuotas pagal EKD miokardo ir perikardo ligų darbo grupės nustatytą kriterijų: imunohistochemiškai diagnozuotą židininę ar difuzinę uždegiminių ląstelių infiltraciją endomiokardo bioptate ($\geq$14 leukocitų/mm^2, bet iš jų ne daugiau nei 4 monocitai/mm^2 bei $\geq$7 ląstelės/mm^2 CD3 teigiamų T limfocitų) [13]. Uždegiminė endotelio aktyvacija buvo diagnozuota, jei atlikus imunohistocheminę analizę buvo nustatytos daugiau nei 3 ląstelės/mm^2, turinčios adhezijos molekules, t. y. ICAM-1 (CD54) ir (arba) ŽLA-DR [227].

2.10. Virusų genomų miokarde diagnozavimas

Genominės DNR ir visos RNR išskyrimui iš miokardo bioptatų buvo naudojamas „ZR-Duet DNA/RNA Miniprep“ rinkinys („Zymo Research“, Irvi-

nas, Kalifornija, JAV). Naudojant atsitiktinius heksamerus ir „First Strand cDNA Synthesis Kit“ („Thermo Fisher Scientific“, Vilnius, Lietuva), buvo atlikta atvirkštinė RNR (1 µg) transkribcija į 20 µl reakcijų tūrius ir po reakcijos atskiesta dejonizuotu vandeniu (iki 100 µl). Lizdinės PGR pradmenys buvo naudojami nustatant adenovirusą [228], 1 ir 2 *herpes simplex* virusus, *varicella-zoster* virusą (VZV), citomegalovirusą (CMV), parvovirusą B19 (B19V), Epšteino–Bar (*Epstein–Barr*) virusą (EBV), hepatito C virusą (HCV), enterovirusą (EV) ir raudonukės virusą (metodika aprašyta kitur [229]), taip pat žmogaus herpesvirusą 6 (HHV-6, GenBank prisijungimo Nr. NC001664.2 ir NC000898.1), Kirsteno žiurkių sarkomos viruso onkogeno homologą (KRAS, GenBank prisijungimo Nr. NM033360) bei ubikvitiną C (UBC, GenBank prisijungimo Nr. NM021009) (pradmenų sekos pateikiamos 1 lentelėje). Tiesioginiai pradmenys antrojo PGR etapo buvo pažymėti 6-karboksifluoresceinu FAM nuo 5′ galo. Visus pradmenis susintetino „Metabion Company“ (Martinsriedas, Vokietija).

1 lentelė. Žmogaus herpesviruso 6 (HHV6), Kirsteno žiurkių sarkomos viruso onkogeno homologo (KRAS) ir ubikvitino C (UBC) aptikimo pradmenys

Pradmuo	**Seka (5′–3′)**
Tiesioginis HHV6-N1	ACCCGAGAGATGATTTTGCGTG
Atvikštinisi HHV6-N1	GCAGAAGACAGCAGCGAGATAG
Tiesioginis HHV6-N2	CATAGCAACCTTTTCTAGCTTTGAC
Atvirkštinis HHV6-N2	TCTATAACATAAATGACCCCTGGGA
Tiesioginis UBC-N1	TTCTTTCCAGAGAGCCGAAC
Atvirkštinis UBC-N1	CCCATCTTCCAGCTGTTTTC
Tiesioginis UBC-N2	TGGGTCGCAGTTCTTGTTTG
Atvirkštinis UBC-N2	CCTTCCTTATCTTGGATCTTTGCC
Tiesioginis KRAS-N1	CTTTGGAGCAGGAACAATGTCT
Tiesioginis KRAS-N2	AATCCAGACTGTGTTTCTCCCT
Atvirkštinis KRAS-N1/N2	TACACAAAGAAAGCCCTCCCC

Visi PGR tyrimai buvo atliekami „TProfessional Standard“ („Biometra“, Getingenas, Vokietija) termocikleryje, kaip aprašyta Allard ir kt. publikacijoje [228]. KRAS ir UBC nustatymas buvo panaudotas nukleorūgščių ekstrakcijai validuoti ir buvo atliekamas lygiagrečiai atitinkama tvarka DNR ir RNR virusams. Galutiniai PGR produktai buvo praskiesti 10 kartų ir analizuojami kapiliarinės elektroforezės būdu analizatoriumi „Genetic Analyzer 3130xl“,

PGR dydžiams nustatyti naudojant „GeneScan 600 LIZ™ Size Standard“ ir „Gene Mapper Software v4.1“ („Applied Biosystems“, Foster City, CA, JAV). Esant teigiamiems rezultatams, buvo tiriami ir genominės DNR arba RNR mėginiai iš periferinio kraujo mėginių, kad būtų galima atmesti užterštumą.

2.11. Su uždegimu susijusių serologinių žymenų biocheminiai tyrimai

Plazmos mėginiai buvo laikomi –80 °C temperatūroje. Uždegiminiai serumo citokinai TNF-α ir IL-6 buvo matuojami kietafaziais chemiliuminescenciniais imunometriniais tyrimais naudojant IMMULITE/Immulite 1000 sistemas („Immulite“, „Siemens“) pagal gamintojų rekomendacijas: TNF-α (katalogo numeris LKNFZ (50 testų) ir LKNF1 (100 testų)), IL-6 (katalogo numeris LK6PZ (50 testų) ir LK6P1 (100 testų)) ir išreikšti pg/ml. Adiponektinas buvo matuojamas naudojant „Millipore Adiponectin“ testą pagal gamintojo rekomendacijas (Millipore, JAV) ir išreiškiamas mg/ml. Žmogaus tirpaus urokinazės tipo plazminogeno aktyvatoriaus receptoriaus (suPAR) lygis buvo įvertintas ELISA testu pagal gamintojo rekomendacijas (Abbexa). Absorbcija matuota 450 nm bangos ilgio spektrofotometru (Varioskan®Flash, Thermo Scientific). Galutinė suPAR koncentracija išreikšta ng/mg baltymo.

2.12. Pacientų stebėjimas

Pacientai buvo stebimi penkerius metus nuo įtraukimo į tyrimą. Tyrimo baigtis buvo sudėtinė, įtraukianti tris galimus kardiovaskulinius įvykius: mirtį dėl kardiovaskulinių priežasčių, KS pavaduojančio prietaiso (KSPP) implantavimą arba širdies transplantaciją. Šios baigtys buvo pasirinktos dėl to, kad visos jos atspindi tą pačią klinikinę ir patofiziologinę pažengusio širdies nepakankamumo būklę, kai daugiau nėra perspektyvių gydymo alternatyvų. Kai kurie iš šių pacientų patyrė kelis kardiovaskulinius įvykius, tačiau į analizę buvo įtraukta pirmojo iš jų data. Nepageidaujami kardiovaskulinius įvykiai buvo patvirtinti medicininiais įrašais, nacionalinio mirties registro įrašais arba pokalbiais telefonu su pacientų šeimomis.

2.13. Statistinė analizė

Pirmo–trečio (I–III) straipsnių duomenų analizė apėmė visą kohortą – 57 pacientus. Tačiau IV straipsnyje, skirtame GLS prognostinei vertei nustatyti,

tyrimo kohortą sudarė 41 pacientas, nes jų echokardiografiniai vaizdai buvo pakankamos kokybės tolesnei dvimatės miokardo deformacijos analizei atlikti.

Duomenų analizė atlikta naudojant R paketą (1.0.143, 3.5.1 ir 4.0.3 versijos) ir SPSS paketą (23.0 versija Windows; IBM.SPSS statistics). Statistiškai reikšminga buvo laikoma p reikšmė <0,05. Šapiro ir Vilko (*Shapiro–Wilk*) kriterijus buvo naudotas nustatyti tolydžiųjų kintamųjų reikšmių pasiskirstymui pagal normalųjį skirstinį. Normaliai pasiskirstę tolydieji kintamieji buvo išreikšti kaip vidurkis ± standartinis nuokrypis. Kiti tolydieji kintamieji buvo išreikšti mediana (25-asis procentilis, 75-asis procentilis), o kategoriniai duomenys – skaičiais ir procentais. Tolydieji kintamieji buvo lyginami taikant nepriklausomą Stjudento t-kriterijų, kai jie pasiskirstę normaliai, arba Mano ir Vitnio (*Mann–Whitney*) U testą, kai jie nepasiskirstę pagal normalųjį skirstinį. Kategorinių kintamųjų palyginimai tarp grupių atlikti naudojant chi kvadrato (χ^2) kriterijų arba Fišerio (*Fisher*) tikslųjį kriterijų, jei tikėtinos reikšmės buvo <5. Koreliacija tarp echokardiografinių ir hemodinaminių parametrų įvertinta naudojant Spirmeno (*Spearman*) koreliacijos koeficientą.

ROC analizė buvo naudojama siekiant nustatyti optimalią kiekvieno su uždegimu susijusio biožymens ir echokardiografinių parametrų prognozavimo ribinę vertę bei siekiant įvertinti šių žymenų tikslumą prognozuojant sudėtines klinikines bagtis. Skirtumai tarp plotų po kreive (AUC) buvo tikrinami taikant savirankos (*bootstrap*) metodą. Kaplano ir Mejerio (*Kaplan–Meier*) analizė buvo naudota siekiant palyginti kumuliacinį išgyvenamumą tarp NI-DKM sergančių pacientų pogrupių, stratifikuotų pagal kiekvieno su uždegimu susijusio biožymens, GLS ar KSIF ribines vertes arba pagal kardiotropinio viruso ar uždegimo buvimą miokarde. Skirtumų tarp kreivių statistiniam reikšmingumui įvertinti naudotas logranginis (*log-rank*) kriterijus.

Siekiant įvertinti, kurie pradiniai parametrai buvo susiję su blogomis sudėtinėmis baigtimis, buvo atlikta Kokso (*Cox*) proporcingos rizikos regresijos analizė. Pirmiausia atlikome vienlypę Kokso regresijos analizę visiems pradiniams kintamiesiems. Tada tie kintamieji, kurių p-reikšmė buvo <0,1 vienalypėje analizėje, buvo įtraukti į daugialypę Kokso regresijos analizę, kuri buvo atlikta taikant laipsnišką grįžtamąją eliminaciją.

Svarbu pažymėti, kad su uždegimo žymenys nebuvo įtraukti į IV straipsnio statistinę analizę, GLS – į II straipsnio statistinę analizę. Todėl atliekant daugialypę Kokso regresijos analizę II ir IV straipsniuose nebuvo vertinama atitinkamai GLS ir uždegimo žymenų prognostinė reikšmė.

3. REZULTATAI

3.1. Pagrindinės pacientų charakteristikos (I–IV straipsniai)

Tyrime dalyvavo 57 pacientai (vidutinis amžius 47,3 ± 10,9 metų; 45 (79 %) vyrai), sergantys NI-DKM. Vidutinis KSGDD buvo 6,8 ± 0,9 cm, vidutinė KSIF – 26,08 ± 9,5 %, vidutinis PKPS – 21,8 ± 8,9 mm Hg. 41 (72 %) pacientas priklausė III NYHA funkcinei klasei, o 10 (17 %) – IV NYHA funkcinei klasei. Įtraukimo į tyrimą metu pacientų vidutinis sistolinis kraujospūdis buvo 116 ± 20 mm Hg, o diastolinis – 80 ± 10 mm Hg. GLS buvo sumažėjęs (–8,1 ± 3,7 %). Širdies nepakankamumo trukmės mediana (25 procentilis, 75 procentilis) buvo 12 (5, 60) mėnesių.

3.2. Širdies uždegimo ir kardiotropinių virusų paplitimas miokarde (I straipsnis)

3.2.1. Miokardo uždegimo paplitimas (I straipsnis)

Imunohistocheminės analizės būdu buvo įvertinti 55 pacientų endomiokardo bioptatai. Miokardo uždegimas nustatytas 31 iš 55 (54 %) NI-DKM pacientų. Pacientai buvo suskirstyti į dvi grupes: uždegiminės dilatacinės kardiomiopatijos (uDKM) ir neuždegiminės dilatacinės kardiomiopatijos (ne-uDKM). Abiejų grupių aprašomosios charakteristikos nesiskyrė ($p > 0,05$), išskyrus mažesnį sistolinį kraujospūdį ir didesnę BNP koncentraciją uDKM grupėje ($p \leq 0,05$).

Uždegiminė endotelio aktyvacija (padidėjusi ŽLA-DR ir ICAM-1 [≥ 3 ląstelės/mm^2] raiška) imunohistocheminiu būdu nustatyta 50 (91 %) pacientų. Tačiau šių žymenų raiška nesiskyrė tarp uDKM ir ne-uDKM grupių ($p > 0,05$). Vis dėlto endotelio aktyvacijos žymenis reikėtų interpretuoti atsargiai, nes šie baltymai aptinkami ne tik ant endotelio, bet ir ant imuninių ląstelių paviršiaus.

3.2.2. Kardiotropinių virusų paplitimas

Atliekant polimerazės grandininės reakcijos tyrimą buvo analizuojami 56 pacientų bioptatai. Virusų genomai aptikti 29 (52 %) iš 56 NI-DKM sergančių pacientų miokarde. Iš šių 29 pacientų 25 (86 %) buvo nustatytas B19V genomas, o 5 (17 %) – HHV6 genomas. Kiti virusai (VZV, CMV, EBV, HCV, EV) aptikti pavieniais atvejais (n = 1 [3 %] kiekvieno tipo). Trims pacientams (10 %) nustatyta dviguba infekcija, o vienam (3 %) – triguba infekcija (vy-

ravo B19V ir HHV6 derinys (n = 3 [10 %]). Likusiems 27 (48 %) pacientams virusinio genomo miokarde nebuvo aptikta. Daugelis aprašomųjų charakteristikų nesiskyrė tarp pacientų, kuriems buvo ir nebuvo aptiktas virusinis genomas ($p > 0,05$), išskyrus didesnę BNP ir IL-6 koncentraciją, blogesnius hemodinamikos parametrus ir didesnį uždegiminių CD68+ ląstelių skaičių miokarde pacientams, kurių miokarde viruso neaptikta.

3.2.3. Idiopatinės NI-DKM pogrupiai (I straipsnis)

PGR ir imunohistocheminė analizė buvo atlikti 54 NI-DKM pacientams. Remiantis virusinio genomo aptikimu – kartu su teigiama arba neigiama imunohistochemija – buvo išskirti keturi pacientų pogrupiai:

- virusinio (lėtinio) miokardito (15 (28 %) pacientų): diagnozuotas ir kardiotropinis virusas, ir miokardo uždegimas;
- autoreaktyvios uždegiminės dilatacinės kardiomiopatijos (16 (30 %) pacientų): kardiotropinio viruso neaptikta, tačiau diagnozuotas miokardo uždegimas;
- virusinės dilatacinės kardiomiopatijos (14 (26 %) pacientų): aptiktas virusinis genomas, tačiau miokardo uždegimo požymių nenustatyta;
- neuždegiminės dilatacinės kardiomiopatijos (9 (17 %) pacientų): neaptikta nei viruso genomo, nei uždegimo miokarde.

3.3. Uždegimo žymenų ir GLS ryšys su širdies nepakankamumo sunkumu (II–IV straipsniai)

3.3.1. Ryšys tarp uždegimo žymenų ir širdies nepakankamumo (II–III straipsniai)

Įvertinome sąsajas tarp uždegimo žymenų ir parametrų, atspindinčių širdies nepakankamumo sunkumą. Leukocitai kraujyje, suPAR, uždegiminės infiltracinės CD68+, CD4+, CD54+ ir ŽLA-DR+ ląstelės nekoreliavo nė su vienu iš širdies nepakankamumo sunkumo parametrų. Silpna, bet reikšminga teigiama koreliacija nustatyta tarp uždegiminių infiltracinių CD3+ ir CD45ro+ ląstelių ir BNP koncentracijos, o dj-CRB reikšmingai, bet silpnai koreliavo su KSIF ir BNP. Be to, IL-6, TNF-α ir adiponektino koncentracija didėjo priklausomai nuo NYHA funkcinės klasės ir koreliavo su visais širdies nepakankamumo sunkumo rodikliais (KSIF, vidutiniu PAS, PKPS ir BNP), išskyrus TNF-α, kuris nekoreliavo su PKPS. Galiausiai IL-6 ir adiponektino koncentracija stipriausiai koreliavo su širdies nepakankamumo sunkumo parametrais, ypač su BNP.

Taip pat įvertinome sąsajas tarp miokardo uždegiminių ląstelių ir uždegimo žymenų serume. Tačiau uždegiminės infiltracinės ląstelės nekoreliavo su sisteminio uždegimo žymenimis, išskyrus vidutinę koreliaciją tarp CD68+ ląstelių/mm^2 ir IL-6.

3.3.2. Ryšys tarp GLS ir širdies nepakankamumo sunkumo parametrų (IV straipsnis)

Įvertinome GLS ir kitų įprastinių echokardiografinių parametrų ryšį su invaziniu būdu nustatytais hemodinamikos rodikliais. GLS, taip pat vidutinis E/e', TV nesandarumo greitis, KPTi ir KSIF reikšmingai koreliavo su invaziniais hemodinamikos rodikliais (2 lentelė). Taip pat įvertinome koreliacijas tarp GLS ir serumo žymenų (BNP ir troponinino T). GLS stipriai koreliavo su BNP (r = 0,66, p < 0,001), bet ne su troponinu T.

2 lentelė. Koreliacijos tarp echokardiografinių ir invazinių hemodinamikos parametrų

	PKPS, mmHg		**Vidutinis PAS, mmHg**		**PKP, Vudo vienetai**		**ŠI, l/min/m^2**	
	r	p	r	P	r	p	r	p
DT, ms	-0,13	0,42	-0,17	0,28	**-0,4**	0,01	0,21	0,21
E/A	0,22	0,21	**0,38**	0,03	**0,62**	<0,0001	**-0,55**	<0,01
Vidutinis E/e‘	**0,37**	0,02	**0,36**	0,02	**0,31**	0,048	-0,02	0,92
TV nesandarumo greitis, m/s	**0,54**	<0,001	**0,59**	<0,0001	**0,5**	<0,001	-0,19	0,24
KPTi, ml/m^2	**0,40**	<0,01	**0,47**	<0,01	**0,6**	<0,0001	**-0,45**	<0,01
KSIF, %	**-0,38**	0,01	**-0,41**	<0,01	**-0,45**	<0,01	0,26	0,11
GLS, %	**0,38**	0,01	**0,35**	0,02	**0,46**	<0,01	-0,31	0,06

Paryškintas šriftas reiškia statistinį reikšmingumą (p < 0,05).
Santrumpos: DT – deceleracijos laikas, E/A – santykis kairiojo skilvelio ankstyvojo prisipildymo greičio (E) ir maksimalaus tėkmės per mitralinį vožtuvą greičio susitraukiant prieširdžiams, E/e' – kairiojo skilvelio ankstyvojo prisipildymo greičio (E) ir ankstyvojo mitralinio žiedo diastolinio greičio (e') santykis, GLS – bendra išilginė KS miokardo deformacija, KPTi – indeksuotas kairiojo prieširdžio tūris, KSIF – kairiojo skilvelio išstūmimo frakcija, PAS – plaučių arterijos spaudimas, PKP – plaučių kraujagyslių pasipriešinimas, PKPS – plaučių kapiliarų pleištinis spaudimas, TV – triburis vožtuvas.

3.4. Klinikinės baigtys (I, II, IV straipsniai)

Iš 57 pacientų 27 (47 %) per penkerių metų stebėjimo laikotarpį patyrė bent vieną iš sudėtinių klinikinių baigčių:

- 10 (18 %) pacientų mirė;
- 9 (16 %) pacientams buvo atlikta širdies transplantacija (skubos būklė pagal širdies paskirstymo politiką (angl. *Heart allocation policy*) [230]: 6 pacientai atitiko I-III kategoriją, o 3 pacientai – IV kategoriją);
- 8 (14 %) pacientams buvo implantuotas KSPP (7 pacientai turėjo 1–3 INTERMACS profilį [231], 1 pacientas – 4 profilį).

Kitiems pacientams toliau buvo taikomas įprastinis medikamentinis širdies nepakankamumo gydymas. Kumuliacinis penkerių metų išgyvenamumo rodiklis buvo 53 %.

Remdamiesi šiais rezultatais, kohortą suskirstėme į dvi grupes: blogų baigčių (n=27) ir gerų baigčių (n=30). Pacientus, priklausančius blogų baigčių grupei, širdies nepakankamumo simptomai iki įtraukimo į tyrimą vargino ilgiau, nei pacientus, priklausančius gerų baigčių grupei, tačiau šis skirtumas nebuvo statistiškai reikšmingas. Blogų baigčių grupės pacientams buvo statistiškai reikšmingai mažesnis sistolinis kraujospūdis, blogesnė KS sistolinė ir diastolinė funkcija, didesnis GLS ir labiau išsiplėtęs dešinysis skilvelis. Be to, blogų baigčių grupėje buvo didesnė BNP ir uždegimo žymenų (IL-6, TNF-α ir adiponektino) koncentracija, negu gerų baigčių pacientų grupėje.

3.4.1. Kardiotropinių virusų ir miokardo uždegimo prognostinė vertė (I straipsnis)

Kaplano ir Mejerio išgyvenamumo kreivės parodė, kad viruso genomo aptikimas miokarde nėra susijęs su blogomis baigtimis ($p > 0,05$). Taip pat uDKM diagnozė nebuvo susijusi su blogomis baigtimis ($p > 0,05$).

3.4.2. Uždegimo žymenų prognostinė vertė (II straipsnis)

Atlikome ROC analizę, siekdami įvertinti uždegimo žymenų prognostinį tikslumą bei nustatyti šių žymenų optimalią ribinę vertę, leidžiančią geriausiai prognozuoti pacientų baigtis (3 lentelė). Tiksliausiai blogas baigtis prognozavo IL-6, TNF-α ir adiponektinas (AUC atitinkamai 0,77, 0,65 ir 0,70).

3 lentelė. Blogas baigtis prognozuojančių uždegimo žymenų ribinės vertės, nustatytos atlikus ROC analizę

	Ribinė vertė	Specifiškumas, %	Jautrumas, %	AUC (95 % PI)
Leukocitai $\times 10^9$/l	10,66	32	88	0,56 (0,39–0,72)
dj-CRB, mg/l	4,62	65	65	0,61 (0,44–0,77)
IL-6, pg/ml	4,53	83	64	**0,77 (0,64–0,89)**
TNF-α, pg/ml	7,81	53	80	**0,65 (0,50–0,79)**
Adiponektinas, µg/ml	17,14	73	60	**0,70 (0,55–0,73)**
suPAR, ng/mg baltymo	0,85	80	35	0,51 (0,34–0,69)
CD3+, ląstelės/mm^2	13	75	41	0,59 (0,43–0,74)
CD45ro+, ląstelės/mm^2	11,5	89	26	0,53 (0,37–0,68)
CD68+, ląstelės/mm^2	9	96	11	0,51 (0,36–0,66)
CD54+, ląstelės/mm^2	2,5	93	7	0,43 (0,29–0,56)
CD4+, ląstelės/mm^2	3,5	61	56	0,53 (0,37–0,69)
ŽLA-DR+, ląstelės/mm^2	5,5	68	41	0,53 (0,38–0,68)

Paryškintas šriftas reiškia statistinį reikšmingumą ($p < 0,05$). Santrumpos: AUC – plotas po kreive, CD3+ – T limfocitų receptorius, CD4+ – T pagalbinkų receptorius, CD45ro+ – atminties T ląstelių receptorius, CD68+ – monocitų / makrofagų receptorius, dj-CRB – didelio jautrumo C reaktyvusis baltymas, ICAM-1/CD54+ – viduląstelinės adhezijos molekulės, IL-6 – interleukinas 6, PI – pasikliautiniai intervalai, suPAR – tirpus urokinazės plazminogeno aktyvavimo receptorius, TNF-α – naviko nekrozės faktorius α.

Tada atlikome Kaplano ir Mejerio analizę, siekdami įvertinti išgyvenamumo skirtumus tarp pacientų, suskirstytų į grupes pagal gautas ribines vertes. Didesnės uždegimo žymenų koncentracijos serume (dj-CRB >4,6 µg/ml, IL-6 >4,53 pg/ml, TNF-α >7,81 pg/ml, adiponektinas >17,14 g/ml) ir didesnis uždegiminių ląstelių skaičius miokarde (CD3+ >13 ląstelių/mm^2 ir CD45ro+ >11,5 ląstelių/mm^2) buvo susijęs su blogesnėmis klinikinėmis baigtimis ($p < 0,05$). Kiti uždegimo žymenys nebuvo susiję su blogomis baigtimis.

Vienalypė Kokso regresinė analizė parodė, kad sistolinis ir diastolinis kraujospūdis, echokardiografiniai parametrai (KSIF, DS galinis diastolinis diametras, sunki DS sistolinė disfunkcija), invaziniai hemodinamikos rodikliai (vidutinis PAS, PKPS), uždegimo žymenys (dj-CRB >4,62 µg/ml, IL-6 >4,53 pg/ml, TNF-α >7,81 pg/ml ir adiponektinas >17,14 g/ml), taip pat uždegiminės ląstelės miokarde (CD3+ >13 ląstelių/mm^2 ir CD45ro+ >11,5 ląstelių/mm^2) prognozavo blogas klinikines baigtis. Rodikliai ir žymenys, kurių p-reikšmė buvo <0,1 vienalypėje Kokso regresinėje analizėje, buvo įtraukti į tolesnę daugialypę Kokso regresinę analizę. Daugialypė Kokso regresi-

nė analizė parodė, kad IL-6 >4,53 pg/ml (HR 6,26 (PI 95 % 2,52–15,58), p < 0,0001), adiponektinas >17,14 g/ml (5,43 (1,01–10,33), p < 0,001), dj-troponinas T (HR 1,005 (PI 95 % 1,0–1,01), p = 0,04), CD45ro+ >11,5 ląstelių/mm^2 (HR 3,23 (PI 95 % 1,01–10,33), p = 0,048) ir diastolinis kraujospūdis (HR 0,95 (PI 95 % 0,91–0,99), p = 0,01) gali nepriklausomai prognozuoti blogas klinines baigtis.

3.4.2.1. Kombinuota uždegiminių citokinų prognostinė vertė (II straipsnis)

Tolesnė Kaplano ir Mejerio analizė buvo atlikta su trimis citokinais, nes jie tiksliausiai prognozavo blogas klinikines baigtis. Visi pacientai (n = 12), kurių IL-6 <4,53 pg/ml, adiponektinas <17,14 g/ml ir TNF-α <7,81 pg/ml, išgyveno (100 %) visą penkerių metų stebėjimo laikotarpį, o visi pacientai (n = 10), kurių šių trijų citokinų koncentracija viršijo ribines vertes, patyrė bent vieną iš sudėtinių baigčių. Pacientų, kurių vieno ar dviejų citokinų koncentracija viršijo ribinę vertę, penkerių metų išgyvenamumas buvo panašus: atitinkamai 50 % ir 40 %.

3.4.3. GLS prognostinė vertė (IV straipsnis)

Atlikome ROC analizę, siekdami nustatyti GLS tikslumą prognozuojant blogas klinikines baigtis. Palyginimui su GLS pasirinkome KSIF – klinikinėje praktikoje plačiausiai naudojamą prognostinį echokardiografinį parametrą. ROC analizė parodė, kad GLS (AUC 0,81 (PI 95 % 0,65–0,94)) ir KSIF (AUC 0,77 (PI 95 % 0,61–0,91)) prognozuoja blogas klinikines baigtis penkerių metų stebėjimo laikotarpiu. Tačiau skirtumai tarp GLS ir KSIF kreivių nebuvo reikšmingi. Be to, analizės metu nustatytos optimalios ribinės vertės: –5,96 % GLS (60 % jautrumas, 100 % specifiškumas) ir 24,5 % KSIF (80 % jautrumas, 81 % specifiškumas).

Atlikome Kaplano ir Mejerio analizę siekdami įvertinti GLS ir KSIF įtaką išgyvenamumui. Išgyvenamumo analizė parodė, kad pacientų, kurių GLS reikšmės buvo didesnės už ribinę vertę, o KSIF mažesnės už ją, išgyvenamumas buvo reikšmingai mažesnis (p < 0,001) per penkerius stebėjimo metus. Iš grafiko matyti, kad daugiau nei pusė pacientų, kurių GLS vertė viršijo ribinę vertę, per pirmuosius stebėjimo metus patyrė sudėtines baigtis. Be to, vienalypė Kokso regresinė analizė parodė, kad GLS reikšmei viršijant ribinę vertę rizika patirti sudėtinę baigtį yra 12 kartų (HR 12,47; 95 % PI 4,6–33,82, p < 0,0001) didesnė penkerių metų laikotarpiu.

Atsižvelgiant į tai, kad KSIF yra gerai žinomas prognostinis žymuo, buvo atlikta išgyvenamumo analizė siekiant įvertinti, ar GLS turi papildomą prognostinę vertę pacientams, stratifikuotiems pagal KSIF ribinę vertę. Pacientams, kurių KSIF reikšmės buvo didesnės už ribinę vertę, GLS neturėjo papildomos vertės stratifikuojant pacientus ($p > 0,05$): visų pacientų GLS reikšmės buvo mažesnės už ribinę vertę. Tačiau pacientų, kurių KSIF vertė buvo mažesnė už ribinę vertę, GLS papildomai stratifikavo pacientų riziką. Pacientų, kurių GLS buvo didesnė už ribinę vertę (>–5,96 %), sudėtinių baigčių dažnis labai sumažėjusios KSIF grupėje buvo reikšmingai didesnis.

Paskui atlikome vienalypę Kokso regresinę analizę, kad įvertintume GLS, kaip tolydaus kintamojo, prognostinę reikšmę. Į analizę buvo įtraukti visi aprašomosios charakteristikos kintamieji. Analizė parodė, kad GLS, KSIF, BNP, dj-troponino T, invaziniai hemodinamikos parametrai ir dešiniojo skilvelio padidėjimas buvo susiję su sudėtinėmis baigtimis penkerių metų stebėjimo laikotarpiu. GLS padidėjimas 1 procentu buvo susijęs su 41 procentu didesne sudėtinių baigčių rizika.

Kintamieji, kurie reikšmingai susiję su blogomis klinikinėmis baigtimis vienalypėje Kokso regresinėje analizėje, buvo įtraukti į daugialypę Kokso regresinę analizę, kuri buvo atlikta taikant laipsnišką grįžtamosios eliminacijos metodą. Daugialypėje Kokso regresinėje analizėje GLS išliko reikšmingas prognostinis žymuo (koreguotas HR 1,25 (95 % PI 1,01–1,55); $p = 0,04$).

4. IŠVADOS

1. Šis tyrimas parodė, kad NI-DKM pacientams dažnai aptinkama kardiotropinių virusų miokarde ir diagnozuojama uždegiminė dilatacinė kardiomiopatija. Kardiotropiniai virusai ir miokardo uždegimas taip pat padeda nustatyti NI-DKM pogrupius. (I straipsnis)
2. Kardiotropinių virusų aptikimas miokarde ir uždegiminės kardiomiopatijos diagnozė nebuvo susiję su blogesnėmis NI-DKM pacientų klinikinėmis baigtimis. (I straipsnis)
3. Didesnė T ląstelių (CD3+) ir atminties T ląstelių (CD45ro+) infiltracija miokarde leido numatyti blogesnes penkerių metų ligos baigtis pacientams, sergantiems NI-DKMP. (I–II straipsniai)
4. Didesnės CRB, IL-6, TNF-α ir adiponektino koncentracijos tyrimo pradžioje buvo susijusios su blogesne ligos baigtimi pacientams, sergantiems

NI-DKM. Padidėjusios visų trijų uždegiminių citokinų – serumo IL-6, TNF-α ir adiponektino – koncentracijos galėtų būti papildomas prognozės žymuo atrenkant NI-DKM sergančius pacientus, turinčius blogiausią prognozę ir širdies transplantacijos būtinybę. (II–III straipsniai)

5. Blogėjanti bendra išilginė deformacija susijusi su blogomis klinikinėmis baigtimis ir galėtų padėti nustatyti didelės rizikos pacientus, sergančius NI-DKM. (IV straipsnis)

5. PRAKTINĖS REKOMENDACIJOS

6. Labai sutrikusi bendra išilginė KS miokardo deformacija (GLS >–5,96 %) ir padidėjusi visų trijų uždegiminių citokinų (IL-6 <4,53 pg/ml, adiponektino <17,14 g/ml ir TNF <7,81 pg/ml) koncentracija gali būti papildomi žymenys, padedantys nustatyti didelės rizikos NI-DKM pacientus, kuriems reikalinga širdies transplantacija.
7. Tolesnis žingsnis būtų atlikti didesnės imties tyrimą, kuriuo būtų galima patikrinti šio tyrimo rezultatus. Be to, didesnės imties tyrimas leistų atlikti išsamesnę pogrupių analizę: tyrimą, kuriame būtų lyginami keturi skirtingi etiopatogenetiniai pogrupiai, arba tyrimą, kuriame būtų analizuojami NI-DKM sergantys pacientai, kurių ligos sunkumas skiriasi (pvz. skirtingos NYHA klasės) arba kuriems reikalinga širdies transplantacija ar pagalbinės mechaninės asistuojančios kraujotakos priemonės (pvz. skirtingas INTERMACS profilis ar širdies transplantacijos skubos statusas).

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ABOUT THE AUTHOR

Ieva Kažukauskienė obtained a diploma of medicine doctor at Vilnius University in 2012. In 2016 she completed her cardiology residency at Vilnius University and continued working as a cardiologist at Vilnius University Hospital Santaros Klinikos. In 2017 she entered the doctoral study program at Vilnius University. During her Ph.D. studies, she started working as a lecturer at Vilnius University, Faculty of Medicine, Department of Pathology, Forensic Medicine and Pharmacology.

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LIST OF PUBLICATIONS NOT INCLUDED IN THE THESIS

1. Bironaitė D, **Kažukauskienė I**, Bogomolovas J, Daunoravičius D, Jakubauskas A, Vitkus D, Žurauskas E, Ručinskas K, Labeit S, Grabauskienė V. *Molecular Mechanisms behind Persistent Presence of Parvovirus B19 in Human Dilated Myocardium.* Advances in experimental medicine and biology, 10.1007/5584_2021_702. 14 Jan. 2022. https://doi.org/10.1007/5584_2021_702
2. Baltrūnienė V, Rinkūnaitė I, Bogomolovas J, Bironaitė D, **Kažukauskienė I,** Šimoliūnas E, Ručinskas K, Puronaitė R, Bukelskienė V, Grabauskienė V. *The role of cardiac T-cadherin in the indicating heart failure severity of patients with non-ischemic dilated cardiomyopathy*. Medicina. Kaunas; Basel: LSMU, MDPI AG. ISSN 1010-660X. eISSN 1648-9144. 2020, vol. 56, no. 1, art. no 27, p. [1-14]. DOI: 10.3390/medicina56010027. https://doi.org/10.3390/medicina56010027
3. Bironaitė D, Daunoravičius D, Bogomolovas J, Čibiras S, Vitkus D, Žurauskas E, **Žąsytytė I**, Ručinskas K, Labeit S, Venalis A, Grabauskienė V. *Molecular mechanisms behind progressing chronic inflammatory dilated cardiomyopathy.* BMC Cardiovascular Disorders (2015) 15:26. ISSN 1471-2261. https://doi.org/10.1186/s12872-015-0017-1

LIST OF PRESENTATIONS

1. **Kažukauskienė I**, Baltrūnienė V, Besusparis J, Daunoravicius D, Balčiūnaitė G, Žurauskas E, Maneikienė V.V, Grabauskienė V. *Left ventricle global longitudinal strain is associated with myocardial inflammation and fibrosis.* European Journal of Heart Failure (2021) 23 (Suppl. S2) 111. doi:10.1002/ejhf.2297. E-poster presentation. Heart Failure 2021 and the World Congress on Acute Heart Failure Congress online June 29 – July 1, 2021. https://doi.org/10.1002/ejhf.2297
2. **Kažukauskienė I,** Baltrūnienė V, Bironaitė D, Čibiras S.V, Ručinskas K, Grabauskienė V. *Prognostic significance of carboxy-terminal telopeptide (ICTP) and Caspase-3 in patients with non-ischemic dilated cardiomyopathy* // European heart journal: vol. 40, suppl. 1: ESC Congress 2019 together with World Congress of Cardiology 31 August – 4 September 2019, Paris - France. Oxford : Oxford University Press. ISSN 0195-668X.

eISSN 1522-9645. 2019, vol. 40, suppl. 1, art. no. P5447, p. 3331. DOI: 10.1093/eurheartj/ehz746.0403. Poster presentation. Paris, France. https://doi.org/10.1093/eurheartj/ehz746.0403

3. **Kažukauskienė I,** Balčiūnaitė G, Baltrūnienė V, Ručinskas K, Maneikienė V.V, Grabauskienė V. *Left ventricle global longitudinal strain is an independent predictor of poor outcome in patients with nonischemic dilated cardiomyopathy* // European heart journal cardiovascular imaging. Oxford: Oxford University Press. ISSN 2047-2404. eISSN 2047-2412. 2019, vol. 20, suppl. 1, i627. DOI: 10.1093/ehjci/jey267. Poster presentation. EuroEcho-Imaging 2018 Congress, Milan, Italy, December 5-8, 2018. https://doi.org/10.1093/ehjci/jey267
4. Baltrūnienė V, Bironaitė D, **Kažukauskienė I,** Augulis R, Grabauskienė V. *Serum Adiponectin Level as an Independent Predictor of Mortality in Patients with Inflammatory Cardiomyopathy and Advanced Heart Failure.* Oral presentation. Baltic Conference Series, Stokholm, Sweeden, October 8-11, 2017.
5. **Kažukauskienė I,** Baltrūnienė V, Čelutkienė J, Grabauskienė V. *Pulmonary hypertension is associated with readmission due to heart failure in patients with non-ishemic dilated cardiomyopathy*. Oral presentation. The 3rd. International Conference of Evolutionary Medicine: Pre-existing Mechanisms and Patterns of Current Health Issues. ISBN 978-609-459-720-6, p. 93. Vilnius, Lithuania, June 14-19, 2016.
6. Baltrūnienė V, **Žąsytytė I,** Bironaitė D, Grabauskienė V. *Adiponectin can predict mortality and readmission for heart failure.* Poster presentation. The 8th Baltic Morphology Conference: Interdisciplinary Nature of Contemporary Morphology. P.76. Vilnius, Lithuania, November 12-14, 2015.
7. Bironaitė D, Baltrūnienė V, **Žąsytytė I,** Grabauskienė V. *The role of collagen and adiponectin in virus induced dilated cardiomyopathy.* Poster presentation. The 8th Baltic Morphology Conference: Interdisciplinary Nature of Contemporary Morphology. P.79; Vilnius, Lithuania, November 12-14, 2015.
8. **Žąsytytė I,** Vitkus D, Žurauskas E, Bironaitė D, Ručinskas K, Čelutkienė J, Karčiauskaitė D, Grabauskienė V. *Prognostic biomarkers of mortality in patients with non-ischemic dilated cardiomyopathy.* // European Journal of Heart Failure (2015) 17 (Suppl. S1) P181, doi: 10.1002/ejhf.277. Poster presentation. Heart Failure 2015 and the 2nd World Congress on Acute Heart Failure, Seville, Spain, May 23-26, 2015.

9. Bironaitė D, Daunoravičius D, Bogomolovas J, Jakubauskas A, Vitkus D, Žurauskas E, **Žąsytytė I** Ručinskas K, Venalis A, Grabauskienė V. *The role of collagen in virus-positive dilated cardiomyopathy.* Global heart. 2014, vol. 9, iss. 1, suppl. ISSN 2211-8160 p. e71. doi: 10.1016/j.gheart.2014.03.1461. Poster presentation. World Congress of Cardiology, Melbourne, Australia, May 5-7, 2014. https://doi.org/10.1016/j.gheart.2014.03.1461
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12. Bironaitė D, Daunoravičius D, Bogomolovas J, Čibiras S.V, Vitkus D, Žurauskas E, **Žąsytytė I,** Labeit S, Venalis A, Grabauskienė V. *Mechanism of fibrosis in inflammatory dilated cardiomyopathy.* Journal of cardiac failure. Philadelphia: Churchill Livingstone. ISSN 1071-9164. 2014, vol. 80, no. 8, suppl, p. s81. https://doi.org/10.1016/j.cardfail.2014.06.229

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Prevalence and prognostic relevance of myocardial inflammation and cardiotropic viruses in non-ischemic dilated cardiomyopathy

Ieva Kažukauskienė[1], Vaida Baltrūnienė[1], Artūras Jakubauskas[2], Edvardas Žurauskas[1], Vytė Valerija Maneikienė[3], Dainius Daunoravičius[4], Jelena Čelutkienė[3], Kęstutis Ručinskas[3], Virginija Grabauskienė[1, 3]

[1]Department of Pathology, Forensic Medicine and Pharmacology, Faculty of Medicine, Vilnius University, Vilnius, Lithuania
[2]Hematology, Oncology and Transfusion Medicine Center, Vilnius University Hospital Santaros Klinikos, Vilnius, Lithuania
[3]Clinic of Cardiac and Vascular Diseases, Institute of Clinical Medicine, Faculty of Medicine, Vilnius University, Vilnius, Lithuania
[4]Clinicus Vilnius, Lithuania

Abstract

Background: *Non-ischemic dilated cardiomyopathy (DCM) is a heterogeneous disease with a spectrum of etiological factors. However, subsets of the disease are not well-characterized with respect to these factors. The aim of this study was to evaluate the prevalence of myocardial inflammation and cardiotropic viruses in DCM patients and their impact on clinical outcome.*

Methods: *Fifty-seven patients with DCM underwent endomyocardial biopsy between 2010 and 2013. Biopsies were analyzed by polymerase chain reaction (PCR) for the presence of cardiotropic viruses, and inflammatory cell infiltration was assessed by immunohistochemistry. During a 5-year follow-up, 27 (47%) patients reached the composite outcome measure: heart transplantation, left ventricle assist device implantation or cardiovascular-related death.*

Results: *Thirty-one (54%) patients had myocardial inflammation and cardiotropic viruses were detected in 29 (52%). The most frequent viruses were parvovirus B19 and human herpesvirus type-6. Four specific sub-groups were distinguished by PCR and immunohistochemistry: virus-positive (chronic) myocarditis, autoreactive inflammatory DCM, viral DCM, non-inflammatory DCM. The presence of a viral genome in myocardium or diagnosis of inflammatory DCM did not predict the outcome of composite outcome measures ($p > 0.05$). However, univariate Cox regression and survival function estimation revealed an association between inflammation by a high number of T-cells and poor prognosis.*

Conclusions: *This study has shown that two markers — cardiotropic viruses and myocardial inflammation — are prevalent among DCM patients. They are also helpful in identifying sub-groups of DCM. An increased number of T-lymphocytes in the myocardium is a predictor of poor mid-term and long-term prognosis.* (Cardiol J)

Key words: dilated cardiomyopathy, chronic heart failure, myocardial inflammation, viruses, prognosis

Address for correspondence: Ieva Kažukauskienė, MD, Department of Pathology, Forensic Medicine and Pharmacology, Faculty of Medicine, Vilnius University, M. K. Ciurlionio 21, LT-03101 Vilnius, Lithuania, tel: +37052398728, e-mail: ieva.zasytyte@gmail.com

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Introduction

Non-ischemic dilated cardiomyopathy (DCM) is a chronic heart disease. It presents with left ventricle (LV) dilatation and impaired ventricle function (left or both ventricles), which is not caused by coronary artery disease or abnormal loading conditions [1]. DCM is a heterogeneous disease with a spectrum of etiologic factors such as infectious agents, genetic abnormalities, autoimmune mechanisms, drugs, and toxins [2]. DCM causes heart failure, leading to heart transplantation or death [3].

Over the past few decades, the definition of DCM has developed [1, 4–6]. Endomyocardial biopsy, analyzed by immunohistochemistry and viral polymerase chain reaction (PCR), became an essential procedure for diagnosing the cause of DCM [1, 7, 8]. Consequently, cardiotropic viruses are recognized as a crucial etiologic factor of heart failure and are found in the myocardium of up to 67% of DCM patients [10, 11]. The data concerning the impact of the presence of cardiotropic viruses on clinical significance and prognosis remains under debate [12, 13].

Diagnostic criteria for inflammation in the myocardium were updated several times [1, 4, 5, 9]. Myocardial inflammation, confirmed by endomyocardial biopsy, is also known as a significant causal factor, and is responsible for progression of LV dilatation [14–17]. However, the prognostic role of myocardial inflammation on clinical outcome varies in different studies due to diverse diagnostic criteria [12, 18–21]. The latest definition of myocardial inflammation was endorsed by the European Society of Cardiology (ESC) Working Group on Myocardial and Pericardial Diseases [1]. However, there is a shortage of data, which demonstrate the prognostic relevance of myocardial inflammation defined by this criterion. In addition, immunohistochemistry and viral PCR are used to characterize etiopathogenetic subsets of DCM patients, but prospective data are lacking for these subsets. Thus, the clinical value of research of etiopathogenetic factors may be of paramount importance to prognosis assessment and may help to further the development of treatment strategies.

The aim of this study was to use immunohistochemistry and PCR — to evaluate the prevalence of myocardial inflammation and cardiotropic viruses — in DCM patients. Further, to investigate their impact on the clinical outcome; and to clarify the impact of different myocardial inflammatory cells on mid-term and long-term prognosis.

Methods

Study population. Inclusion and exclusion criteria

A prospective cohort study was done in our center between January 2010 and December 2013. 57 patients admitted to this institution with heart failure and reduced LV ejection fraction (LVEF) (with unknown etiology of LV dilatation) for diagnostic evaluation were enrolled. Inclusion criteria were clinical signs and symptoms of heart failure, accompanied by echocardiographic evidence of LV dilatation (LV end-diastolic diameter [LVEDD] > 117% of the predicted value, corrected for age and body surface area [> 2 standard deviations] of the predicted normal limit +5%) and reduced (< 45%) LVEF [22, 23].

Exclusion criteria were: 1) Significant coronary artery disease, defined as at least 50% proximal stenosis of a coronary artery or a history of myocardial infarction; 2) Known causes of heart failure, such as primary valvular or heart muscle disease, hypertensive heart disease, endocrine disease, advanced renal insufficiency, drug or alcohol abuse; 3) Acute myocarditis (new-onset symptoms during the past 3 months) or acute myocardial infarction suspected by clinical presentation or in diagnostic testing.

All patients provided written informed consent. Ethical approval was obtained from the local Lithuanian Bioethics Committee (license numbers 158200-09-382-l03; 158200-382-PP1-23; and 158200-17-891-413).

All patients were treated according to the ESC guidelines [24, 25]. At the time of inclusion, none of the patients were treated with inotropic agents. Specific etiology-directed treatment was not administered.

Medical examinations

All patients underwent a medical interview, physical examination, and routine laboratory studies. Additionally, the proinflammatory serum cytokine interleukin-6 (IL-6) was tested as described elsewhere [26].

Echocardiography was performed for all patients to obtain conventional echocardiographic parameters. Cardiac magnetic resonance with late gadolinium enhancement was performed for 33 patients.

Mandatory investigations included coronary angiography to exclude coronary artery disease, right heart catheterization for hemodynamic evaluation. During the same procedures, right ventricle

endomyocardial biopsy was performed for the immunohistochemical evaluation and the detection of viruses by PCR. Three endomyocardial biopsy procedures were discontinued because of complications (arrhythmias or right ventricular perforation). Due to the lack of biopsy samples, PCR was performed for two of the above-mentioned patients and immunohistochemical analysis for one.

Histological and immunohistochemical assessment

Storage of the endomyocardial biopsy samples, and histological and immunohistochemical analyses were performed as described previously [26]. In brief, we detected antibodies (Santa Cruz Biotechnology, Inc.) against: T-lymphocyte CD3 (DAKO A0452 Rabbit 1, Hamburg, Germany), active-memory T-lymphocyte CD45Ro (DAKO Hamburg), macrophage CD68 (DAKO M0876 Mouse 1, Hamburg), T-helper cell CD4 (DAKO Hamburg, Germany), intracellular adhesion molecule-1 (ICAM-1) CD54 (NovocastraTM Lyophilized Mouse Monoclonal Antibody CD54 Clone 23G12), and MHC class II cell surface receptor HLA-DR (DAKO Hamburg, Germany). Positive cells were registered by an experienced pathologist and expressed as the number of cells per mm^2. Myocardial inflammation was diagnosed according to the criterion established by the ESC Working Group on Myocardial and Pericardial Diseases. This criterion is immunohistochemical detection of significant focal or diffuse cellular infiltration in the endomyocardial biopsy (≥ 14 leucocytes/mm^2, including up to 4 monocytes/mm^2 with the presence of CD3 positive T-lymphocytes ≥ 7 cells/mm^2) [1]. Inflammatory endothelial activation was diagnosed if immunohistochemical analysis revealed ≥ 3 cells expressing adhesion molecules, i.e., ICAM-1 (CD54) and/or HLA-DR [27].

Detection of viral genomes

Genomic DNA and total RNA were extracted simultaneously using the ZR-Duet DNA/RNA Miniprep kit (Zymo Research, Irvine, CA, USA). RNA (1 μg) was reverse transcribed in 20 μL reaction volumes using random hexamers and the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Vilnius, Lithuania) according to the vendor's recommendations and diluted up to 100 μL with deionized water after the reaction.

Nested PCR primers for the detection of adenovirus [28], herpes simplex viruses 1 and 2, varicella-zoster virus (VZV), cytomegalovirus (CMV), parvovirus B19 (B19V), Epstein-Barr virus (EBV), hepatitis C virus (HCV), enterovirus (EV), and rubella virus [29] are described elsewhere. Primer sequences for the nested PCR of human herpes virus 6 (HHV-6, GenBank accession no. NC001664.2 and NC000898.1); Kirsten rat sarcoma viral oncogene homolog (KRAS, GenBank accession no. NM033360); and ubiquitin C (UBC, GenBank accession no. NM021009) are presented in Table 1. Forward primers for the second PCR step (N2Fw) were labeled with 6-carboxyfluorescein at the 5' end. All primers were synthesized by the Metabion Company (Martinsried, Germany).

Table 1. Primers for detection of human herpes virus 6 (HHV6), Kirsten rat sarcoma viral oncogene homolog (KRAS) and ubiquitin C (UBC).

Primer	Sequence (5'–3')
HHV6-N1 Forward	ACCCGAGAGATGATTTTGCGTG
HHV6-N1 Reverse	GCAGAAGACAGCAGCGAGATAG
HHV6-N2 Forward	CATAGCAACCTTTTCTAGCTTTGAC
HHV6-N2 Reverse	TCTATAACATAAATGACCCCTGGGA
UBC-N1 Forward	TTCTTTCCAGAGAGCCGAAC
UBC-N1 Reverse	CCCATCTTCCAGCTGTTTTC
UBC-N2 Forward	TGGGTCGCAGTTCTTGTTTG
UBC-N2 Reverse	CCTTCCTTATCTTGGATCTTTGCC
KRAS-N1 Forward	CTTTGGAGCAGGAACAATGTCT
KRAS-N2 Forward	AATCCAGACTGTGTTTCTCCCT
KRAS-N1/N2 Reverse	TACACAAAGAAAGCCCTCCCC

All PCRs were run on a TProfessional Standard thermocycler (Biometra, Göttingen, Germany), as described by Allard et al. [28]. KRAS and UBC detection was used to validate the extraction of nucleic acids and was performed in parallel according to the conditions for viral DNA and RNR, respectively. Final PCR products were diluted 10-fold and sized 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 positive results, the genomic DNA or RNR

specimens from peripheral blood samples were also tested to exclude contamination.

Follow-up

All patients were followed-up for 5 years after the endomyocardial biopsy. The composite outcome measures were composite and combined three outcomes: cardiovascular death, LV assist device implantation, or heart transplantation. The time of the first event was included in the analysis. Follow-up events were confirmed by medical records or telephone interview with the patients' families.

Statistical analysis

Data management and analysis were performed using the R studio package (3.5.1 version) at a 5% significance level. Continuous variables were tested by the Shapiro-Wilk statistic for normal distribution. Normally distributed continuous variables were expressed as the mean ± standard deviation. Other continuous variables were expressed as the median (interquartile range), and categorical data as counts and percentages. Continuous variables were compared by the Student independent t-test when normally distributed, or by the Mann-Whitney-U test when non-normally distributed. Comparisons of categorical variables between the groups were made using the χ^2 test or the Fisher Exact test if expected values were < 5.

Univariate Cox proportional hazards regression analysis was performed to evaluate which inflammatory cells or clinical parameters were associated with poor mid-term and long-term composite outcome measures after 2-year and 5-year follow-up. The optimal cut-off point was determined using the receiver operating characteristic (ROC) curve. The Kaplan-Meier analysis was used to compare cumulative survival rates between different subgroups of DCM patients after a 2-year and 5-year follow-up. The log-rank statistic was used to evaluate the statistical significance of differences between the curves.

Results

Baseline patients' characteristics

Fifty-seven DCM patients (mean age 47.3 ± 10.9 years; 45 [79%] males) with chronic heart failure participated in the study. The average LVEDD was 6.8 ± 0.9 cm, average LVEF — 26.1 ± 9.5%, and average pulmonary artery wedge pressure — 22 ± 9 mmHg. Of these patients, 41 (72%) were in New York Heart Association (NYHA) III functional class and 10 (17%) were NYHA IV functional class. The median (interquartile range) heart failure duration was 12 (55) months. Baseline characteristics are shown in Table 2.

Of the 57 patients, 27 (47%) reached the composite outcome measure during the 5-year follow-up period: 10 (18%) patients died, 9 (16%) underwent heart transplantation, and 8 (14%) LV assist device implantation. Other patients remained on conventional medical heart-failure therapy. The 5-year cumulative survival rate was 53%. In univariate Cox regression models, most hemodynamic parameters, echocardiographic parameters and IL-6 were associated with poor clinical outcomes (Table 2).

The prevalence of cardiac inflammation

Immunohistochemical analysis was performed on 55 endomyocardial biopsies. Myocardial inflammation was detected in 31 of the 55 (54%) DCM patients. Patients were divided into two groups: inflammatory DCM (iDCM) and non-inflammatory DCM (non-iDCM). No difference was observed in baseline characteristics of the two groups ($p > 0.05$; Table 2), except for lower systolic blood pressure and higher level of B-type natriuretic peptide (BNP) in the iDCM group ($p \leq 0.05$).

Kaplan-Meier analysis showed no difference between survival curves of patients with iDCM and non-iDCM ($p > 0.05$; Fig. 1).

However, univariate Cox regression analysis revealed an association between a higher CD45ro+ cell count in the myocardium and poor mid-term prognosis. Higher CD3+ cell count in the myocardium was associated with poor mid-term and long-term prognosis. This was not the case for CD68+ inflammatory infiltrative cells (Table 3). ROC curves determined the cut-off values for CD3+ and CD45ro+ cells (Fig. 2). The cohort was divided into two groups according to whether their CD3+ and CD45ro+ cell counts were above or below the cut-off value (13 cells/mm^2 and 11.5 cells/mm^2, respectively). Univariate Cox regression analysis showed that cell counts above cut-off values were associated with worse mid-term and long-term clinical outcome (Table 3). Estimation of survival curves demonstrated that patients with CD3+ and CD45ro+ cell counts above the cut-off values had lower survival rates (Fig. 3). Lower p-values in survival analysis and higher hazard ratio (95% confidence interval) in Cox regression analysis revealed that inflammatory cells predict better mid-term than long-term outcomes.

Inflammatory endothelial activation (increased expression of HLA-DR and ICAM [≥ 3 cells/mm^2]) was detected by immunohistochemistry in

Table 2. Baseline characteristics for the study population. A comparison of baseline characteristics of non-inflammatory dilated cardiomyopathy (non-iDCM) and inflammatory dilated cardiomyopathy (iDCM) patients, and patients with and without the viral genome. Univariate Cox analysis showing the association between the various clinical parameters and poor long-term clinical outcome.

Variable	All patients (n = 57)	Non-iDCM (n = 24)	iDCM (n = 31)	P	Virus--negative (n = 27)	Virus--positive (n = 29)	P	HR (95% CI)	P
Clinical characteristics									
Age, years	47.3 ± 10.9	48.3 ± 13	46.6 ± 9,6	0.58	48.44 ± 12.68	46.07 ± 9.28	0.43	0.98 (0.95–1.01)	0.26
Male gender	45 (79%)	17 (71%)	26 (82%)	0.25	23 (85%)	21 (72%)	0.23	1.41 (0.53–3.73)	0.49
BMI [kg/m^2]	26.84 (8.13%)	27.3 (8.2%)	25.7 (8%)	0.45	28.1 (7.2%)	25.2 (8.7%)	0.24	0.98 (0.91–1.05)	0.54
Systolic BP [mmHg]	116 ± 20	123 ± 20	110 ± 17	**0.01**	115 ± 20	118 ± 22	0.63	**0.97 (0.95–0.99)**	**0.002**
Diastolic BP [mmHg]	80 (10%)	78 (13%)	80 (10%)	0.43	80 (10%)	80 (10%)	0.76	**0.96 (0.92–0.99)**	**0.02**
Heart rate [bpm]	77 (27%)	73 (22%)	86 (32%)	0.18	79 (23%)	76 (34%)	0.83	1.01 (0.99–1.03)	0.35
Atrial fibrillation	11 (19%)	3 (13%)	8 (26%)	0.31	6 (22%)	5 (17%)	0.64	0.95 (0.36–2.52)	0.92
LBBB	14 (25%)	6 (25%)	8 (26%)	0.99	10 (37%)	4 (14%)	0.15	1.66 (0.72–3.79)	0.23
NYHA III–IV class	51 (90%)	20 (83%)	29 (94%)	0.64	24 (89%)	26 (90%)	1	4 (0.54–29.53)	0.17
Follow-up time [months]	60 (37%)	60 (32%)	60 (47%)	0.4	48 (46%)	60 (15%)	0.14		
Biomarkers/blood testing									
Hemoglobin [g/L]	142 ± 16	137.7 ± 13.7	144.7 ± 14.7	0.08	142.7 ±16.6	141.3 ±14.6	0.73	1 (0.98–1.02)	0.72
eGFR [mL/min/1.73 m^2]	87 ± 24	83 ± 27	91 ± 22	0.2	86 ± 22	88 ± 26	0.73	1 (0.99–1.02)	0.43
BNP [ng/L]	728 (1797%)	214 (1445%)	1017 (2432%)	0.05	916 (2571%)	228 (1329%)	**0.04**	1 (1–1)	0.12
CRP [mg/L]	4.6 (14.2%)	5.2 (6.6%)	4.5 (15.3%)	0.74	6.6 (13.5%)	2.4 (10.3%)	0.12	1.01 (0.99–1.02)	0.39
IL-6 [pg/mL]	2.5 (4.7%)	2.2 (3.1%)	2.9 (7.4%)	0.21	4.62 (6.3%)	2.01 (2.6%)	**0.04**	**1.04 (1.01–1.06)**	**0.004**
Echocardiographic parameters									
LVEF [%]	26.08 ± 9.5	25.6 ±11.8	26.7 ±7.1	0.69	26.9 ± 9.2	26.7 ± 9.9	0.94	**0.93 (0.89–0.98)**	**0.004**
LVEDD [cm]	6.8 ± 0.9	6.8 ± 0.9	6.9 ± 0.9	0.57	7.0 ± 0.9	6.7 ± 0.8	0.12	1.49 (0.94–2.36)	0.09
LV diastolic function (n = 54):									
Grade I	14 (26%)	7 (30%)	7 (23%)	0.79	6 (22%)	9 (33%)	0.61		
Grade II	16 (30%)	6 (26%)	10 (33%)		8 (30%)	8 (30%)		1.32 (0.44–3.93)	0.62
Grade III	23 (43%)	10 (44%)	13 (43%)		13 (48%)	10 (37%)		2.4 (1.15–6.07)	0.02
Functional MR ≥ moderate*	32 (56%)	13 (54%)	19 (61%)	0.6	17 (63%)	14 (48%)	0.27	**2.4 (1.08–5.33)**	**0.03**
RV end-diastolic diameter [cm]	3.3 ± 0.6	3.2 ± 0.8	3.4 ± 0.5	0.3	3.4 ± 0.6	3.3 ± 0.6	0.63	**2.75 (1.56–4.84)**	**< 0.001**

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Table 2. (cont.) Baseline characteristics for the study population. A comparison of baseline characteristics of non-inflammatory dilated cardiomyopathy (non-iDCM) and inflammatory dilated cardiomyopathy (iDCM) patients, and patients with and without the viral genome. Univariate Cox analysis showing the association between the various clinical parameters and poor long-term clinical outcome.

Variable	All patients (n = 57)	Non-iDCM (n = 24)	iDCM (n = 31)	P	Virus--negative (n = 27)	Virus--positive (n = 29)	P	HR (95% CI)	P
RV systolic function:*									
Normal	21 (37%)	11 (46%)	9 (29%)	0.11	6 (22%)	15 (52%)	0.13		
Mildly impaired	9 (16%)	4 (17%)	5 (16%)		5 (19%)	4 (14%)		2.04 (0.6–6.41)	0.23
Moderately impaired	11 (19%)	1 (4%)	9 (29%)		6 (22%)	5 (17%)		1.72 (0.55–5.4)	0.35
Severely impaired	16 (28%)	8 (33%)	8 (26%)		10 (37%)	5 (17%)		**3.67 (1.42–9.53)**	**0.008**
TAPSE (n = 33)	15 (6.3%)	15 (5.5%)	13 (5.5%)	0.64	15 (4.5%)	16 (5.5%)	0.83	**0.7 (0.56–0.87)**	**0.001**
Functional TR ≥ moderate*	20 (35%)	9 (38%)	11 (36%)	0.88	11 (41%)	9 (31%)	0.45	**2.98 (1.41–6.3)**	**0.004**
Cardiac magnetic resonance									
Mid-wall late gadolinium enhancement (n = 33)	22 (73%)	7 (54%)	14 (78%)	0.25	10 (72%)	11 (61%)	0.71	1.59 (0.44–5.78)	0.48
Hemodynamic measurements (n = 54)									
PAWP [mmHg]	21.8 ± 8.9	21.5 ± 9.6	22.6 ± 8.8	0.65	25 ± 9	19 ± 8	**0.02**	**1.07 (1.02–1.12)**	**0.004**
Mean RAP [mmHg]	11 (6.5%)	10 (6%)	11 (10%)	0.96	12 (8%)	8 (8%)	0.09	**1.07 (1.02–1.13)**	**0.007**
Mean PAP [mmHg]	29 ± 18	31 ± 12	32 ± 11	0.67	35 ± 11	28 ± 9	**0.02**	**1.05 (1.02–1.09)**	**0.005**
PVR [mmHg]	2 (2%)	1.5 (1.6%)	2.3 (1.6%)	0.08	2.4 (2.7%)	1.6 (1.4%)	**0.04**	**1.22 (1–1.49)**	**0.05**
Pulmonary hypertension	36 (68%)	14 (60%)	21 (75%)	0.28	18 (72%)	17 (63%)	0.49	**3.37 (1.15–9.9)**	**0.03**
Concomitant cardiac medication									
ACEI/ARB	41 (72%)	19 (79%)	20 (65%)	0.24	16 (59%)	24 (83%)	0.06	0.83 (0.38–1.84)	0.65
Beta-blocker	54 (95%)	24 (100%)	28 (90%)	0.25	24 (89%)	29 (100%)	0.11	0.42 (0.1–1.79)	0.24
MRA	51 (90%)	20 (83%)	30 (97%)	0.16	26 (96%)	24 (83%)	0.2		0.99
Diuretics	53 (93%)	21 (88%)	30 (97%)	0.31	25 (93%)	27 (93%)	1	2.46 (0.33–18)	0.38
Virus-positive endomyocardial biopsies									
Total		15 (63%)	14 (47%)	0.25	0	29 (52%)		0.64 (0.3–1.39)	0.26
B19V		13 (54%)	12 (40%)	0.3	0	25 (45%)			
EBV		0 (0%)	1 (3%)	1	0	1 (2%)			
EV		0 (0%)	1 (3%)	1	0	1 (2%)			
HHV-6		2 (8%)	3 (10%)	1	0	5 (9%)			
VZV		1 (4%)	0 (0%)	0.44	0	1 (2%)			
HCV		0 (0%)	1 (3%)	1	0	1 (2%)			

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Table 2. (cont.) Baseline characteristics for the study population. A comparison of baseline characteristics of non-inflammatory dilated cardiomyopathy (non-iDCM) and inflammatory dilated cardiomyopathy (iDCM) patients, and patients with and without the viral genome. Univariate Cox analysis showing the association between the various clinical parameters and poor long-term clinical outcome.

Variable	All patients (n = 57)	Non-iDCM (n = 24)	iDCM (n = 31)	P	Virus-negative (n = 27)	Virus-positive (n = 29)	P	HR (95% CI)	P
Immunohistological markers of endothelial activation (n = 55)									
ICAM-1/CD54+ [cells/mm^2]	0 (1%)	0 (1%)	0 (1.5%)	0.45	0 (2%)	0 (1%)	0.26		
HLA DR [cells/mm^2]	5 (2%)	4.5 (2%)	5.0 (2%)	0.1	5 (1%)	5 (2%)	0.23		
CD3+ [cells/mm^2]	10 (9%)	7 (2%)	15 (8%)	**< 0.001**	10 (9%)	9 (5%)	0.66		
CD45+ [cells/mm^2]	7 (5%)	5 (2%)	10 (6%)	**< 0.001**	7 (5%)	6 (3%)	0.42		
CD68+ [cells/mm^2]	4 (2%)	3 (2%)	5 (4%)	**< 0.001**	5 (3%)	3 (3%)	**0.01**		

*Evaluated visually. Values are expressed as: median (interquartile range), mean ± standard deviation or number (%); hazard ratio (HR) and 95% confidence interval (CI). Significant at the p-value of < 0.05 (bold value),(bold value). ACEI — angiotensin-converting enzyme inhibitor; ARB — angiotensin II receptor blocker; BP — blood pressure; BMI — body mass index; BNP — B-type natriuretic peptide; B19V — parvovirus B19; CRP — C-reactive protein; eGFR — estimated glomerular filtration rate; EBV — Epstein-Barr virus; EV — enterovirus; HCV — hepatitis C virus; HHV-6 — human herpes virus 6; VZV — varicella zoster virus; IL-6 — interleukin 6; LBBB — left bundle branch block; LV — left ventricle; LVEF — left ventricular ejection fraction; LVEDD — left ventricular end-diastolic diameter; MRA — mineralocorticoid receptor antagonist; MR — mitral regurgitation; RV — right ventricle; NYHA — New York Heart Association; PAWP — pulmonary capillary wedge pressure; RAP — right atrial pressure; PAP — pulmonary arterial pressure; PVR — pulmonary vascular resistance; TAPSE — tricuspid annular plane systolic excursion; TR — tricuspid regurgitation

50 (91%) patients. The expression did not, however, differ between the iDCM and non-iDCM groups ($p > 0.05$). However, it should be interpreted with caution, while these proteins are not only markers for endothelial activation, but are also found on the surface of immune cells.

Prevalence of cardiotropic viruses

Polymerase chain reaction analysis was performed on 56 endomyocardial biopsies. Viral genomes were detected in the myocardium of 29 (52%) of the 56 DCM patients. Of these 29 patients, 25 (86%) had the B19V genome, and other 5 (17%) had HHV6 genome. Other viruses (VZV, CMV, EBV, HCV, EV), were detected in single cases (n = 1 [3%] of each type). Three (10%) of virus-positive patients had a double infection and one of them (3%) a triple infection. Co-detection of B19V and HHV6 prevailed (n = 3 [10%]).

The remaining 27 (48%) patients were virus-negative. There were no differences in most baseline parameters between the virus-positive and virus-negative groups ($p > 0.05$), except for higher BNP and IL-6 levels, worse hemodynamic parameters (Table 2), and a higher number of infiltrative CD68+ cells in the virus-negative group (Fig. 4).

Kaplan-Meier survival curves demonstrated no difference in survival rates of patients in virus-positive and virus-negative groups ($p > 0.05$; Fig. 5).

Sub-groups of idiopathic DCM

Both PCR analysis and immunohistochemical evaluation were performed on 54 DCM patients. Based on the detection of viral genome — in combination with positive or negative immunohistochemistry — four specific sub-groups of patients were distinguished:

- Virus-positive (chronic) myocarditis (15 [28%] patients): both cardiotropic virus and myocardial inflammation was present;
- Autoreactive iDCM (16 [30%] patients): no cardiotropic virus was detected but myocardial inflammation was present;
- Viral DCM (14 [26%] patients): viral genome was detected but no signs of myocardial inflammation;
- Non-inflammatory DCM (9 [17%] patients): neither viral genome nor inflammation was detected.

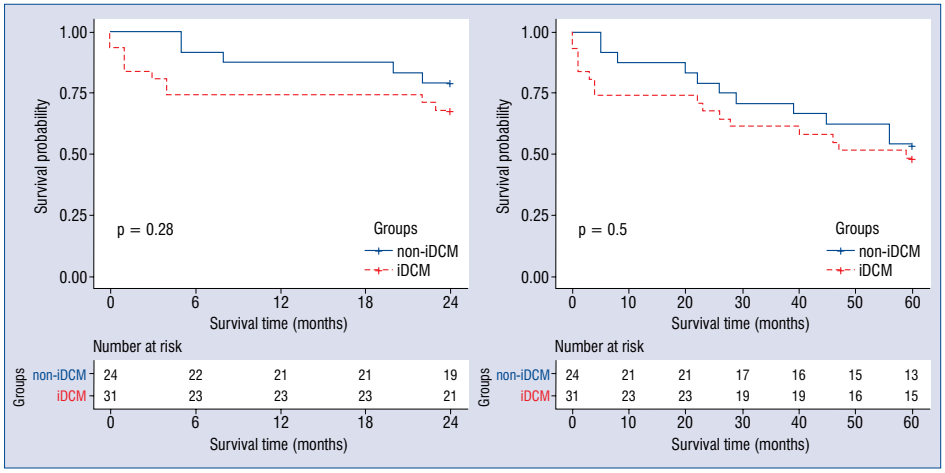

Figure 1. Kaplan-Meier analysis of the inflammatory dilated cardiomyopathy (iDCM) and non-inflammatory dilated cardiomyopathy (non-iDCM) groups.

Table 3. Univariate Cox analysis showing the association between the number of myocardial inflammatory infiltrates and poor clinical outcome after 2-year and 5-year follow-up.

Cardiac inflammatory infiltration and endothelial activation markers [cells/mm^2]	HR (95% CI)			
	2-year follow-up	P	5-year follow-up	P
CD3+	**1.085 (1.04–1.132)**	**< 0.001**	**1.061 (1.02–1.103)**	**0.003**
CD45ro+	**1.079 (1.025–1.136)**	**0.004**	1.048 (0.998–1.101)	0.06
CD68+	1.075 (0.862–1.34)	0.523	1.029 (0.867–1.223)	0.74
CD4+-	1.01 (0.91–1.13)	0.82	1.013 (0.908–0.13)	0.82
CD54+	0.82 (0.53–1.25)	0.35	0.816 (0.532–1.254)	0.35
HLA-DR+	1.02 (0.91–1.14)	0.7	1.022 (0.915–1.143)	0.7
CD3+ ≥ 13 cells/mm^2	**4.481 (1.588–12.64)**	**0.005**	**2.181 (1.009–4.711)**	**0.047**
CD45ro+ ≥ 11.5 cells/mm^2	**5.261 (1.854–14.93)**	**0.002**	**2.892 (1.217–6.871)**	**0.016**

Values are expressed as hazard ratio (HR) and 95% confidence interval (CI). Significant at the p-value of < 0.05 (bold value).

Discussion

This prospective study summarizes an experience identifying etiopathogenetic markers of idiopathic DCM for diagnosis of distinct disease sub-entities, and evaluates their prognostic value. In this study, the criterion defined by the ESC Working Group on Myocardial and Pericardial Diseases [1] for diagnosing iDCM was used. iDCM was diagnosed in 54% of the patients by immunohistochemistry. The rate of the iDCM was similar when compared to the study by Palecek et al. [30]. The prognostic value of myocardial inflammation and different inflammatory cells varies in different studies, possibly due to the diversity of diagnostic protocols [31]. Though, according to available research, we found no study which evaluated the prognostic value of iDCM diagnosed by ESC criterion. In the present cohort, iDCM had no impact on clinical outcomes. However, a higher count of CD3+ and CD45ro+ cells were associated with a poor clinical outcome.

The current study found a high prevalence of cardiotropic viruses (52% of patients), of which

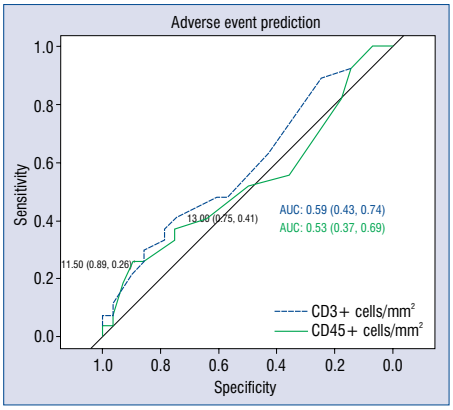

Figure 2. Receiver operating characteristic curve analysis of CD3+ and CD45ro+ cells for predicting composite endpoint. The best cut-off values were 13 CD3+ cells/ /mm^2 (sensitivity, 41%; specificity, 75%) and 11.5 CD45ro+ cells/mm^2 (sensitivity, 26%; specificity, 89%).

the most frequently detected were B19V and HHV6. Three (10%) patients were co-infected with B19V and HHV6. These findings support previous studies, in which B19V and HHV6 were the most frequent viruses [10, 32, 33]. Furthermore, the high prevalence of B19V suggests that chronic DCM might have developed from the previous B19V-associated myocarditis [34]. However, there is conflicting evidence about the viral genome's impact on the long-term prognosis. Several studies revealed that viral genomes were associated with worsening LV function, the need for heart transplantation, and death [10, 35]. While other studies report that the existence of viral genomes *per se* is not associated with poor clinical outcome [12, 36–38]. In the present cohort, detection of a virus had no impact on clinical outcome.

However, half of the virus-positive patients had no myocardial inflammation. This finding is also reported by Kühl et al. [10], in whose study DCM patients had symptoms of heart failure and viral genome, but no evidence of inflammation. Kindermann et al. [12] have also reported that the frequency of inflammation is independent of any evidence of the virus genome. Several studies detected B19V in healthy people's hearts with no evidence of inflammation [38–40].

In contrast, half of the current iDCM patients had no viral genome, and cause of inflammation remains unknown. This finding supports the idea that myocardial inflammation could be maintained by an autoimmune process leading to the deterioration of LV function [41]. Contrarily, this high prevalence of cardiac inflammation might be due to the advanced DCM phenotype. The question remains whether this inflammation is a consequence of this advanced stadium or if it acts as a causal factor.

Interestingly, the macrophage count was higher in the virus-negative group. It could be hypothesized that CD68+ macrophages have an impact on DCM pathogenesis because of their pro-inflammatory activity. As known from previous studies, macrophages can cause or maintain persistent LV systolic dysfunction and LV remodeling [42]. While recent studies have noted the importance of macrophage profiles and their function in heart diseases, much is still unknown about their impact on DCM pathogenesis [43].

Virus-negative patients had worse hemodynamic parameters and higher BNP levels than the virus-positive patients, although echocardiographic parameters did not differ between the two groups. Worse hemodynamic status might be explained by chronic immune activation and myocardial inflammation, given that higher numbers of macrophages and higher levels of IL-6 were detected in this virus-negative group. Macrophages secrete IL-6 [44], which might increase the severity of pulmonary hypertension [45].

Although heart failure treatment has become more effective, there are still many refractory DCM patients who do not respond to any available treatment. Therefore, developing alternative therapies is essential. Four etiopathogenetic groups were distinguished, for whom the specific therapeutic strategy selected could be suitable [1, 6, 46] or novel treatment options established [13]. Treatment strategies based on the etiopathogenetic approach to the disease might improve LV function, prevent progression of heart failure, and, in some cases, exclude patients from the heart transplant list.

Limitations of the study

First, small sample size did not allow for differentiation of patients based on the type of infectious agent. Second, the study had no control group, due to a shortage of healthy donor hearts which were not suitable for transplantation. Third, as a result of financial considerations, neither virus replication nor the viral load for distinguishing active from incidental infection were investigated [47], nor was autoantibody testing performed or genetic screening for pathogenic DCM mutations. Fourth, due to limited-experience in specific DCM treatment and

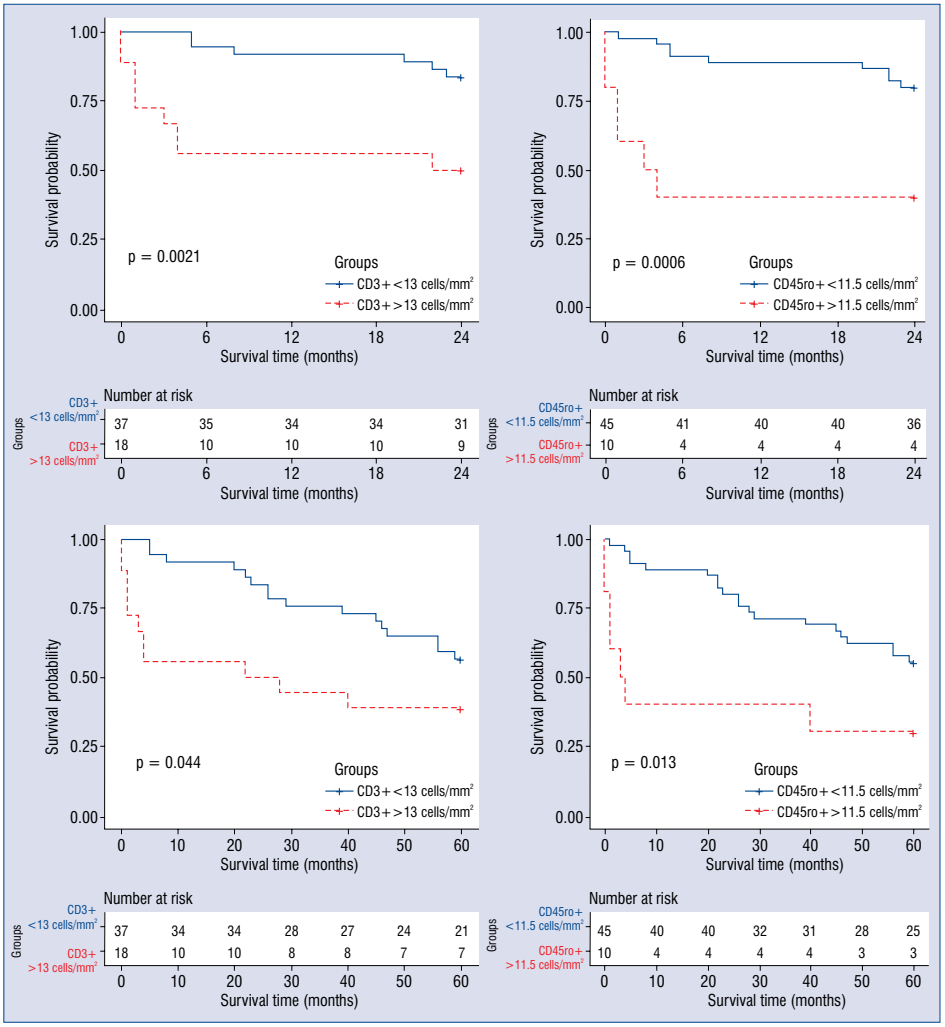

Figure 3. Survival curves according to the count of CD3+ and CD45ro+ cells. Patient groups with a higher number of infiltrative cells (CD3+ > 13 cells/mm^2 and CD45ro+ > 11.5 cells/mm^2) had a significantly lower survival rate than groups with lower cell counts.

the treatment costs, none of the patients received etiology-directed treatment. Finally, the study was held at a time when right ventricle was a "forgotten" ventricle, therefore it was limitedly assessed. In spite of its limitations, the study certainly provides a basis for a more extensive diagnostic and treatment studies — based on etiopathogenetic sub-entities which include a control group.

Conclusions

This study has shown that two markers, cardiotropic viruses and myocardial inflammation, are prevalent among DCM patients and are helpful in identifying sub-groups of DCMs. An increased number of T-lymphocytes in the myocardium is a predictor of poor mid-term and long-term

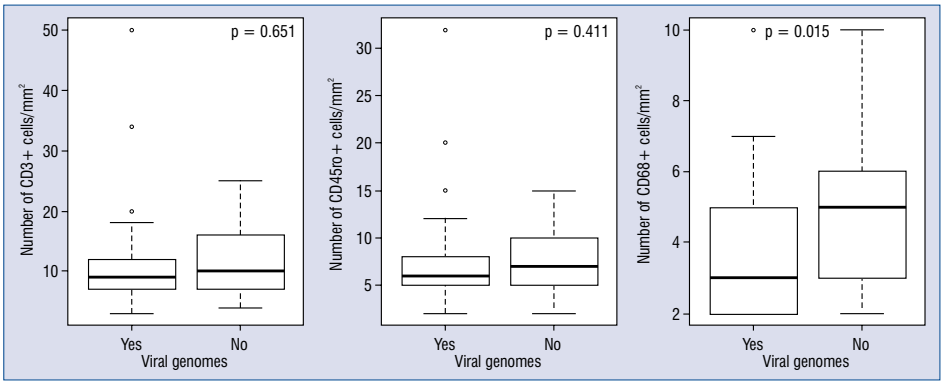

Figure 4. Comparison of inflammatory cell (CD3+, CD45ro+, and CD68+) counts in the virus-negative and virus--positive biopsies.

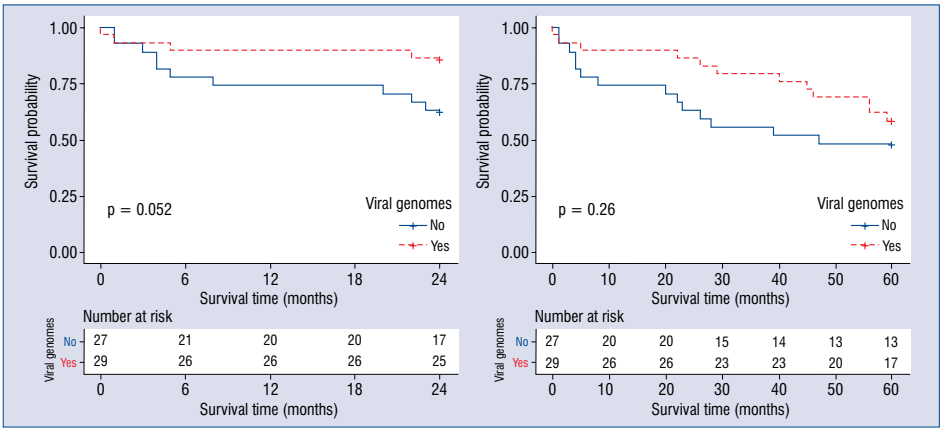

Figure 5. The Kaplan-Meier curves for virus-positive and virus-negative groups.

prognosis. The finding of specific inflammatory cells as a prognostic marker could be of value in determining new definitions of cardiac inflammation. A natural continuation of this work would be further analysis of specific etiologic DCM subgroups and a search for etiology-directed treatment strategies.

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Inflammation-Related Biomarkers Are Associated with Heart Failure Severity and Poor Clinical Outcomes in Patients with Non-Ischemic Dilated Cardiomyopathy

Ieva Kažukauskienė [1,*], Vaida Baltrūnienė [1], Ieva Rinkūnaitė [2], Edvardas Žurauskas [1], Dalius Vitkus [3], Vytė Valerija Maneikienė [4], Kęstutis Ručinskas [4] and Virginija Grabauskienė [1,4]

[1] Department of Pathology, Forensic Medicine and Pharmacology, Faculty of Medicine, Vilnius University, M. K. Čiurlionio 21, LT-03101 Vilnius, Lithuania; vaida.baltruniene@mf.vu.lt (V.B.); Edvardas.Zurauskas@vpc.lt (E.Ž.); virginija.grabauskiene@santa.lt (V.G.)
[2] Department of Biological Models, Life Sciences Center, Institute of Biochemistry, Vilnius University, 7 Saulėtekio av., LT-10257 Vilnius, Lithuania; ieva.rinkunaite@bchi.vu.lt
[3] Department of Physiology, Biochemistry, Microbiology and Laboratory Medicine, Faculty of Medicine, Vilnius University, M. K. Čiurlionio 21, LT-03101 Vilnius, Lithuania; dalius.vitkus@santa.lt
[4] Clinic of Cardiac and Vascular Diseases, Institute of Clinical Medicine, Faculty of Medicine, Vilnius University, M. K. Čiurlionio 21, LT-03101 Vilnius, Lithuania; vyte.maneikiene@santa.lt (V.V.M.); Kestutis.Rucinskas@santa.lt (K.R.)
* Correspondence: ieva.kazukauskiene@mf.vu.lt; Tel.: +370-52398728

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Abstract: Inflammation-related biomarkers are associated with clinical outcomes in mixed-etiology chronic heart failure populations. Inflammation-related markers tend to be higher in ischemic than in non-ischemic dilated cardiomyopathy (NI-DCM) patients, which might impact their prognostic performance in NI-DCM patients. Therefore, we aimed to assess the association of inflammation-related biomarkers with heart failure severity parameters and adverse cardiac events in a pure NI-DCM patient cohort. Fifty-seven patients with NI-DCM underwent endomyocardial biopsy. Biopsies were evaluated by immunohistochemistry for CD3+, CD45ro+, CD68+, CD4+, CD54+, and HLA-DR+ cells. Blood samples were tested for high-sensitivity C-reactive protein (hs-CRP), interleukin-6, tumor necrosis factor-α (TNF-α), soluble urokinase-type plasminogen activator receptor and adiponectin. During a five-year follow-up, twenty-seven patients experienced at least one composite adverse cardiac event: left ventricle assist device implantation, heart transplantation or death. Interleukin-6, TNF-α and adiponectin correlated with heart failure severity parameters. Patients with higher levels of interleukin-6, TNF-α, adiponectin or hs-CRP, or a higher number of CD3+ or CD45ro+ cells, had lower survival rates. Interleukin-6, adiponectin, and CD45ro+ cells were independently associated with poor clinical outcomes. All patients who had interleukin-6, TNF-α and adiponectin concentrations above the threshold experienced an adverse cardiac event. Therefore, a combination of these cytokines can identify high-risk NI-DCM patients.

Keywords: biomarkers; endomyocardial biopsy; inflammation; non-ischemic dilated cardiomyopathy; prognosis

1. Introduction

Heart failure is a multi-etiologic clinical syndrome that affects up to 2% of the adult population in developed countries [1]. One of the leading causes of heart failure is non-ischemic dilated cardiomyopathy (NI-DCM), affecting approximately one-third of heart failure patients [2]. Due to its etiopathogenetic diversity, the progression of NI-DCM varies between individuals. Despite the use of guideline-directed treatment, a significant proportion of NI-DCM patients deteriorate (progressively), either until death or until they receive a heart transplant. NI-DCM is the most common indication for heart transplantation worldwide, accounting for more than 50% of all heart transplants in patients younger

than 60 years [3]. However, there are no prognostic strategies for risk stratification in patients who have developed NI-DCM. Furthermore, Dziewiecka et al. showed that most risk assessment models, which have been created for heterogeneous heart failure populations, have suboptimal accuracy for NI-DCM patients [4]. Therefore, there is a need for biomarkers that can aid in risk assessment and help identify disease progression, as well as to facilitate the search for specific therapeutic targets.

Infiltration of inflammatory cells in the myocardium and concurrent systemic inflammation are important factors in the development and progression of chronic heart failure [5]. Various inflammation-related biomarkers can predict poor clinical outcomes in the chronic heart failure population [6]. However, this population consists of multi-etiologic diseases, primarily NI-DCM and ischemic heart disease. Ischemic heart disease patients stand out not just because of a poorer prognosis than patients with non-ischemic heart failure [7,8] but also due to a higher expression and concentration of inflammation-related biomarkers [9–11]. Thus, the prognostic role of inflammation-related biomarkers in chronic heart failure studies might be primarily determined by patients with ischemic etiology. Although various studies demonstrate a distinct role for inflammation-related biomarkers in the pathogenesis of NI-DCM, there is a lack of data on the predictive value of such markers in NI-DCM patients. Therefore, we aimed to assess the association of inflammation-related myocardial and serum biomarkers with parameters of heart failure severity, and evaluate their predictive potential in a pure NI-DCM patient cohort.

2. Materials and Methods

2.1. Study Population and Protocol

A prospective cohort study with retrospective analysis was done in the Vilnius University Hospital Santaros Klinikos. We enrolled 57 patients with suspected NI-DCM that were admitted to the hospital for diagnostic evaluation between January 2010 and December 2013. The median duration of heart failure symptoms before enrollment was 12 (5–60) months. The inclusion criteria were symptoms and signs of heart failure, with echocardiographic evidence of left ventricular dilation and reduced left ventricular ejection fraction (LVEF) ($\leq$45%). The main goal of the study was to look for etiopathogenetic factors in NI-DCM, such as cardiotropic viruses and myocardial inflammation, by analyzing various biomarkers in serum and endomyocardial biopsies [12]; and to find the differences in pathogenesis between non-inflammatory and inflammatory NI-DCM [13], which might have an impact on future research or the patient treatment process.

Exclusion criteria were:

1. Significant coronary artery disease, defined as at least 50% proximal stenosis of a coronary artery, or a history of myocardial infarction;
2. Other causes of heart failure, such as heart muscle or primary valvular disease, hypertensive heart disease, advanced chronic kidney disease, endocrine disease, alcohol or drug abuse;
3. Acute myocarditis (onset in the previous three months), or acute coronary syndrome as suspected by clinical presentation or diagnostic evaluation.

Study patients underwent a detailed medical interview, physical examination and routine laboratory tests, including complete blood count, high sensitivity C-reactive protein (hs-CRP), creatinine (CKD-EPI creatinine equation was used to estimate glomerular filtration rate (GFR)), high-sensitivity troponin T (hs-troponin T) and B-type natriuretic peptide (BNP). All patients underwent standard transthoracic echocardiography to obtain conventional echocardiographic measurements within 24 h before interventional procedures. The methodology has been described in detail elsewhere [14], except severe LV diastolic dysfunction, which matched grade III according to recommendations [15]. Coronary angiography was performed to exclude coronary artery disease. Right heart catheterization was performed as described previously [14]. In brief, the procedure was carried out for cardiac pressures (pulmonary capillary wedge pressure (PCWP), mean pulmonary arterial

pressure (mPAP)) and cardiac index assessment, followed by endomyocardial biopsy from the interventricular septum.

All patients were treated according to the guidelines of the European Society of Cardiology [16] and provided informed consent. Ethical approval was obtained from the local Lithuanian Bioethics Committee (license numbers 158200-382-PP1-23; 158200-09-382-l03; and 158200-17-891-413).

2.2. Endomyocardial Biopsy

Endomyocardial biopsy procedure, storage of the biopsy samples, histological and immunohistochemical analyses were performed as described previously [12]. In brief, we detected infiltrative inflammatory cells in the myocardium using the following antibodies: T-lymphocyte CD3 (Agilent DAKO, Hamburg, Germany), active-memory T-lymphocyte CD45ro (Agilent DAKO, Hamburg, Germany), macrophage CD68 (Agilent DAKO, Hamburg, Germany), T-helper cell CD4 (Agilent DAKO, Hamburg, Germany), intracellular adhesion molecule 1 (ICAM-1) CD54 (Leica Biosystems, Newcastle, United Kingdom) and MHC class II cell surface receptor HLA-DR (Agilent DAKO, Hamburg, Germany). Positive cells were registered by an experienced pathologist and expressed as the number of cells per mm^2. Three endomyocardial biopsy procedures were discontinued because of arrhythmias or right ventricular perforation, and as a result, immunohistochemical analysis was not performed for two of these patients due to a lack of biopsy material.

2.3. Biochemical Assays of Serologic Inflammation-Related Markers

Plasma samples were stored at −80 °C until analysis. The proinflammatory serum cytokines TNF-α and IL-6 were measured by solid-phase, chemiluminescent immunometric assays using IMMULITE/Immulite 1000 systems (Immulite, Siemens) according to the manufacturer's instructions: TNF-α (Catalog number LKNFZ (50 tests) and LKNF1 (100 tests)), IL-6 (Catalog number LK6PZ (50 tests) and LK6P1 (100 tests)) and expressed as pg/mL. Adiponectin was measured using the Millipore Adiponectin assay according to the manufacturer's recommendations (Millipore, Burlington, MA, USA) and expressed as mg/mL. Levels of human soluble urokinase-type plasminogen activator receptor (suPAR) were estimated by ELISA assay according to the manufacturer's recommendations (Abbexa). Absorbance was measured at 450 nm with a spectrophotometer (Varioskan®Flash, Thermo Fisher Scientific, Vantaa, Finland). Final concentrations of suPAR are expressed as ng/mg protein.

2.4. Follow-Up

Patients were followed up for five years after enrollment in the study. The clinical outcome measure was defined retrospectively and was a composite endpoint of left ventricle assist device implantation, heart transplantation, or cardiovascular death. The time of the first event was included in the analysis. Adverse cardiac events were confirmed by medical records, national death registry records, or telephone interviews with the patients' families. The clinical relevance of choosing these outcomes as a composite endpoint was based on the idea that all these outcomes are clinically relevant and reflect the same clinical and probably pathophysiological state of advanced heart failure when there are no more viable alternatives of treatment.

2.5. Statistical Analysis

Data analysis was performed using the R studio package (4.0.3 version). A p-value of <0.05 was considered statistically significant. Continuous variables are expressed as the median (25th percentile, 75th percentile) and categorical data as counts and percentages. The Mann–Whitney U test compared continuous variables between two groups, Kruskal-Wallis test—between three groups. Categorical variables were compared between the groups by the chi-square test or Fisher's exact test if expected values were <5. The association

between inflammation-related and heart failure severity parameters was assessed using the Spearman correlation.

The receiver operating characteristic (ROC) curve was used to identify the optimal cut-off value for the outcome prediction of each inflammation-related biomarker and to estimate their accuracy for predicting composite outcome measures. Kaplan–Meier analysis was used to compare the cumulative survival rates between the two groups of NI-DCM patients stratified by cut-off values of each inflammation-related biomarker. The log-rank statistic was used to evaluate the statistical significance of differences between the curves. Univariate Cox proportional hazards regression analysis was run for all baseline variables to evaluate their association with poor composite outcomes. All variables with a p-value < 0.1 in the univariate analysis were included in multivariate Cox regression analysis, which was performed using the stepwise backward elimination.

3. Results

3.1. Baseline Patient Characteristics

The baseline characteristics of our cohort are shown in Tables 1 and 2. The median (25th percentile, 75th percentile) age of the patients was 47 (44–53) years, and 45 (79%) were men. The majority (90%) were classified as New York Heart Association (NYHA) functional class III-IV. Patients tended to have elevated cardiac pressures and impaired cardiac index, as well as elevated levels of BNP, hs-troponin T and TNF-α.

Table 1. Baseline characteristics for the study population, stratified by outcome.

Variable	All Patients (n = 57)	Event-Free Group (n = 30)	Event Group (n = 27)	p
Clinical characteristics				
Age, years	47 (44–53)	48 (47–53)	46 (39–54)	0.19
Male gender, n (%)	45 (79%)	23 (79)	22 (79)	0.95
NYHA III-IV class, n (%)	51 (90%)	24 (83)	27 (96)	0.19
Body mass index, kg/m^2	26.84 (23.4–31.6)	27.8 (22.7–32.4)	26.6 (23.6–30.4)	0.8
Systolic blood pressure, mmHg	114 (100–130)	123 (110–130)	106 (94–116)	<0.01
Diastolic blood pressure, mmHg	80 (70–80)	80 (70–80)	70 (69–80)	0.11
Atrial fibrillation, n (%)	11 (19)	6 (20)	5 (19)	0.89
Dyslipidemia, n (%)	10 (18)	6 (20)	4 (15)	0.73
Rheumatologic disease, n (%)	4 (7)	1 (3)	3 (11)	0.34
Duration of heart failure symptoms, months	12 (5–60)	10 (4–48)	48 (10–72)	0.08
Laboratory findings				
Hemoglobin, g/L	144 (130–151)	146 (137–154)	143 (126–150)	0.41
eGFR, mL/min/1.73 m^2	85 (73–105)	83 (73–100)	93 (73–107)	0.27
BNP, ng/L	728 (90–1887)	248 (46–893)	1280 (343–2681)	<0.01
hs-troponin T, pg/mL	29.9 (18.5–48.5)	25.8 (16.2–45.1)	31 (22.7–59.7)	0.34
Concomitant cardiac medication				
ACE-I/ARB, n (%)	41 (72%)	21 (73)	19 (68)	0.71
Beta-blocker, n (%)	54 (95%)	28 (97)	26 (93)	0.61
MRA, n (%)	51 (90%)	24 (83)	27 (96)	0.19
Loop diuretics, n (%)	53 (93%)	26 (90)	27 (96)	0.61
Echocardiographic parameters				
LVEF, %	24 (2–32)	30 (21–35)	22 (18.8–26)	0.03
LV end-diastolic diameter, cm	6.9 (6.2–7.3)	6.7 (6.2–7.1)	6.9 (6.5–7.5)	0.17
Severe LV diastolic dysfunction, n (%)	23 (40)	7 (24)	16 (57)	0.01
Severe RV systolic dysfunction, n (%)	16 (28)	5 (17)	11 (39)	0.06
RV end-diastolic diameter, cm	3.3 (2.9–3.6)	3.1 (2.6–3.4)	3.6 (3.1–3.9)	<0.01
Hemodynamic measurements (n = 54)				
PCWP, mmHg	20 (15–30)	18 (14.8–24.8)	23 (16–34)	0.11
mPAP, mmHg	29 (21–39)	25 (21–37.3)	34 (27–43)	0.06
Cardiac index, L/min/m^2	2.2 (1.6–2.8)	2.27 (1.93–2.81)	2.0 (1.5–2.49)	0.14

Values are expressed as: median (25th percentile, 75th percentile) or n (%). ACE-I—angiotensin-converting enzyme inhibitor, ARB—angiotensin II receptor blocker, BNP—B type natriuretic peptide, eGFR—estimated glomerular filtration rate, LV—left ventricle, LVEF—left ventricle ejection fraction, mPAP—mean pulmonary arterial pressure, MRA—mineralocorticoid receptor antagonist, NYHA—New York Heart Association, PCWP—pulmonary capillary wedge pressure, RV—right ventricle.

Table 2. Inflammation-related biomarkers of the study population, stratified by outcome.

Variable	All Patients (n = 57)	Event-Free Group (n = 30)	Event Group (n = 27)	p
Inflammation-related serum biomarkers				
WBC × 10^9/L (n = 57)	7.92 (5.92–10.06)	8.33 (6.19–10.75)	7.83 (5.7–9.13)	0.48
hs-CRP, mg/L (n = 56)	4.6 (1.5–15.7)	2.4 (1.3–15)	6.6 (2.7–16.1)	0.27
IL-6, pg/mL (n = 55)	2.5 (4.7)	2.0 (2.0–3.8)	5.24 (2.0–13.7)	0.002
TNF-α, pg/mL (n = 55)	8.6 (6.6–10)	7.5 (6.1–9.3)	9.0 (7.9–12.9)	0.03
Adiponectin, µg/mL (n = 55)	14.2 (7.97–28.85)	10.9 (5.63–18.96)	23.4 (11.1–32.9)	0.01
suPAR, ng/mg protein (n = 45)	1.79 (0.84–2.65)	1.77 (0.92–2.84)	1.79 (0.79–2.65)	0.92
Inflammatory cells in myocardium (n = 55)				
CD3+, cells/mm^2	10 (7–16)	10 (7–13)	10 (7–50)	0.39
CD45ro+, cells/mm^2	7 (5–10)	7 (5–9)	6.5 (5–10.5)	0.71
CD68+, cells/mm^2	4 (3–5)	4 (3–5)	3.5 (3–5)	0.96
CD4+, cells/mm^2	4 (2–6)	4 (2–6)	3 (2–8)	0.71
CD54+, cells/mm^2	0 (0–1)	0 (0–2)	0 (0–1)	0.29
HLA-DR+, cells/mm^2	5 (4–6)	5 (4–6)	5 (4–7)	0.71

Values are expressed as: median (25th percentile, 75th percentile). hs-CRP—high-sensitivity C-reactive protein, IL-6—interleukin 6, suPAR—soluble urokinase-type plasminogen activator receptor, TNF-α—tumor necrosis factor α, WBC—white blood cell.

Twenty-seven patients experienced adverse cardiac events during the five-year follow-up:

Ten (18%) patients died;

Nine (16%) underwent heart transplantation (the urgency status according to heart allocation policy [17]: six patients had status 1–3 and three patients had status 4);

Eight (14%) had an LVAD implantation (seven patients had 1–3 INTERMACS profile [18], one patient had profile 4).

The other patients remained on conventional medical heart-failure therapy. Based on these outcomes, we divided the cohort into two groups: event (n = 27) and event-free (n = 30) groups. Patients in the event group tended to have a longer duration of heart failure symptoms before enrollment than patients in the event-free group, but the difference was not statistically significant. Patients who experienced adverse outcomes had significantly lower systolic blood pressure, more impaired LV function, and enlarged right ventricle (Table 1). In addition, there were higher levels of BNP and inflammation-related cytokines (IL-6, TNF-α and adiponectin) in the event group compared to patients that remained event-free (Table 2).

3.2. Association between Inflammation-Related Biomarkers and Heart Failure Severity

We evaluated the correlations between inflammation-related biomarkers and parameters that reflect heart failure severity (Table 3). WBC, suPAR, infiltrative CD68+, CD4+, CD54+, and HLA-DR+ cells did not correlate with any of the heart failure severity parameters. A weak but significant positive correlation was found between infiltrative CD3+ and CD45ro+ cells and BNP levels, while hs-CRP significantly but weakly correlated with LVEF and BNP. Furthermore, levels of IL-6, TNF-α and adiponectin increased according to NYHA class (Figure 1) and correlated with all heart failure severity parameters (LVEF, mPAP, PCWP, and BNP), except TNF-α, which did not correlate with PCWP. Finally, IL-6 and adiponectin levels correlated most strongly with parameters of heart failure severity, particularly BNP.

Then we evaluated the correlations between inflammatory cells in the myocardium and inflammation-related biomarkers in serum (Table 4). Inflammatory cells did not correlate with biomarkers of systemic inflammation, except moderate correlation between CD68+ cells/mm^2 and IL-6.

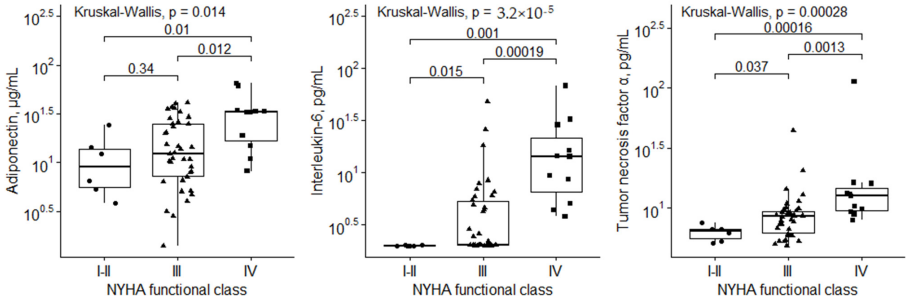

Figure 1. Serum cytokine levels based on NYHA functional class.

Table 3. Correlations between inflammatory-related biomarkers and parameters of heart failure severity.

	LVEF		mPAP		PCWP		BNP	
	r	*p*	r	*p*	r	*p*	r	*p*
WBC × 10^9/L	−0.09	0.5	0.23	0.12	0.05	0.7	0.01	0.97
hs-CRP, mg/L	−0.3	0.03	0.19	0.19	0.17	0.2	0.33	0.02
IL-6, pg/mL	−0.56	<0.0001	0.48	<0.001	0.42	<0.01	0.66	<0.0001
TNF-α, pg/mL	−0.29	0.03	0.39	<0.01	0.25	0.08	0.5	<0.01
Adiponectin, µg/mL	−0.37	<0.01	0.43	<0.01	0.39	<0.01	0.65	<0.0001
suPAR, ng/mg protein	−0.07	0.66	0.2	0.21	0.11	0.48	0.25	0.11
CD3+, cells/mm^2	−0.01	0.96	0.12	0.41	0.12	0.4	0.31	0.02
CD45ro+, cells/mm^2	−0.04	0.78	0.06	0.67	0.03	0.84	0.28	0.04
CD68+, cells/mm^2	−0.16	0.23	0.09	0.51	0.06	0.69	0.22	0.11
CD4+, cells/mm^2	0.18	0.18	−0.03	0.81	0.02	0.9	−0.01	0.98
CD54+, cells/mm^2	−0.1	0.7	0.1	0.57	0.12	0.39	0.15	0.28
HLA-DR+, cells/mm^2	−0.19	0.16	−0.02	0.88	0.04	0.76	0.12	0.38

BNP—B type natriuretic peptide, hs-CRP—high sensitivity C-reactive protein, IL-6—interleukin 6, LVEF—left ventricle ejection fraction, mPAP—mean pulmonary arterial pressure, NYHA—New York Heart Association, PCWP—pulmonary capillary wedge pressure, suPAR—soluble urokinase-type plasminogen activator receptor, TNF-α—tumor necrosis factor α, WBC—white blood cell. NYHA—New York Heart Association.

Table 4. Correlations between inflammatory cells in the myocardium and inflammatory-related biomarkers in serum.

	CD3+, cells/mm^2		CD45ro+, cells/mm^2		CD68+, cells/mm^2		CD4+, cells/mm^2		CD54+, cells/mm^2		HLA_DR+, cells/mm^2	
	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*
WBC × 10^9/L	−0.12	0.42	0.1	0.52	0.12	0.23	−0.06	0.67	0.1	0.48	−0.14	0.35
hs-CRP, mg/L	0.19	0.18	0.23	0.12	0.26	0.07	0.003	0.99	0.01	0.97	0.12	0.39
IL-6, pg/mL	0.21	0.12	0.09	0.51	0.35	0.009	−0.22	0.11	−0.03	0.89	0.09	0.53
TNF-α, pg/mL	0.21	0.14	0.01	0.94	0.2	0.15	−0.07	0.64	−0.05	0.72	0.1	0.49
Adiponectin, µg/mL	0.14	0.3	0.11	0.42	0.05	0.72	−0.2	0.87	0.02	0.91	0.13	0.37
suPAR, ng/mg protein	0.08	0.62	0.16	0.3	0.13	0.42	0.19	0.23	0.1	0.52	0.09	0.55

hs-CRP—high sensitivity C-reactive protein, IL-6—interleukin 6, suPAR—soluble urokinase-type plasminogen activator receptor, TNF-α—tumor necrosis factor α, WBC—white blood cell.

3.3. Inflammation-Related Biomarkers and Risk Prediction

We performed ROC analysis to identify the best cut-off value for each inflammation-related biomarker for predicting outcomes. Cut-off values are presented in Table S1. IL-6, TNF-α and adiponectin predicted adverse cardiac events with the highest accuracy (AUC 0.77, 0.65 and 0.70, respectively).

Kaplan–Meier survival analyses with log-rank tests were subsequently performed to reveal alterations in survival probability among patient groups based on the obtained cut-off values (Figure 2). Higher levels of inflammation-related markers (hs-CRP > 4.6 µg/mL,

IL-6 > 4.53 pg/mL, TNF-α > 7.81 pg/mL, adiponectin > 17.14 µg/mL) and higher numbers of infiltrative inflammatory cells (CD3+ > 13 cells/mm^2 and CD45ro+ > 11.5 cells/mm^2) were associated with lower survival rates (Figure 2). No significant differences in survival probability were obtained for the other inflammation-related biomarkers.

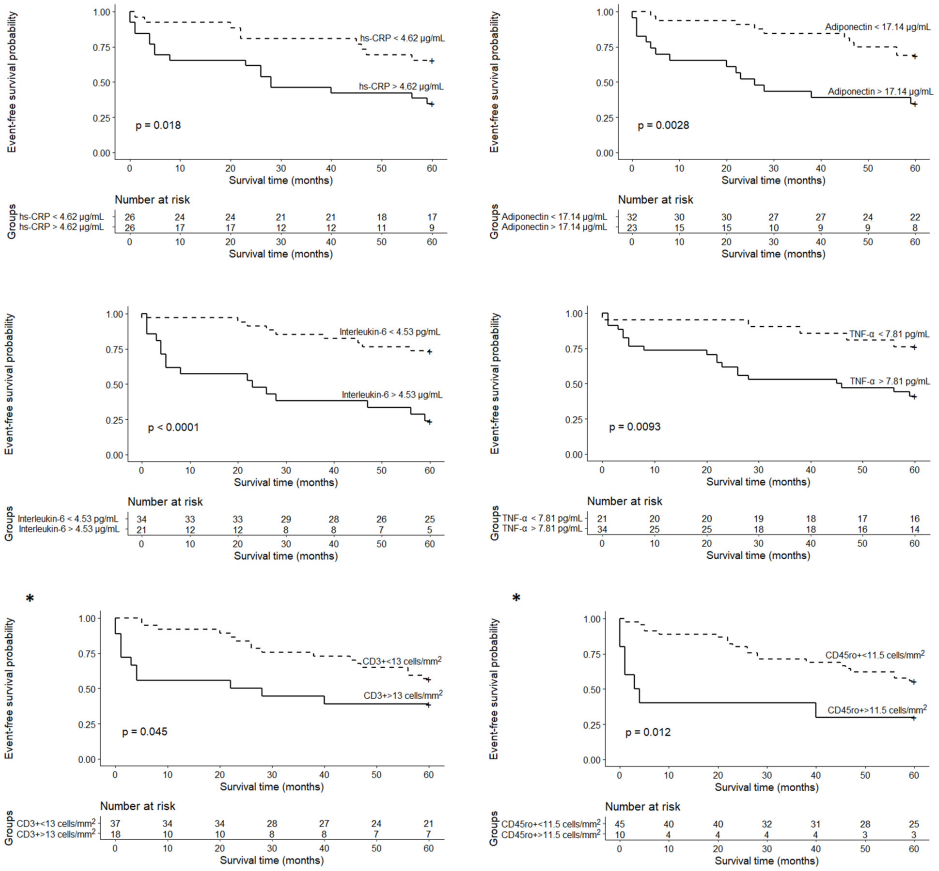

Figure 2. Survival curves stratified by inflammation-related biomarkers during the five-year follow-up. hs-CRP—high-sensitivity C reactive protein, TNF-α—tumor necrosis factor α. * Adapted with permission from https://doi.org/10.5603/cj.a2020.0088 (accessed on 19 September 2021) [12].

Univariate Cox regression analysis showed that systolic and diastolic blood pressure, echocardiographic parameters (LVEF, severe LV diastolic dysfunction, RV end-diastolic diameter, severe RV dysfunction), right heart catheterization measurements (mPAP, PCWP), inflammation-related serum biomarkers (hs-CRP > 4.62 µg/mL, IL-6 > 4.53 pg/mL, TNF-α > 7.81 pg/mL and adiponectin > 17.14 µg/mL) as well as inflammatory CD3+ > 13 cells/mm^2 and CD45ro+ > 11.5 cells/mm^2 in the myocardium predicted adverse cardiac events. Parameters with a *p*-value < 0.1 in univariate Cox regression analysis are shown in Table 5. Multivariate Cox analysis revealed that IL-6 > 4.53 pg/mL, adiponectin > 17.14 µg/mL,

hs-troponin T, CD45ro+ > 11.5 cells/mm^2 and diastolic blood pressure could independently predict adverse cardiac events.

Table 5. Results of Cox regression analysis for predictors of adverse cardiac events.

	Univariate		Multivariate	
Variable	**HR (95% CI)**	***p***	**HR (95% CI)**	***p***
Systolic blood pressure, mmHg	0.97 (0.95–0.995)	0.02		
Diastolic blood pressure, mmHg	0.96 (0.93–1.0)	0.05	0.95 (0.91–0.99)	0.01
LVEF, %	0.93 (0.88–0.97)	0.003		
Severe RV systolic dysfunction	2.81 (1.29–6.11)	0.009		
Severe LV diastolic dysfunction	3.15 (1.45–6.84)	0.003		
RV end-diastolic diameter, cm	2.65 (1.4–5.0)	0.003		
PCWP, mmHg	1.06 (1.01–1.11)	0.01		
mPAP, mmHg	1.05 (1.01–1.08)	0.01		
log BNP, ng/L	1.47 (1.16–1.86)	0.002		
hs-troponin T, pg/mL	1.004 (1–1.01)	0.068	1.005 (1.0–1.01)	0.04
IL-6 > 4.53 pg/mL	4.78 (2.09–10.89)	0.0002	6.26 (2.52–15.58)	<0.0001
hs-CRP > 4.62 µg/mL	2.57 (1.14–5.79)	0.02		
TNF-α > 7.81 pg/mL	3.42 (1.28–9.15)	0.01		
Adiponectin > 17.14 µg/mL	3.2 (1.43–7.16)	0.005	5.43 (1.01–10.33)	<0.001
CD3+ > 13 cells/mm^2	2.18 (1.01–4.7)	0.048		
CD45ro+ > 11.5 cells/mm^2	2.89 (1.22–6.87)	0.012	3.23 (1.01–10.33)	0.048

BNP—B type natriuretic peptide, CI—confidence interval, HR—hazard ratio, hs—high-sensitivity, hs-CRP—high-sensitivity C reactive protein, IL-6—interleukin 6, LV–left ventricle, LVEF—left ventricle ejection fraction, mPAP—mean pulmonary arterial pressure, PCWP—pulmonary capillary wedge pressure, RV—right ventricle, TNF-α—tumor necrosis factor α.

3.4. Combined Assessment of Inflammation-Related Cytokines

As all three cytokines that were measured predicted adverse cardiac events with the highest accuracy (Table S1), they were subjected to further analysis. Figure 3 shows a risk stratification based on the three cytokines. All patients (n = 12) with IL-6 < 4.53 pg/mL, adiponectin < 17.14 µg/mL and TNF < 7.81 pg/mL had a 100% five-year event-free survival, whereas all patients (n = 10) with concentrations of these three cytokines above the cut-off values experienced adverse cardiac events. Patients with one or two cytokines above the cut-off value had a similar five-year survival: 50% and 40% event-free survival rate, respectively.

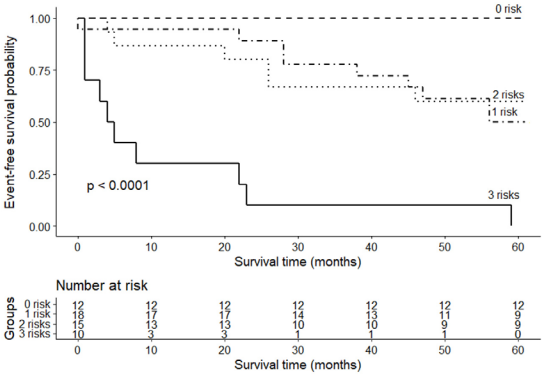

Figure 3. Event-free survival from adverse cardiac events according to a triple in Figure > 17.14 µg/mL, IL-6 > 4.53 pg/mL and TNF > 7.81 pg/mL as unfavorable risk factors.

4. Discussion

This study evaluates the association of inflammation-related biomarkers with heart failure severity, as well as their prognostic value, in a cohort of NI-DCM patients. The main findings are that:

hs-CRP is associated with certain heart failure severity parameters (LVEF, BNP) and adverse cardiac events;

suPAR is not associated with heart failure severity or poor clinical outcomes in NI-DCM patients;

Higher counts of CD3+ T lymphocytes and CD45ro+ memory T cells correspond to a poorer clinical outcome.

Higher levels of inflammation-related cytokines (IL-6, TNF-α, adiponectin) are associated with heart failure severity and predict poor clinical outcomes.

An increase in serum levels of all three cytokines (IL-6 > 4.53 pg/mL, TNF-α > 7.81 pg/mL and adiponectin > 17.14 mg/mL) can be helpful in identifying high-risk patients.

hs-CRP is an acute-phase protein produced mainly by hepatocytes under the influence of cytokines. Elevated hs-CRP levels are detected and associated with poor clinical outcomes in chronic heart failure patients [19,20]. However, the predictive value of hs-CRP in NI-DCM patients has not been resolved. Lamblin et al. [21] evaluated hs-CRP predictive value in 546 patients with heart failure with reduced ejection fraction (non-ischemic etiology ~60%). hs-CRP was a predictor of mortality in the whole cohort and ischemic heart failure subgroup during the median 2.7-year follow-up but not in non-ischemic patients. Li et al. [22] found that hs-CRP was an independent predictor of mortality during an average of 2.6 years of follow-up in 622 NI-DCM patients. Ishikawa et al. [23]. also demonstrated that hs-CRP was an independent predictor of poor clinical outcome in a cohort of 84 NI-DCM patients during 42 months of follow-up. In our study, which employed a longer follow-up period than previous studies, we found that hs-CRP was associated with long-term adverse cardiac events, although it did not remain a significant predictor following multivariate analysis.

suPAR is a proinflammatory marker originating from proteolytic cleavage and releasing of the membrane-bound urokinase-type plasminogen activator receptor from vascular endothelial and immune cells [24]. It is associated with infectious diseases, systemic inflammation, malignancies [25–27] and cardiovascular diseases, predominantly ischemic heart disease [28]. Several studies have analyzed suPAR in chronic heart failure patients, but the results have been inconsistent. Lichtenauer et al. [29] found no difference in suPAR concentrations between NI-DCM (n = 65) and ischemic heart failure (n = 59) patients. However, they observed an increase in suPAR according to NYHA stage, which reached a plateau at NYHA stage III. In another study, Koller et al. [30] found that suPAR was associated with adverse cardiac events in 319 chronic heart failure patients (55% NYHA class II, 43% class III). van den Berg et al. [31] found suPAR was associated with adverse cardiac events in 263 mixed-etiology chronic heart failure patients (26% had NYHA III-IV class) during a median of 2.2 years of follow-up. In contrast to earlier findings, we did not detect any association between suPAR and heart failure severity parameters or adverse cardiac events. A possible explanation for this might be that most patients in our cohort were classified as NYHA III-IV when suPAR levels would be expected to reach a plateau [29]; consequently, this marker possibly loses its prognostic value in our more advanced NI-DCM patient cohort.

The presence of infiltrative inflammatory cells in the myocardium can be assessed to diagnose inflammation [32]. However, only a few studies have estimated the prognostic value of distinct inflammatory cells, with inconsistent results [33,34]. Zuern et al. [33] found no association between CD3+ and CD68+ cells and clinical outcome in 227 patients with congestive heart failure (79% chronic myocarditis or non-inflammatory DCM) during three-years of follow-up. Nakayama et al. [34] evaluated CD3+ and CD68+ cells in the myocardium in 182 DCM patients. During an average of 6.9 years of follow-up, both inflammatory cell types were associated with poor clinical outcomes but did not remain

significant in a multivariate approach. Our results demonstrate that CD3+ and CD45ro+ cells, but not CD68+, CD4+, CD54+ or HLA-DR+ cells, were associated with poor clinical outcomes during the five-year follow-up period. Higher counts of CD45ro+ cells remained an independent predictor of adverse cardiac events in the multivariate analysis. These divergent results might be influenced by differences in statistical analysis and follow-up duration, unequal proportions of different etiopathogenetic phenotypes in the NI-DCM cohorts [12], as well as biopsy collection occurring at different time points over the course of the disease [35].

Adiponectin is an adipocyte-derived cytokine, which acts in obesity-linked diseases [36], renal failure, and various cardiovascular diseases [37]. However, its actions are multifaceted and controversial [38]. Adiponectin has cardioprotective [39], antiatherogenic [40] and anti-inflammatory effects [41,42] in the context of atherosclerotic cardiovascular diseases. Despite these beneficial effects, adiponectin levels increase with the clinical worsening of chronic heart failure [43,44], and are associated with higher mortality [45,46]. Our study results support previous findings demonstrating an association of adiponectin with heart failure severity parameters and adverse clinical outcomes in NI-DCM patients. Previously, Wojciechowska et al. [47] found that elevated adiponectin levels were associated with poor clinical outcomes in 129 NI-DCM patients during three-years of follow-up. Our results are consistent with these findings and show that adiponectin remains a significant predictor in NI-DCM patients during the five-year follow-up.

IL-6 and TNF-α are proinflammatory cytokines produced mostly by activated monocytes and macrophages. The proinflammatory role of these cytokines in systemic inflammation is well established in various pathologies, including cardiovascular diseases such as atherosclerotic cardiovascular disease and chronic heart failure [48,49]. In addition, the prognostic role of IL-6 and TNF-α in chronic heart failure patients has been demonstrated in previous studies [50,51]. However, approximately half of these cohorts were composed of ischemic heart failure patients, which might have had a significant impact on the predictive value of these cytokines, considering that serum concentrations of IL-6 and TNF-α are significantly higher in ischemic heart failure patients than in patients with NI-DCM [9,10]. To the best of our knowledge, the prognostic value of IL-6 and TNF-α has not previously been estimated in a NI-DCM patient population. We found an association between the cytokines, IL-6 and TNF-α, and adverse cardiac events during the five-year follow-up period. In addition, IL-6 > 4.53 pg/mL was an independent predictor of long-term poor clinical outcomes.

The present study also revealed that inflammation-related cytokines (IL-6, TNF-α and adiponectin) were associated with heart failure severity. These results are consistent with those of previous studies, which showed an association between circulating cytokines and heart failure progression (i.e., NYHA functional class and/or BNP levels) [9,44,52]. Additionally, our results supplement and consolidate previous findings, in which, besides having an association with NYHA and BNP, cytokines also correlated with other heart failure severity parameters, such as LVEF, PCWP (except TNF-α), and mPAP. Moreover, these cytokines showed the highest accuracy in predicting clinical outcomes out of all the inflammation-related biomarkers that we investigated. Our most important clinically relevant finding was that the combination of all three cytokines could help identify high-risk patients. Patients who had low concentrations of all three biomarkers did not experience adverse cardiac events. In contrast, all patients with higher concentrations than the cut-off values of all three cytokines experienced adverse cardiac events during follow-up, mainly within the first two years. Therefore, the use of inflammation-related cytokines might aid in identifying high-risk NI-DCM patients, which could lead to changes in follow-up intensity, the timing for device therapy, or heart transplantation.

Overall, this research evaluated both biomarkers of myocardial and systemic inflammation. A significant finding to emerge from this study is that inflammatory cells in the myocardium did not correlate with biomarkers of systemic inflammation, except CD68+ cells with IL-6. There is a lack of consistent data about inflammatory cells in an advanced disease phase [53] because they are mainly studied in patients with acute myocarditis,

but not in a more advanced disease phase–NI-DCM. Therefore, we hypothesize that the absence of the association may partly be explained by the existence of different etiopathogenetic sub-groups in this cohort with different myocardial inflammation statuses [12]. The other possible explanation for this might be that pathological processes in the myocardium, including inflammation, cause malfunction and remodeling of the heart, which triggers a systemic inflammatory response that proceeds to a low-grade inflammation (para-inflammation) state leading to further disease progression [54]. This hypothesis might be supported by another significant finding from this study: systemic inflammation-related biomarkers (cytokines) predicted clinical outcomes more significantly and with higher accuracy than infiltrative inflammatory cells. This finding also reflects the importance of chronic low-grade systemic inflammation in the disease progression.

Limitations

Our study's main limitation is the single-center small sample size, which prevents us from making generalizations about independent predictors of poor clinical outcomes or exact cut-off values for each inflammation-related biomarker in patients with NI-DCM. Small-sample size also limited deeper subgroup analysis (e.g., urgent versus non-urgent heart transplantation or LVAD implantation) and the ability to perform more extent survival analysis—competing risks regression models. In addition, we did not evaluate the dynamics of the biomarkers during follow-up, which could help to understand the pathologic processes leading to heart failure progression more comprehensively. Although we demonstrated the ability of risk stratification by three inflammation-related cytokines, a larger study is needed to verify and build on the findings of the present study.

5. Conclusions

Increased myocardial and systemic inflammation is associated with poor long-term outcomes. IL-6, adiponectin and memory T cells appear to be independent predictors of poor clinical outcomes. IL-6, TNF-α and adiponectin are associated with disease severity, and a combination of these can be helpful in identifying high-risk NI-DCM patients. Future larger-sample research is necessary to confirm and validate these findings.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/life11101006/s1, Table S1: Cut-off values identified by ROC analysis of inflammation-related biomarkers for predicting adverse cardiac events.

Author Contributions: I.K., conceptualization, formal analysis, data curation, writing—initial draft preparation, visualization; V.B., data curation, writing—reviewing and editing; I.R., investigation, writing—reviewing and editing; E.Ž.—investigation, writing–reviewing and editing; D.V., investigation, writing—reviewing and editing; V.V.M., investigation, writing—reviewing and editing; K.R., investigation, writing—reviewing and editing; V.G., methodology, investigation, supervision, project administration, funding acquisition, writing—reviewing and editing. All the authors have read and approved the manuscript version submitted. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The datasets during and/or analyzed during the current study available from the corresponding author on reasonable request.

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Baltrūnienė V, Bironaitė D, **Kažukauskienė I,** Bogomolovas J, Vitkus D, Ručinskas K, Žurauskas E, Augulis R, Grabauskienė V.

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Research Article

The Role of Serum Adiponectin for Outcome Prediction in Patients with Dilated Cardiomyopathy and Advanced Heart Failure

Vaida Baltrūnienė,[1] Daiva Bironaitė,[2] Ieva Kažukauskienė,[1,3] Julius Bogomolovas,[1,4] Dalius Vitkus,[5] Kęstutis Ručinskas,[3] Edvardas Žurauskas,[1] Renaldas Augulis,[1] and Virginija Grabauskienė[1,3]

[1]*Department of Pathology, Forensic Medicine and Pharmacology, Faculty of Medicine, Vilnius University, M. K. Ciurlionio 21, LT-03101 Vilnius, Lithuania*
[2]*Department of Regenerative Medicine, State Research Institute, Center for Innovative Medicine, Santariskiu 5, LT-08406 Vilnius, Lithuania*
[3]*Department of Cardiology and Angiology, Vilnius University Hospital Santaros Klinikos, Santariskiu 2, LT-08661 Vilnius, Lithuania*
[4]*Department of Integrative Pathophysiology, Medical Faculty Mannheim, Mannheim, Germany*
[5]*Department of Physiology, Biochemistry, Microbiology and Laboratory Medicine, Faculty of Medicine, Vilnius University, Vilnius, Lithuania*

Correspondence should be addressed to Vaida Baltrūnienė; vaida.baltruniene@gmail.com

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Clinical interpretation of patients' plasma adiponectin (APN) remains challenging; its value as biomarker in dilated cardiomyopathy (DCM) is equivocal. We evaluated whether circulating APN level is an independent predictor of composite outcome: death, left ventricle assist device (LVAD) implantation, and heart transplantation (HT) in patients with nonischemic DCM. 57 patients with nonischemic DCM (average LV diastolic diameter 6.85 cm, LV ejection fraction 26.63%, and pulmonary capillary wedge pressure 22.06 mmHg) were enrolled. Patients underwent echocardiography, right heart catheterization, and endomyocardial biopsy. During a mean follow-up of 33.42 months, 15 (26%) patients died, 12 (21%) patients underwent HT, and 8 (14%) patients were implanted with LVAD. APN level was significantly higher in patients who experienced study endpoints (23.4 versus 10.9 ug/ml, $p = 0.01$). APN was associated with worse outcome in univariate Cox proportional hazards model (HR 1.04, CI 1.02–1.07, $p = 0.001$) but lost significance adjusting for other covariates. Average global strain (AGS) is an independent outcome predictor (HR 1.42, CI 1.081–1.866, $p = 0.012$). Increased circulating APN level was associated with higher mortality and may be an additive prognostic marker in DCM with advanced HF. Combination of serum (APN, BNP, TNF-α) and echocardiographic (AGS) markers may increase the HF predicting power for the nonischemic DCM patients.

1. Introduction

In recent years, the concept of chronic heart failure (CHF) pathogenesis has changed dramatically. It became clear that CHF is not simply a hemodynamic failure and even not a problem of impaired neuroendocrine activation; it is a far more complex process, a systemic disorder, which involves immune activation, metabolic alterations, and pathologic processes in skeletal muscle [1].

Adiponectin (APN) is an adipocyte-derived cytokine (adipokine), which is also synthesized in cardiac muscle cells and connective tissue cells within the heart [2]. APN has a critical signaling function in the heart which is particularly important in patients with heart failure.

Its beneficial cardioprotective effects leave no doubt. APN has antiapoptotic, fibrosis reducing, and oxidative stress diminishing properties in myocardium [3, 4]. Lower serum APN is an independent cardiovascular risk factor in coronary

artery disease [5–8]. Low serum APN levels also increase cardiovascular risk and inflammation in hypertension, coronary artery disease, obesity, and insulin resistance [5, 9, 10] and correlates with left ventricular (LV) hypertrophy [11, 12]. On the other hand, high APN levels are associated with increased risk of recurrent cardiovascular events [13] and mortality in patients with acute myocardial infarction [14] and heart failure [15, 16]. In patients with systolic HF, APN levels are increased and correlate with mortality, disease severity, and HF symptoms [17, 18]. Adiponectin concentration increases with increasing HF severity and parallels NYHA functional class [19–22]. It is still a question of debate whether APN loses its cardioprotective function in CHF or it fails to control progression of disease.

APN has also been investigated as a prognostic marker in CHF. Although it seems that APN has an additive role in predicting the disease course [23–25], it has not been officially recognized as a biomarker in HF with reduced ejection fraction [26].

Tamura and coauthors show that APN can be an independent predictor of mortality in patients with ischemic CHF. But they did not find significant impact of high serum APN level on the mortality of patients with nonischemic CHF [25].

Nonischemic DCM is an important cause of HF and heart transplantation (HT). So there is a compelling need for markers predicting the prognosis and disease course of the end-stage heart failure caused by DCM. It is also of great importance in prioritizing patients' list for transplantation.

In this study, we investigated the predictive potential of serum APN with regard to LVAD implantation, HT, and mortality in a cohort of patients with nonischemic dilated DCM and advanced HF and analyzed the associations between APN and other biomarkers of CHF.

2. Material and Methods

2.1. Patients. Our study cohort was composed of patients admitted to Vilnius University Hospital "Santaros klinikos" with suspected diagnosis of idiopathic DCM. The patient inclusion criteria were exacerbation of heart failure symptoms, accompanied by LV dilation, reduced LV ejection fraction (LVEF < 45%) and the absence of significant coronary artery disease (stenosis of coronary arteries of more than 50%), a history of myocardial infarction, and other specific heart muscle diseases (primary valvular heart disease, toxic cardiomyopathy, arterial hypertension, renal failure, and abuse of alcohol or illicit drugs).

All patients underwent a careful history and physical examination, routine laboratory studies, including high-sensitivity C reactive protein (CRP), brain natriuretic peptide (BNP), adiponectin, tumor necrosis α (TNF-α), interleukin-6 (IL-6), and cardiac troponin T (hsTnT) as well as echocardiography to evaluate LV function and EF. NYHA class was assigned prior to echocardiographic investigation. Mandatory investigations included coronary angiography to exclude significant coronary disease, right heart catheterization for hemodynamic evaluation, and EMB for evaluation of inflammatory infiltrates in myocardium.

57 patients with nonischemic DCM (average LV end diastolic diameter (LVEDD) 6.85 ± 0.86 cm, LVEF 26.4 ± 9.45%, and mean pulmonary capillary wedge pressure (PCWP) 22.06 ± 8.97 mmHg) were enrolled into the study consistent with primary DCM. 49 (90%) of the patients were ranked as NYHA III and IV classes and all had increased BNP values.

All patients had long duration of HF symptoms: at enrollment, the average duration of observed symptoms was 40 ± 53 months.

At the moment of enrollment to the study, the patients were normotensive to hypotensive: the average of systolic blood pressure was 116 ± 20 mmHg and average of diastolic pressure was 80 ± 10 mmHg. Patients with long standing arterial hypertension in anamnesis were not included in this study. Patients diagnosed with diabetes were not enrolled.

All patients received pharmacological HF therapy according to the guidelines of European Society of Cardiology [27]: ACE inhibitors or blockers of angiotensin receptors, β-blockers, mineralocorticoid receptors blockers, digitalis (in case of atrial fibrillation), diuretics, anticoagulant (in case of atrial fibrillation, LVEF < 40%), and antiarrhythmic (class III: amiodarone). Thiazolidinedione was not administered to any patients at the time of blood sample collection. Clinical decision regarding cardiac resynchronization therapy, radiofrequency ablation, implantation of LVAD, or cardioverter-defibrillator was made after coronary angiography and right heart catheterization. In case of histologically proven acute myocarditis, patients were excluded from the study. Baseline characteristics of all patients are presented in Tables 1 and 2.

2.2. Follow-Up Period. Patients were followed up for a mean of 33.42 ± 21 months. The first date of the follow-up was the date of taking EMB. The endpoint of this study was composite and combined three possible outcomes: death from cardiovascular causes, LVAD implantation, or HT. The rationale to use this composite end point was that all those states meant either death or a very severe cardiac state of the patient with exhausted therapeutic measures. Some of the patients experienced several outcomes. The time of the endpoint was the time of the first event.

During the follow-up period, 25 patients (43.8%) reached endpoint of the study (died or underwent HT or LVAD implantation). Patients were subdivided into two groups according to their outcomes: those who have reached follow-up endpoint $n = 25$ and the ones who did not – $n = 32$ in order to see if there is a significant difference in level of APN in both groups.

All deaths and other endpoint outcomes were confirmed by medical records or telephone interview with the patients' families.

2.3. Biochemical Assays of APN and Other Serological Markers. Blood samples data were obtained shortly after admission. Blood was drawn at the same day as cardiac catheterization.

The proinflammatory serum cytokines TNF-α and IL-6 were measured by solid-phase, chemiluminescent immunometric assays using IMMULITE/Immulite 1000 systems (Immulite, Siemens) according to manufactures instructions:

Table 1: Baseline characteristics and treatment of patients.

Parameter	Value	Total number of cases
Sex	M: 45 (78.95%) F: 12 (21.05%)	57
NYHA class		55
I	1 (1.81%)	
II	5 (9.09%)	
III	38 (69.09%)	
IV	11 (20.01%)	
iDCM	30 (55.56%)	55
Medications received		
ACE inhibitors	31 (54%)	57
Diuretics and mineralocorticoids receptor blockers	55 (96%)	57
ß-Blockers	52 (91%)	57
Digitalis (in atrial fibrillation)	18 (32%)	57
Anticoagulation (atrial fibrillation, EF < 40%)	33 (58%)	57
Antiarrhythmic (class III: amiodarone)	10 (18%)	57

iDCM: inflammatory dilated cardiomyopathy, ACE: angiotensin converting enzyme.

Table 2: Baseline characteristics of patients.

Variable	Mean ± SD or median ± IQR*	Total number of cases
Age (years)	47.3 ± 10.9	57
BMI (kg/m^2)	26.84 ± 8.39*	57
GFR (ml/min)	108.6 ± 38.6	54
Systolic BP (mmHg)	116 ± 20	57
Diastolic BP (mmHg)	80 ± 10	57
Duration of symptoms before enrollment	40 ± 53	57
Glucose (mmol/l)	5.37 ± 1.25	34
APN (μg/ml)	14.2 ± 20.8*	55
BNP (pg/ml)	727.7 ± 1796.8*	56
Il-6 (pg/ml)	2.4 ± 4.7*	55
TNF-α (pg/ml)	8.6 ± 3.37*	55
CRP (μg/ml)	4.6 ± 14.2*	52
hsTnT (pg/ml)	29.92 ± 30.04*	55
LVEF (%)	26.08 ± 9.5	57
LVEDD (cm)	6.8 ± 0.8	57
Average global strain (%)	−8.07 ± 3.5	41
Mean RAP (mmHg)	11 ± 6.5*	51
Mean PAP (mmHg)	29 ± 18*	53
Mean PCWP (mmHg)	21.8 ± 8.9	54
CO (l/min)	4.00 ± 1.88	51
CI (l/min/m^2)	2.16 ± 1.14	51
CD3+ (cells/mm^2)	10 ± 9*	55
CD45ro+ (cells/mm^2)	7 ± 5*	55
CD68+ (cells/mm^2)	4 ± 2	55

BMI: body mass index, GFR: glomerular filtration rate, systolic BP: systolic blood pressure, diastolic BP: diastolic blood pressure, APN: adiponectin, BNP: brain natriuretic peptide, IL-6: interleukin-6, TNF-α: tumor necrosis factor α, CRP: C reactive protein, hsTnT: high sensitivity troponin T, LVEF: left ventricular ejection fraction, LVEDD: left ventricle end diastolic diameter, RAP: right atrial pressure, PAP: pulmonary artery pressure, PCWP: pulmonary capillary wedge pressure, CD3+: T cell receptor, CD45ro+: memory T cell receptor, CD68+: monocyte/macrophage receptor, CO: cardiac output, and CI: cardiac index; *median ± interquartile range.

TNF-α (Catalog number LKNFZ (50 tests) and LKNF1 (100 tests)), IL-6 (Catalog number LK6PZ (50 tests) and LK6P1 (100 tests)). Adiponectin was measured by Millipore Adiponectin assay according to manufacturers' recommendations (Millipore, USA).

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

Brain natriuretic peptide (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 and washed again and the chemoluminescence initiating mixture was added. Resulting chemiluminescent reaction was measured by chemiluminometer and expressed as relative light units (RLU).

2.4. Echocardiography. Echocardiographic evaluation was accomplished on admission by investigator blinded for the study objectives. GE Vivid 7 and 9 ultrasound systems were used. The standard LV apical (apical 4, apical 2, and apical 3) views and parasternal short axis views at mid-papillary level were acquired at 70–90 frames/s. Conventional echocardiographic parameters such as LVEF, LVEDD, left ventricular end-systolic dimension (LVESD) [28], velocities of E and A waves (E and A) and their ratio (E/A), and E deceleration time (DcT) were obtained. All images were stored in PACS for subsequent analysis. Quantification of myocardial deformation values was performed by 2D speckle tracking using Echopac PCBT08 (GE Healthcare) software. After the manual selection, speckles were assumed automatically and then confirmed by the investigator. Longitudinal, circumferential, and radial strain and strain rate parameters were extracted using semiautomatic postprocessing. Global strain is presented as the mean value of all valid segments. Global strain in our measurements showed an interobserver variability of 1.1 + 0.9% and an interobserver variability of 1.3 + 1.2%.

2.5. Cardiac Catheterization and Endomyocardial Biopsy. All patients signed written informed consent for cardiac catheterization and EMB and coronary angiography, which included resulting analysis to elucidate a possible origin of the myocardial and coronary artery diseases. Each patient underwent coronary angiography to exclude significant coronary artery disease (stenosis > 50%) and right heart catheterization to assess hemodynamic parameters: mean pulmonary artery pressure (PAP), right atrial pressure (RAP), pulmonary capillary wedge pressure (PCWP), and cardiac output (CO).

Right ventricular EMB was obtained using a flexible bioptome *(Westmed)* via the right femoral vein [29]. Biopsies were drawn from the right interventricular septum. At least 3 EMBs were subjected to conventional histologic and immunohistochemical evaluation and 2 EMBs were stored at −70 C in the biobank as retained biosamples. EMBs were immediately placed on ice and investigated within 24 hours.

2.6. Histological and Immunohistochemical Assessment of EMBs. 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 Hematoxylin and Eosin (H&E) according to the standard protocol for the routine histological evaluation. Histological diagnosis was based on the Dallas criteria [30, 31]. The experienced pathologist evaluated endocardium (thickness, subendocardial fat, fibrosis, and inflammation); myocardium (muscle fiber number, size, and damage); interstitium (fibrosis, fat, edema, and inflammation); and intramural vessels (size, signs of inflammation, damage, and luminal stenosis). Immunohistological assessment of EMBs was carried out as described elsewhere [32]. Autoantibodies (Santa Cruz Biotechnology, Inc.) against CD3+ (DAKO A0452 Rabbit 1, Hamburg, Germany), CD45Ro (DAKO Hamburg), and CD68+ (DAKO M0876 Mouse 1, Hamburg) were used for immunohistochemical staining. The number of positively stained cells in each biopsy sample was scored by an experienced pathologist and expressed as number of positive cells/mm2. EMB were considered to be inflamed if IHC staining revealed significant inflammatory cellular infiltrates (≥14 leucocytes/mm2 including up to 4 monocytes/mm2 with the presence of CD3 positive T-lymphocytes ≥7 cells/mm2) [33, 34].

2.7. Statistical Analysis. Statistical analyses were performed using the SPSS package (version 23.0 for Windows; IBM.SPSS statistics) and R studio package (version 1.0.143 – © 2009–2016 RStudio, Inc.) at not higher than 5% significance level. The normality of the data distribution was tested by the Shapiro-Wilk test. Variables which did not follow normal distribution were expressed as medians (interquartile ranges). All the other continuous variables were expressed as means ± SD.

Significance of measurements was tested by Student's t-test or the Wilcoxon–Mann–Whitney rank sum nonparametric test.

For comparative purposes, Spearman's correlation coefficient was used. Testing the differences between parameter values in the subgroups of nonischemic DCM patients (good versus bad outcome groups, high versus low adiponectin groups) Student's t-test or the nonparametric Mann–Whitney U test (serum adiponectin, BNP, IL-6, TNF-α, TnT, CRP, etc.) was used. Differences between categorical variables were tested using Chi-squared test.

Kaplan–Meier analysis was used to compare the cumulative survival rates between the 2 subgroups of nonischemic DCM patients stratified according to the median serum APN levels. Differences between the survival times were tested using a log-rank analysis for APN. Univariate analysis with the Cox proportional hazards model was used to assess the association of each variable with patient survival. Multivariate analysis with the Cox proportional hazards model was used to assess the independence of the predictors of composite endpoint. The covariates included (1) the parameters with $p < 0.05$ in the univariate analysis (APN, IL-6, average global strain, CD3+ cell number in myocardium, and PCWP) and (2) the established predictors of mortality

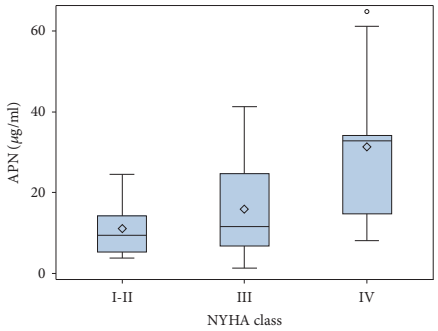

Figure 1: Adiponectin according to NYHA classes.

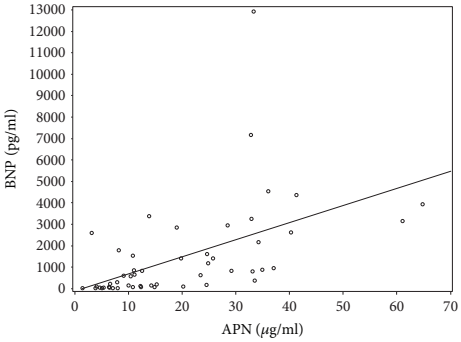

Figure 2: APN correlation with BNP.

in CHF patients (gender, age, EF, NYHA class IV versus I–III, and GFR). Stepwise selection procedure was used for choosing the independent predictors of outcome.

For the search of a set of variables which could be a reflection of some more global parameter or be a good combination for predicting patient outcome factor analysis was used.

2.8. Ethical Approval. The study was approved by the local Lithuanian Bioethics Committee (license numbers 158200-09-382-l03; 158200-382-PP1-23; and 158200-17-891-413). Informed consent was signed by all study patients. Our study was conducted in accordance with the Declaration of Helsinki.

3. Results

3.1. Correlations between Serum Adiponectin Level, Cardiac Hemodynamics, and Inflammation in Patients with Nonischemic DCM. The median of adiponectin in our cohort of patients was 14.2 ± 20.8 μg/ml. There was a tendency for APN concentration to rise in each subsequent NYHA functional class. (Figure 1). APN concentration was statistically significantly higher in NYHA functional class IV in comparison to NYHA functional class III ($p = 0.014$). We found no difference in APN means between I + II and III NYHA functional classes ($p = 0.712$).

The significant correlation between APN and BNP (rho 0.65, $p = 0.001$) was found and is shown in (Figure 2). APN has also had a positive correlation with inflammatory cytokine TNF-α (rho 0.331, $p = 0.021$) (Figure 3), although correlation with another inflammatory cytokine IL-6 (rho 0.257, $p = 0.058$) was not statistically significant. Both inflammatory cytokines IL-6 (rho 0.656, $p < 0.001$) and TNF-α (rho 0.504, $p = 0.004$) significantly correlated with BNP.

APN also positively correlated with certain hemodynamic parameters such as mean PCWP (rho 0.38, $p = 0.005$) (Figure 4) and mean PAP (rho 0.434, $p = 0.001$). There was a significant association between APN and LV dysfunction

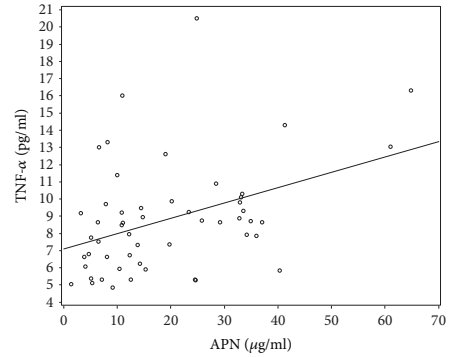

Figure 3: APN correlation with TNF-α.

parameter, average global strain (rho 0.472, $p = 0.002$) (Figure 5). We found no significant correlation with LVEF and negative correlation with BMI did not reach statistical significance ($p = 0.054$). APN correlation data are shown in Table 3.

3.2. Adiponectin Level Is Significantly Higher in the Bad Outcome Group. During the follow-up period, 25 patients (43.8%) reached endpoint of the study: 15 (26%) patients died because of cardiovascular causes, 12 (21%) patients underwent heart transplantation (HT), and 8 (14%) were implanted with LVAD. 3 out of 8 patients with LVAD underwent HT later on.

Kaplan–Meier cumulative survival curve was drawn Figure 6.

The patients were divided into two groups according to their outcome: bad outcome (the ones who reached the composite endpoint) $n = 25$ and good outcome group $n = 32$. The groups did not differ in their age ($p = 0.08$), sex ($p = 0.863$), NYHA class ($p = 0.119$), BMI ($p = 0.51$), and GFR

Table 3: APN correlations with other parameters.

Variables	Correlation coefficient	p value	Number of cases
BMI (kg/m^2)	−0.266	0.054	53
Systolic BP (mmHg)	−0.061	0.66	55
Diastolic BP (mmHg)	−0.315	0.019	55
Serum glucose (mmol/l)	−0.099	0.58	33
BNP (pg/ml)	0.651	**<0.001**	54
IL-6 (pg/ml)	0.257	0.058	55
TNF-α (pg/ml)	0.311	**0.021**	55
LVEF (%)	−0.206	0.139	53
RAP (mmHg)	0.310	**0.030**	49
PAP (mmHg)	0.434	**0.001**	51
PCWP (mmHg)	0.388	**0.005**	51
Average global strain (%)	0.472	**0.002**	40

BMI: body mass index, systolic BP: systolic blood pressure, diastolic BP: diastolic blood pressure, BNP: B-type natriuretic peptide, IL-6: interleukin-6, TNF-α: tumor necrosis factor α, LVEF: left ventricle ejection fraction, RAP: right atrial pressure, PAP: pulmonary artery pressure, and PCWP: pulmonary capillary wedge pressure; significant correlation is bolded. Correlation is significant at the p level 0.05 (2-tailed).

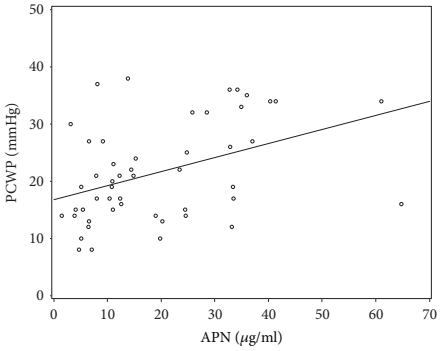

Figure 4: APN correlation with PCWP.

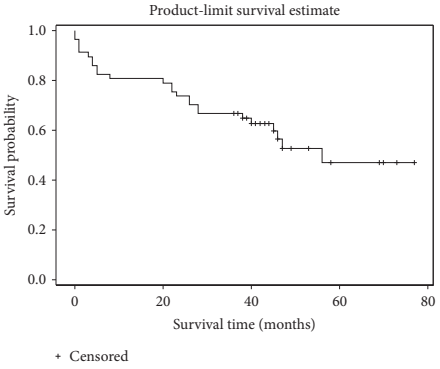

Figure 6: Kaplan–Meier survival curve for the whole study cohort.

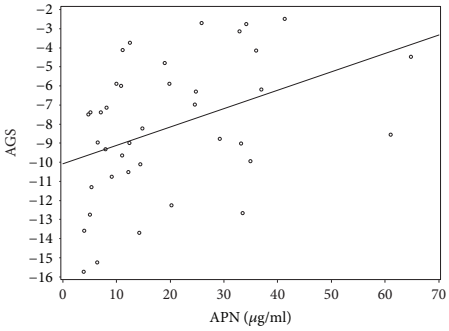

Figure 5: APN correlation with average global strain (AGS).

(p = 0.30) (Table 4). There was significant difference in the baseline concentration of APN between the two groups (10.9 ± 17.87 μg/ml versus. 23.4 ± 23.1 μg/ml, p = 0.01) (Figure 7).

The concentrations of IL-6 and BNP at baseline were also statistically significantly higher in the bad outcome group. Patients in the bad outcome group had worse hemodynamic parameters: lower LVEF (22.42 ± 7.19% versus 29.45 ± 9.9%, p = 0.005), CO (3.51 ± 1.94 ml/min versus 4.47 ± 1.26 ml/min, p = 0.049), CI (1.79 ± 0.80 ml/min/m^2 versus 2.09 ± 0.71 ml/min/m^2, p = 0.036), higher intracardiac pressures: mean PAP (35.5 ± 16.75 mmHg versus 24.0 ± 16.0 mmHg, p = 0.02), and mean PCWP (25.36 ± 9.9 mmHg versus 19.7 ± 7.56 mmHg, p = 0.03). Average of global strain was also significantly lower in the bad outcome group (−5.46 ± 2.3% versus −10.11 ± 2.87%, p = 0.001).

Table 4: Baseline characteristics of good and bad outcome patient groups.

	Good outcome	Number of cases	Bad outcome	Number of cases	p value
Age (years)	49.72 ± 9.57	32	44.24 ± 11.98	25	0,08
Sex	F: 7 (22%) M: 25 (80%)	32	F: 5 (20%) M: 20 (80%)	25	0,863
NYHA functional class	I: 1 (3.3%) II: 4 (13.3%) III: 22 (73.3%) IV: 3 (10.1%)		I: 0 II: 1 (4%) III: 16 (64%) IV: 8 (32%)		0,119
Inflammatory infiltrates in myocardium	Inflammatory DCM Noninflammatory DCM	15 (51.7%) 14 (48.3%)		15 (60%) 10 (40%)	0,541
BMI (kg/m^2)	27.96 ± 5.57	32	27.06 ± 5.17	25	0.51
GFR (ml/min)	108.4 ± 33.83	29	117.98 ± 31.38	25	0.30
Systolic BP (mmHg)	124 ± 21	32	106 ± 15	25	**0.0002**
Diastolic BP (mmHg)	80 ± 13	32	70 ± 15	25	**0.01**
Serum glucose (mmol/l)	5.45 ± 1.49	19	4.94 ± 1.25	15	0.093
APN* (ug/ml)	10.9 ± 17.87	32	23.4 ± 23.1	23	**0.01**
BNP* (pg/ml)	228 ± 915.4	31	1397.1 ± 2500.75	25	**0.004**
IL-6* (pg/ml)	2.01 ± 2.36	32	5.45 ± 12.29	23	**0.002**
TNF-α* (pg/ml)	8.2 ± 3.71	32	8.74 ± 4.73	23	0.239
CRP (ug/ml)	3.25 ± 15.73	28	6.55 ± 14.13	24	0.388
hsTnT* (pg/ml)	24.67 ± 27.8	32	32.98 ± 44.3	23	0.167
LVEDD (cm)	6.65 ± 0,67	32	7.09 ± 0.9	25	0.062
LVEF (%)	29.45 ± 9.9	32	22.56 ± 7.0	25	**0.005**
Average global strain (%)	−10.11 ± 2.87	23	−5.46 ± 2.30	18	**0.001**
Mean RAP* (mmHg)	9.00 ± 7.0	31	13.00 ± 15.5	20	**0.016**
Mean PAP* (mmHg)	24.0 ± 16.0	31	35.5 ± 16.75	22	**0.021**
Mean PCWP (mmHg)	19.7 ± 7.56	31	25.36 ± 9.9	22	**0.03**
CD3+ (cells/mm^2)	10 ± 5	30	10 ± 11	25	0.249
CD45ro+ (cells/mm^2)	7 ± 4	30	7 ± 7	25	0.574
CD68+ (cells/mm^2)	5 ± 2	30	3 ± 2	25	0.716
CO (ml/min)	4.47 ± 1.26	29	3.51 ± 1.94	22	**0.049**
CI (ml/min/m^2)	2.09 ± 0.71	30	1.79 ± 0.80	21	**0.036**

BMI: body mass index, GFR: glomerular filtration rate, systolic BP: systolic blood pressure, diastolic BP: diastolic blood pressure, APN: adiponectin, BNP: B-type natriuretic peptide, IL-6: interleukin-6, TNF-α: tumor necrosis factor α, CRP: C reactive protein, TnT: hs troponin T, LVEF: left ventricular ejection fraction, LVEDD: left ventricle diastolic diameter, RAP: right atrial pressure, PAP: pulmonary artery pressure, PCWP: pulmonary capillary wedge pressure, CD3+: T cell receptor, CD45ro+: memory T cell receptor, CD68+: monocyte/macrophage receptor, CO: cardiac output, and CI: cardiac index; significant values are bolded. Significant at the p level 0.05 (2-tailed). *Median ± interquartile range.

An increased level of circulating APN was associated with worse outcome in patients with nonischemic DCM and advanced HF.

3.3. Cumulative Survival Differs in Patients with High and Low Adiponectin Levels. All patients were divided into two groups according to their APN concentration at baseline: above and equal to APN median ($n = 28$) or below the median ($n = 27$). The baseline characteristics of high and low APN groups are depicted in Table 5.

Patients with APN levels above the median have also had a significantly higher BNP value (113.8 ± 694.35 pg/ml versus 1397 ± 2338.5 pg/ml, $p < 0.001$) and higher TNF-α concentration (7.54 ± 3.18 pg/ml versus 9.09 ± 2.54 pg/ml, $p = 0.029$). Their mean PAP (25.5 ± 13.00 mmHg versus 37.0 ± 19.0 mmHg, $p = 0.036$) was elevated in comparison to lower APN group. Cardiac output was significantly lower in the above the median APN group (3.9 ± 1.32 versus 4.9 ± 2.77, $p = 0.035$). Age, BMI, GFG, and LVEF were comparable between the groups.

Kaplan–Meier survival curve method and log-rank analysis revealed that event-free survival times differ significantly in both groups. Worse outcome was in the higher (≥14.2 μg/ml) APN group ($p = 0.042$) (Figure 8).

The biggest difference between the curves was seen for the first 4 years. Afterwards survival curves started approximating each other. So the prognostic value of APN level could be helpful in the near future.

Table 5: Baseline characteristics in patient groups with high and low APN value.

Parameters	APN < 14,2 μg/ml	APN ≥ 14.2 μg/ml	p value
Sex	M: 23 (85.2%) F: 4 (14.8%)	M: 21 (75%) F: 7 (25%)	0.345
NYHA class	I + II: 4 (15.4%) III: 20 (76.9%) IV: 2 (7.7%)	I + II: 2 (7.4%) III: 16 (59.3%) IV: 9 (33.3%)	0.061
Age (years)	48.19 ± 8.29	46.96 ± 12.89	0.679
BMI* (kg/m^2)	27.92 ± 5.70	24.41 ± 9.00	0.168
GFR (ml/min)	111.78 ± 27.66	110.70 ± 36.64	0.907
Systolic BP (mmHg)	121 ± 21	112 ± 20	<**0.0002**
Diastolic BP (mmHg)	75 ± 10	80 ± 10	0.647
Serum glucose (mmol/l)	5.38 ± 1.07	5.38 ± 2.39	0.913
BNP* (pg/ml)	113.8 ± 694.35	1397 ± 2338.5	<**0,001**
IL-6* (pg/ml)	2,01 ± 4.27	3,18 ± 6.08	0.245
TNF-α* (pg/ml)	7.54 ± 3.18	9.09 ± 2.54	**0.029**
CRP* (ug/ml)	3.05 ± 6.53	6.15 ± 13.23	0.252
TnT* (pg/ml)	23.55 ± 28.28	31,00 ± 31.76	0.368
LV DD (cm)	6.80 ± 0,80	6.95 ± 0.94	0.548
EF (%)	27.15 ± 9.60	26.22 ± 9.50	0.493
Average global strain (%)	−9.26 ± 3.42	−7.09 ± 3.38	0.051
CD3+* (cells/mm^2)	10 ± 5	11 ± 10	0.364
CD45ro+* (cells/mm^2)	6 ± 3	7 ± 5	0.493
CD68+* (cells/mm^2)	5 ± 2	5 ± 2	0.769
Mean RAP* (mmHg)	11.00 ± 8.00	11.50 ± 11.50	0.099
Mean PAP* (mmHg)	25.5 ± 13.00	37.0 ± 19.00	**0.036**
Mean PCWP (mmHg)	19.50 ± 8.26	24.12 ± 8.73	0.083
CO (l/min)*	4.9 ± 2.77	3.9 ± 1.32	**0.035**
CI (l/min/m^2)*	2.29 ± 1.12	2.05 ± 0.89	0.131

BMI: body mass index, GFR: glomerular filtration rate, APN: adiponectin, BNP: brain natriuretic peptide, IL-6: interleukin-6, TNF-α: tumor necrosis factor α, CRP: C reactive protein, hsTnT: high sensitivity troponin T, LVEDD: left ventricle diastolic diameter, LVEF: left ventricle ejection fraction, CD3+: T cell receptor, CD45ro+: memory T cell receptor, CD68+: monocyte/macrophage receptor, RAP: right atrial pressure, PAP: pulmonary artery pressure, PCWP: pulmonary capillary wedge pressure, CO: cardiac output, and CI: cardiac index. *Data presented as median ± interquartile range. Significant at the p level 0.05 (2-tailed).

Patients with higher APN values had a significantly increased composite endpoint risk which was the most evident in the first few years.

3.4. Adiponectin's and Other Parameter Role in Predicting Outcome in Patients with Nonischemic DCM and Advanced HF. Univariate Cox proportional hazard model showed that APN statistically significantly increases the risk of worse outcome (HR 1.04, $p = 0.001$). Other parameters at baseline also influenced survival and increased the risk of reaching the composite endpoint (Table 6).

NYHA functional class IV appeared to be most significantly associated with worse outcome (HR 3.84, $p = 0.005$). Average global strain also proved to be a powerful tool in predicting the risk of reaching the endpoint (HR 1.7, $p < 0.001$).

We also found that increase in IL-6 concentration (HR 1.04, $p = 0.001$), increase in number of CD3+ cells in myocardium (HR 1.06, $p = 0.006$), decrease in LVEF (HR 0.93, $p = 0.01$), and increased PAP (HR 1.06, $p = 0.004$) and PCWP (HR 1.07, $p = 0.004$) raised the risk of reaching the endpoint. What was unexpected is that BNP did not increase the risk in our model.

Table 6: Parameters influencing outcome.

Univariate Cox regression analysis	HR	95% CI for HR	p
NYHA (class IV versus I–III)	3.48	1.452–8.359	**0.005**
APN (μg/ml)	1.04	1.016–1.067	**0.001**
IL-6 (pg/ml)	1.04	1.01–1.07	**0.004**
BNP (pg/ml)	1.00	1.000–1.000	0.061
Average global strain (%)	1.69	1.322–2.180	<**0.001**
CD3+ (cells/mm^2)	1.06	1.015–1.099	**0.006**
LVEF (%)	0.93	0.882–0.983	**0.010**
Mean PAP (mmHg)	1.06	1.018–1.097	**0.004**
Mean PCWP (mmHg)	1.07	1.022–1.127	**0.004**

APN: adiponectin, IL-6: interleukin-6, BNP: brain natriuretic peptide, CD3+: T cell receptor, LVEF: left ventricle ejection fraction, PAP: pulmonary artery pressure, and PCWP: pulmonary capillary wedge pressure.

Table 7: Independent outcome predictors.

	HR	95% CI (L) HR	95% CI (U) HR	p value
Average global strain	1.42	1.081	1.866	0.012
NYHA (IV class)	4.69	1.052	20.872	0.043

Table 8: Relative effect on patient outcome for individual parameters best characterizing Factor 1 versus Factor 1.

Parameter	HR	95% CI	p
APN	1.51	1.149–1.993	0.003
BNP	1.49	1.152–1.952	0.003
TNF-α	671.04	0.122–3698148	0.138
Average global strain	1.70	1.322–2.180	<0.001
Factor 1	2.61	1.437–4.727	0.0016

APN: transformed adiponectin value, BNP: transformed brain natriuretic peptide value, and TNF-α: transformed tumor necrosis factor α value.

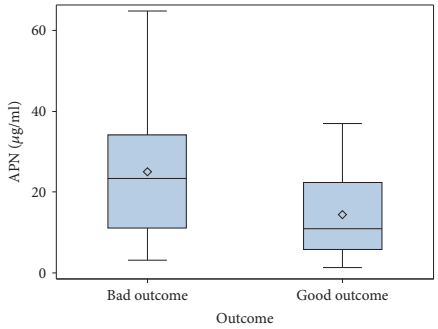

Figure 7: Adiponectin level in good and bad outcome groups.

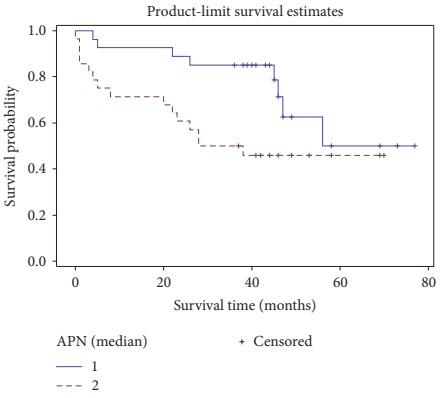

Figure 8: Cumulative survival curve of patient groups stratified by APN median. Straight line: APN < 14.2 ug/ml, dashed line: APN ≥ 14.2 ug/ml.

We looked if the impact of APN on composite endpoint remains after adjusting for other covariates in multivariate Cox regression analysis model. The covariates were age, sex, GFG, LVEF, NYHA class (which are known to have an impact on survival of patients with heart failure), and parameters which appeared to be significant in univariate regression analysis (APN, IL-6, CD 3+ cell count, and average global strain). After adjusting for these covariates, using stepwise model selection, APN lost its significance. NYHA class IV (HR 4.686, $p = 0.012$) and average global strain (HR 1.4, $p = 0.043$) appeared to be independent outcome predictors in our data (Table 7).

3.5. Increasing Predictive Potential of Serum Adiponectin Level in DCM Patients. Factor analysis was performed in order to see if there is a combination of parameters, which could be an expression of a more global factor having impact on patient outcome.

There were 39 cases with complete set of data which were used for the analysis. Continuous variables (age, LVEF, APN, BNP, Troponin T, TNF-α, and average global strain) were enrolled. Variables without normal distribution were transformed using Box-Cox method. Principal component analysis extracted 3 factors with eigenvalues above 1. The rotated factor pattern for all three factors is shown in Figures 9–11.

Factor 1 showed 53,73% of parameter variability and was characterized by a strong loading of APN, BNP, TNF-α, and average global strain. Those are the parameters of proinflammatory status and myocardial dysfunction. There was a significantly worse cumulative survival in Factor 1 above median group (Figure 12).

Cox regression analysis was performed for all parameters with highest Factor 1 loadings separately as well as Factor 1. Factor 1 increased the HR of composite endpoint to 2,6 ($p = 0.0016$, 95% CI 1.437–4.727) and that was more than of any individual parameter (Table 8).

Increased levels of APN and proinflammatory cytokine TNF-α together with the parameters of myocardial function (BNP, average global strain) could be applied in predicting patients' outcome. Combination of those parameters

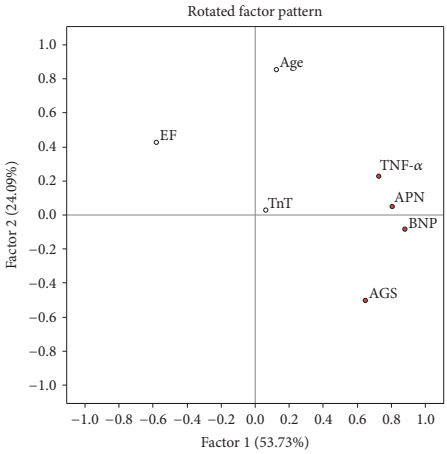

Figure 9: EF: left ventricle ejection fraction, TnT: troponin T, TNF-alpha: tumor necrosis factor α, APN: adiponectin, BNP: brain natriuretic protein, and AGS: average global strain. Parameters with maximal loadings characterizing Factor 1 are marked in red.

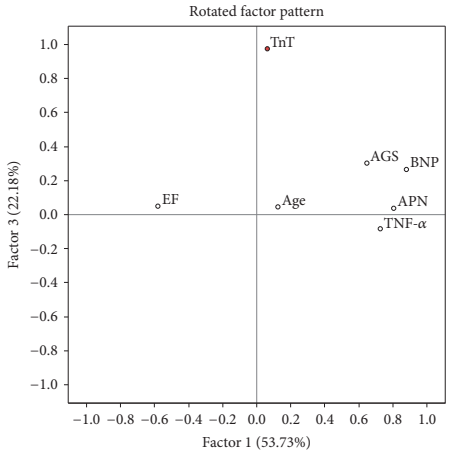

Figure 10: EF: left ventricle ejection fraction, TnT: troponin T, TNF-alpha: tumor necrosis factor α, APN: adiponectin, BNP: brain natriuretic protein, and AGS: average global strain. Parameters with maximal loadings characterizing Factor 3 are marked in red.

significantly increases APN predictive power in patients with DCM and advanced HF.

Factor 2 was best characterized by positive loading of age. Factor 3 was described mostly by troponin T, parameter of myocardial necrosis. Neither Factor 2 nor Factor 3 did show significant changes in hazard ratio or survival curves in our model.

4. Discussion

The purpose of this study was to investigate the predictive potential of APN with regard to LVAD implantation, HT, and mortality in a cohort of patients with nonischemic dilated DCM and advanced HF and also to analyze the association between APN and other biomarkers of CHF.

We found elevated serum APN concentrations in patients with DCM and advanced HF, similar to the ones reported by Huang et al. and Szabo et al. [35, 36]. Our findings are in agreement with previous studies [18, 23, 37]. The mechanisms of high serum APN concentration in HF are not clear; the possible reasons could be a compensatory response to HF progression or APN resistance [38, 39]. APN released from the heart may partly contribute to the increased serum APN level as reported by Takano et al. [40].

Higher circulating levels of APN are associated with increased mortality and disease severity in patients with HF [24, 40]. In our study, Kaplan–Meier survival method and log-rank analyses revealed that overall composite endpoint risk was significantly elevated in the higher serum APN group ($p < 0.042$). Univariate Cox proportional hazard model showed that increase in APN level statistically significantly elevates the risk of worse outcome (HR 1,04, $p = 0,001$), which is relevant to other publications [36]. After adjusting for other covariates (age, sex, GFG, LVEF, NYHA class, APN, IL-6, CD 3+ cell count, and average global strain) in multivariate Cox proportional hazard model APN lost its significance as independent prognostic marker. In agreement with other authors, role of APN as a predictive marker in chronic CHF is highly dependable on various clinical characteristics (age, sex, BMI, NYHA class, treatment received, renal function, type of HF, etc.) [41–44]. For this reason, clinical interpretation of APN level in patient's plasma is not straightforward and not so easily applicable in clinical practice.

Worsening of HF is associated with higher APN concentration, and increase of APN in serum parallels the increase in NYHA class [9, 21, 22, 45]. We could see the same tendency in our data concerning patients with nonischemic DCM with reduced LVEF. APN level in our cohort of patients was statistically significantly higher in NYHA class IV patients compared to NYHA class III. This observation has no easy explanation taking into account the positive functions ascribed to APN in terms of metabolism and cardioprotection. The relation of APN with HF severity confirms the fact that after implantations of LVAD the elevated APN levels have been reported to decline dramatically, in parallel with lowering of systemic and adipose-specific markers of inflammation, as well as improving insulin sensitivity [46].

Serum APN concentration is known to be related to several clinical variables. Our study confirmed correlations of adiponectin with BNP, TNF-α, average global strain, and increased intracardial pressures (PAP, PCWP). There was a strong positive correlation between serum APN and plasma BNP levels in our study. This could confirm the significance of

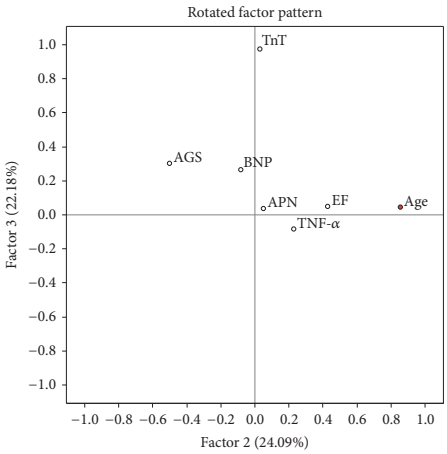

Figure 11: EF: left ventricle ejection fraction, TnT: troponin T, TNF-alpha: tumor necrosis factor α, APN: adiponectin, BNP: brain natriuretic protein, and AGS: average global strain. Parameters with maximal loadings characterizing Factor 2 are marked in red.

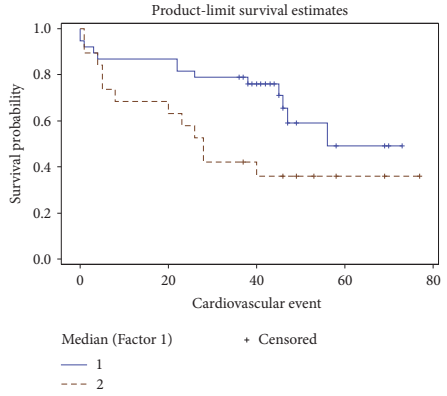

Figure 12: Survival curves according to Factor 1 median. Straight line: Factor 1 below the median, dashed line: Factor 1 above the median.

the circulating APN level as a prognostic marker in patients with DCM and advanced HF. The positive relationship between the 2 molecules can be explained by the results of Tsukamoto et al. study [47] which reported that natriuretic peptides enhance theproduction of APN in humanadipocytes in patients with advance chronic HF. In turn, recognition that natriuretic peptides stimulate APN secretion provides a mechanism linking elevated APN levels to more pronounced cardiac dysfunction and a poorer prognosis [48].

We also found that APN correlates with proinflammatory cytokine TNF-α. Our data do not contradict what is already known about proinflammatory state in patients with chronic HF [6, 9] which means that either APN adds up to ongoing systemic inflammation and acts as a proinflammatory factor or it is unable to overcome the increasing inflammatory milieu [26]. Unfortunately, up to now, the question remains open whether and/or when adiponectin serves as a pro- or anti-inflammatory cytokine in HF [26].

Serum APN was correlated with cardiac geometry and function according to previous studies [46, 49, 50] and was inversely associated with LVEF in elderly adjusted for BMI [51]. However, among all HF patients, there was no significant association between serum concentrations of APN and LVEF. We found significant correlations between APN and average global strain (rho 0.472, $p = 0.002$) on tissue Doppler strain measurement. Our findings are in agreement with previous studies showing that myocardial strain predicted rapid HF progression in end-stage DCM patients [52]. Myocardial strain was helpful for detecting the severity of heart failure as estimated by NYHA functional class [53]. Our data indicate that myocardial strain parameters are superior to LVEF, chamber diameter, and intracardiac pressure in predicting outcome in our patient cohort. According to our knowledge, this is the first report about the association between APN and average global strain, suggesting that serum APN could be a surrogate marker of myocardial dysfunction.

This study has some limitations which have to be pointed out. First, our study was a single center study with a small number of subjects; therefore, a study on a larger scale is warranted to confirm the relationship between worse prognosis and increased serum adiponectin levels in the iDCM patients with advanced HF. Second, the influences of drugs on the serum adiponectin levels should be considered. It is known that angiotensin-converting enzyme inhibitors, angiotensin-II receptor blockers, and β-blockers can improve the survival of CHF patients. All participants of our study were treated with optimal medical HF therapy when their blood samples were collected. Third, the study cohort consisted of patients with advanced HF (NYHA classes III-IV). Thus, future research is needed to confirm the validity of observed clinical correlations in patients with mild HF (NYHA I-II).

In conclusion, increased level of circulating APN was associated with higher risk of worse outcome (death from cardiovascular causes, LVAD, or HT) in nonischemic DCM patients with advanced HF. It did not appear to be an independent outcome predictor in our model. However, the combination of several sera (APN, BNP) and echocardiographic (average global strain) markers increased the outcome predicting power of APN for DCM patients. Elevated serum APN could serve as potential additive clinical prognostic marker uncovering the upcoming need to plan HT for the end-stage HF patients.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Left ventricular global longitudinal strain predicts elevated cardiac pressures and poor clinical outcomes in patients with non-ischemic dilated cardiomyopathy

Ieva Kažukauskienė[1*], Giedrė Balčiūnaitė[2], Vaida Baltrūnienė[1], Jelena Čelutkienė[2], Vytė Valerija Maneikienė[2], Sigitas Čibiras[3], Kęstutis Ručinskas[2] and Virginija Grabauskienė[1,2]

Abstract

Background: Risk stratification in patients with non-ischemic dilated cardiomyopathy (NI-DCM) is essential to treatment planning. Global longitudinal strain (GLS) predicts poor prognosis in various cardiac diseases, but it has not been evaluated in a cohort of exclusively NI-DCM. Although deformation parameters have been shown to reflect diastolic function, their association with other hemodynamic parameters needs further elucidation. We aimed to evaluate the association between GLS and E/GLS and invasive hemodynamic parameters and assess the prognostic value of GLS and E/GLS in a prospective well-defined pure NI-DCM cohort.

Methods and results: Forty-one patients with NI-DCM were enrolled in the study. They underwent a standard diagnostic workup, including transthoracic echocardiography and right heart catheterization. During a five-year follow-up, 20 (49%) patients reached the composite outcome measure: LV assist device implantation, heart transplantation, or cardiovascular death.

Pulmonary capillary wedge pressure (PCWP), mean pulmonary artery pressure, pulmonary vascular resistance (PVR) correlated with GLS and E/GLS ($p < 0.05$). ROC analysis revealed that GLS and E/GLS could identify elevated PCWP ($\geq$ 15 mmHg) and PVR (> 3 Wood units). Survival analysis showed GLS and E/GLS to be associated with short- and long-term adverse cardiac events ($p < 0.05$). GLS values above thresholds of –5.34% and -5.96% indicated 18- and 12-fold higher risk of poor clinical outcomes at one and five years, respectively. Multivariate Cox regression analysis revealed that GLS is an independent long-term outcome predictor.

Conclusion: GLS and E/GLS correlate with invasive hemodynamics parameters and identify patients with elevated PCWP and high PVR. GLS and E/GLS predict short- and long-term adverse cardiac events in patients with NI-DCM. Worsening GLS is associated with incremental risk of long-term adverse cardiac events and might be used to identify high-risk patients.

Keywords: Non-ischemic dilated cardiomyopathy, Global longitudinal strain, Strain-based index, Prognosis, Invasive hemodynamics, Heart failure

*Correspondence: ieva.zasytyte@gmail.com
[1] Department of Pathology, Forensic Medicine and Pharmacology, Institute of Biomedical Science, Faculty of Medicine, Vilnius University, M. K. Čiurlionio 21, 03101 Vilnius, Lithuania
Full list of author information is available at the end of the article

Introduction

Non-ischemic dilated cardiomyopathy (NI-DCM)—one of the main causes of heart failure—eventually leads to a high need for device therapy and heart transplantation. Heart failure, due to NI-DCM, accounts for 51–64% of all heart transplantations in the age group of 18–59 [1]. There is a need for reliable markers for identification of high-risk patients because they require close follow-up and timely decisions regarding advanced treatments.

Left ventricular (LV) global longitudinal strain (GLS) is a well-validated, easily performed echocardiographic parameter for evaluating myocardial deformation. GLS predicts poor prognosis in various cardiac diseases, including ischemic heart disease [2, 3] and heart failure with reduced ejection fraction (HFrEF) [4–6]. However, studies investigating mixed HFrEF populations have also included patients with ischemic heart disease (usually half of the cohort), who have a worse prognosis than patients with non-ischemic heart failure [7, 8]. Therefore, GLS prognostic significance in the HFrEF population has been confounded by the inclusion of ischemic heart failure patients. The predictive value of GLS has not been evaluated in patients with purely NI-DCM.

Evaluation of hemodynamic parameters is essential in prognostication and heart failure management, including device therapy and heart transplantation [9, 10]. Echocardiographic assessment is the main non-invasive diagnostic modality for the estimation of hemodynamic parameters. However, the relationship between various conventional echocardiographic parameters and invasively assessed pressures varies significantly in different studies [11–15], with each parameter having limitations. Thus, there is a need for new non-invasive parameters to judge about cardiac pressures. Several studies have proposed various diastolic strain-based indices that correlate with LV filling pressures [16–21], but variation in the parameters and technical challenges limit their use in clinical practice. Recently, Hayashi et al. [22] proposed a strain-based index of mitral E velocity ratio to GLS (E/GLS). The idea of the E/GLS was developed on the conventional echocardiographic parameter E/e' – a ratio of early-diastolic LV inflow velocity (E) to early-diastolic mitral annular velocity (e´). It is known that e' reflects longitudinal LV wall's expansion rate, but it is angle-dependent, affected by heart translation motion. In comparison, GLS reflects longitudinal deformation of the whole ventricle and does not have the above-mentioned e' limitations. This new index is strongly associated with LV mean diastolic pressure. Their findings encouraged further research in the relationship between invasive hemodynamic parameters and myocardial deformation measures.

Here, we aimed to evaluate the association of both GLS and E/GLS with invasive hemodynamic parameters; to evaluate the prognostic value of GLS and E/GLS for adverse cardiac events in a well-defined cohort of NI-DCM patients.

Methods

Study population and protocol

We enrolled 57 patients with suspected NI-DCM who were admitted to the university hospital for diagnostic evaluation between January 2010 and December 2013. Inclusion criteria were heart failure signs and symptoms, accompanied by echocardiographic evidence of LV dilatation and reduced ($\leq$45%) LV ejection fraction (LVEF). The study's primary aim was to identify etiopathogenetic factors—cardiotropic viruses and myocardial inflammation—of NI-DCM by evaluating various biomarkers in serum and endomyocardial biopsies [23, 24]. Forty-one patients had echocardiographic images of sufficient quality for further two-dimensional myocardial deformation analysis. These patients comprise the cohort of the present echocardiographic sub-study.

Exclusion criteria were: 1) significant coronary artery disease, defined as at least 50% proximal stenosis of a coronary artery, or a history of myocardial infarction; 2) other causes of heart failure, such as primary valvular or heart muscle disease, hypertensive heart disease, endocrine disease, advanced chronic kidney disease, drug or alcohol abuse; or 3) acute myocarditis (onset in the previous three months), or acute myocardial infarction as suspected by clinical presentation or diagnostic tests.

Study patients underwent a clinical evaluation and routine laboratory tests, including complete blood count, creatinine, and high-sensitivity C-reactive protein. Additionally, high-sensitivity troponin T (hs-TnT) was measured in serum using an Elecsys 2010 analyzer (Roche Diagnostics, Indianapolis, Indiana) and B-type natriuretic peptide (BNP) using ARCHITECT i analyzer (Abbott, Illinois, USA). Laboratory tests were performed in the laboratory of our university hospital, which is accredited according to the international standard EN/ISO-IEC 17,025.

Patients underwent transthoracic echocardiography on the same day or day before interventional procedures: coronary angiography and right heart catheterization. All patients were treated according to the guidelines of the European Society of Cardiology [25] and provided informed consent.

Echocardiography

Echocardiographic evaluation was carried out using commercially available ultrasound machines (GE Vivid 7 or 9) with a 2.5-MHz probe. Images were digitally stored

and analyzed offline using EchoPAC version PCBT08. We used a routine protocol of our laboratory for conventional M-mode, two-dimensional, Doppler, and tissue Doppler echocardiographic measurements [26]. LV end-diastolic (LVEDD) diameter was measured from the parasternal long-axis view and indexed to the body surface area. LV end-systolic and end-diastolic volumes were measured, and LVEF was calculated by the Simpson biplane method. Left atrium volume was measured by a biplane area-length method from the apical four- and two-chamber views and indexed to the body surface area. Mitral E and A peak velocity and deceleration time were measured, and the ratio of early-diastolic LV inflow velocity to atrial-systolic velocity (E/A) calculated. The average tissue Doppler-derived early diastolic mitral annular velocity (e') was obtained from the mitral annulus' septal and lateral sides. The average ratio of early-diastolic LV inflow velocity to early-diastolic mitral annular velocity (E/e´) was calculated. Mitral regurgitation and tricuspid regurgitation severity, as well as right ventricular function, were assessed visually.

Myocardial deformation analysis by two-dimensional speckle tracking echocardiography

Echocardiographic images were acquired at 50–70 frames/s (with individual adjustment) for LV GLS analysis. A digital loop was acquired from three apical views (four-, two- and three-chamber views). After the manual cardiac cycle selection, the LV endocardial border was manually traced at the end-systolic frame (aortic valve closure was used for the end-systole timing). The investigator visually assessed the detected region of interest (ROI) and, if necessary, manually modified the ROI to ensure accurate tracking of the speckles. In the case of inaccurate speckle tracking, ROI was readjusted. We calculated the GLS by averaging the mean values of all valid segments. We also calculated a strain-based index, i.e., the ratio of early-diastolic LV inflow velocity (E) to GLS (E/GLS).

Right heart catheterization

A Swan-Ganz catheter was inserted using a femoral approach in a supine position. The zero reference level of fluid-filled transducers was set at the mid-axillary line. Right atrium pressure, mean pulmonary artery pressure (mPAP), and pulmonary capillary wedge pressure (PCWP) were obtained. Wedge position in the PCWP measurement was confirmed by fluoroscopy, waveform changes, and arterial saturation $\geq 95\%$. Cardiac output (CO) was measured using Fick's method and calculated by an equation: CO (l/min) = oxygen consumption (ml/min) / ((aorta SaO_2 – pulmonary artery SvO_2) x hemoglobin $\times 1.34$). Only oxygen consumption (VO_2) was estimated indirectly using VO_2 nomograms based on age, weight and sex. Mixed venous blood was sampled for oximetry from the pulmonary artery, arterial blood – from the aorta. We calculated cardiac index (CO indexed to the body surface area), and pulmonary vascular resistance (PVR) ((mean PA pressure − PA wedge pressure)/CO) [27]. Each pressure measurement was recorded over a brief breath-hold at the end of expiration and was averaged over three consecutive cardiac cycles via computerized analysis [28]. On the basis of literature values, we used the following cut-offs for identifying elevated cardiac pressures: PCWP > 15 mmHg, mPAP, > 20 mmHg, and PVR > 3 Wood units [29].

Follow-up

Patients were followed up for five years after enrollment in the study. The clinical outcome measure was a composite endpoint of LV assist device implantation, heart transplantation, or cardiovascular death. The time of the first event was included in the analysis. Adverse cardiac events were confirmed by medical records, national death registry records, or telephone interviews with the patients' families.

Statistical analysis

Data analysis was performed using the R studio package (4.0.3 version). A p-value of < 0.05 was considered statistically significant. The Shapiro–Wilk statistic tested continuous variables for normal distribution. Normally distributed continuous variables were expressed as the mean ± standard deviation. Other continuous variables were expressed as the median (25th percentile, 75th percentile), and categorical data as counts and percentages. Continuous variables were compared by Student's independent t-test when normally distributed or by the Mann–Whitney-U test when non-normally distributed. Comparisons of categorical variables between the groups were made using the chi-square test or Fisher's exact test if expected values were < 5. The association between echocardiographic and hemodynamic parameters was assessed using Spearman correlation.

The receiver operating characteristic (ROC) curve was used to estimate how well the echocardiographic parameters identified elevated hemodynamic parameters, predicted composite outcome measures, and identified the optimal cut-off value for the prediction. Differences between areas under the curve (AUC) were tested using the bootstrap method. Kaplan–Meier analysis was used to compare the cumulative survival rates between the two groups of NI-DCM patients stratified by the GLS, E/GLS or LVEF cut-off values. The log-rank statistic was used to evaluate the statistical significance of differences between the curves. Cox proportional hazards regression

analysis was performed to evaluate which parameters were associated with poor one-year (short-term) and five-year (long-term) composite outcomes. We performed univariate Cox regression analysis for all baseline variables. The variables that were significant predictors in univariate analysis ($p < 0.05$) were enrolled in multivariate Cox regression analysis, which was performed using the stepwise backward elimination method.

Results

Baseline characteristics of the study population

The study included 41 patients with NI-DCM. The mean age was 47.0 ± 11.6 years and 33 (80%) patients were male. The majority of the patients were in the NYHA III-IV functional class. Patients had elevated PCWP (21 ± 8 mmHg), elevated mPAP (30 ± 12 mmHg), and low cardiac index (2.3 ± 0.7 l/min/m2) (Table 1). The mean LVEDD was 6.8 ± 0.8 cm, with a mean LVEF of 27.0 ± 9.1%. All patients had an impaired GLS (-8.1 ± 3.7%) (Table 2).

During the five-year follow-up, twenty (49%) patients experienced at least one adverse cardiac event: 6 patients underwent LV assist device implantation, 5 had heart transplants, and 9 died. Those who experienced adverse cardiac event had more unfavorable baseline characteristics, such as higher serum B-type natriuretic peptide levels and higher PVR (Table 1). Echocardiographic baseline characteristics, such as LVEF, GLS and E/GLS were more unfavorable in adverse cardiac event groups (Table 2).

Association between GLS, E/GLS and invasively measured cardiac pressures

We evaluated the association between GLS, E/GLS and other conventional echocardiographic parameters with invasive cardiac pressures and cardiac index. GLS as well as average E/e', TR velocity, LAVi and LVEF significantly correlated with cardiac pressures, while E/GLS correlated with cardiac pressures even stronger than GLS. E/GLS correlated with all cardiac pressures and cardiac index. The strongest correlation was between PVR and E/GLS

Table 1 Baseline characteristics of the study population, stratified by outcome

Variables	Total	Event-free group ($n = 21$)	Adverse cardiac event group ($n = 20$)	*p*-value
Clinical characteristics				
Age, years	47.0 ± 11.64	48.8 ± 10.0	45.2 ± 13.2	0.32
Male gender, n (%)	33 (80)	18 (86)	15 (75)	0.42
NYHA III-IV class, n (%)	36 (88)	17 (81)	19 (95)	0.34
Systolic BP, mm Hg	114 (104, 130)	120 (110, 130)	110 (103, 120)	0.09
Diastolic BP, mm Hg	80 (70, 80)	80 (70, 80)	73 (69, 80)	0.21
Concomitant cardiac medication/				
ACE-I/ARB, n (%)	31 (76)	17 (81)	14 (70)	0.48
Beta-blocker, n (%)	39 (95)	20 (95)	19 (95)	1
MRA, n (%)	37 (90)	19 (95)	18 (86)	0.6
Loop diuretics, n (%)	38 (93)	19 (91)	19 (95)	1
Biomarkers				
eGFR, ml/min/1.73 m^2	83 (71, 102)	83 (73, 100)	86 (71, 103)	0.82
BNP, ng/l	809 (79, 1523)	300 (47, 851)	1294 (506. 2920)	< 0.01
Troponin T, pg/ml	29.1 (17.3, 46.5)	23.6 (9.4, 41.7)	33 (25.2, 65.2)	0.09
Hemodynamic measurements				
mPAP, mmHg	28 (21, 38)	23 (21, 34)	33 (27, 40)	0.09
Elevated mPAP (> 20 mmHg), n (%)	33 (80)	17 (81)	16 (80)	1
PCWP, mmHg	19 (15, 27)	17 (14, 22)	22 (16, 33)	0.18
Elevated PCWP (> 15 mmHg), n (%)	27 (66)	12 (57)	15 (75)	0.23
PVR, Wood units	1.9 (1.2, 3.1)	1.6 (1, 2.4)	2.5 (1.8, 3.5)	< 0.05
Elevated PVR (≥ 3 Wood units), n (%)	10 (25)	3 (15)	7 (35)	0.14
Cardiac index, l/min/m^2	2.3 ± 0.7	2.4 ± 0.6	2.2 ± 0.8	0.49

Values are expressed as: mean ± SD, median (25th percentile, 75th percentile) or n (%)

ACE-I Angiotensin-converting enzyme inhibitor, *ARB* Angiotensin II receptor blocker, *BNP* B type natriuretic peptide, *BP* Blood pressure, *eGFR* Estimated glomerular filtration rate, *mPAP* mean pulmonary arterial pressure, *MRA* Mineralocorticoid receptor antagonist, *NYHA* New York Heart Association, *PCWP* pulmonary capillary wedge pressure, *PVR* pulmonary vascular resistance

Table 2 Echocardiographic characteristics of the study population, stratified by outcome

Variables	Total	Event-free group ($n=21$)	Adverse cardiac event group ($n=20$)	*p*-value
LVEF, %	25 (20, 34)	30 (25, 35)	21 (19, 23)	< 0.01
LV GLS, %	-8.1 ± 3.72	-9.9 ± 2.8	-6.2 ± 3.7	< 0.001
E/GLS [× 10^2], cm/s	-8.9 (-18.0, -6.3)	-8.1 (-9.8, -5.8)	-14.8 (-25.6, -8.6)	< 0.01
LVEDD, cm	6.8 ± 0.8	6.6 ± 0.7	7.1 ± 0.9	0.06
LAVi, ml/m^2	66 (50, 77)	55 (53, 76)	73 (46, 81)	0.63
Mitral DT, ms	164 (127, 194)	145 (111, 187)	142 (98, 187)	0.29
Mitral E/A	2.1 (0.9, 2.9)	2 (0.8, 2.7)	2.4 (1.2, 3.3)	0.44
Average E/e'	14.2 (12.3, 15.6)	13.7 (10.9, 15.1)	15.0 (13.1, 17.4)	0.13
Functional mitral regurgitation ≥ moderate, n (%)	24 (58)	11 (52)	13 (65)	0.41
Functional tricuspid regurgitation ≥ moderate, n (%)	16 (39)	6 (29)	10 (50)	0.16
RV end-diastolic diameter, cm	3.3 (3, 3.6)	3.2 (3.0, 3.5)	3.5 (3.1, 3.7)	0.08
Severely impaired RV systolic function, n (%)	13 (32)	5 (24)	8 (40)	0.27
TR systolic jet velocity (m/s)	2.7 (2.5, 3.2)	2.5 (2.4, 2.7)	2.8 (2.5, 3.0)	0.16

Values are expressed as: mean ± SD, median (25th percentile, 75th percentile) or n (%)

DT deceleration time, *E/A* ratio of early-diastolic LV inflow velocity (E) to atrial-systolic velocity (A), *E/e'* ratio of early-diastolic LV inflow velocity (E) to early-diastolic mitral annular velocity (e'), *E/GLS* ratio of early-diastolic LV inflow velocity (E) to global longitudinal strain (GLS), *GLS* global longitudinal strain, *LAVi* left atrial indexed volume, *LVEDD* left ventricular end-diastolic diameter, *LVEF* left ventricular ejection fraction, *TR* tricuspid regurgitation

(Table 3). Correlations between myocardial deformation parameters and invasive hemodynamic measurements are also plotted in Fig. 1. Additionally, we evaluated correlations between GLS, E/GLS and serum biomarkers. Both GLS and E/GLS strongly correlated with BNP, but not with troponin T. The strongest correlation was between E/GLS and BNP (Fig. 1).

We then tested echocardiographic parameters' ability to identify patients with elevated cardiac pressures. ROC analysis revealed that both GLS and E/GLS were good predictors of PCWP ≥ 15 mmHg and PVR > 3 Wood units, but not mPAP > 20 mmHg (Table 4). What stands out in Table 4 is that AUC was significant for GLS and E/GLS, but not for other echocardiographic parameters, except E/A, which predicted high PVR, and LAVi, which predicted elevated mPAP and high PVR.

Prognostic utility of GLS and E/GLS

We then used GLS, E/GLS and LVEF to identify high-risk patients in terms of adverse cardiac events. For comparison, we chose LVEF as the most widely used prognostic echocardiographic parameter in clinical practice. ROC analyses showed, that GLS, E/GLS and LVEF predicted

Table 3 Correlations between echocardiographic parameters and invasive hemodynamic ones

	PCWP, mmHg		mPAP, mmHg		PVR, Wood units		Cardiac index, l/min/m^2	
	r	*p*	*r*	*P*	*r*	*p*	*r*	*p*
DT, ms	-0,13	0.42	-0.17	0.28	**-0.4**	0.01	0.21	0.21
E/A	0.22	0.21	**0.38**	0.03	**0.62**	< 0.0001	**-0.55**	< 0.01
Average E/e'	**0.37**	0.02	**0.36**	0.02	**0.31**	0.048	-0.02	0.92
TR velocity, m/s	**0.54**	< 0.001	**0.59**	< 0.0001	**0.5**	< 0.001	-0.19	0.24
LAVi, ml/m^2	**0.40**	< 0.01	**0.47**	< 0.01	**0.6**	< 0.0001	**-0.45**	< 0.01
LVEF, %	**-0.38**	0.01	**-0.41**	< 0.01	**-0.45**	< 0.01	0.26	0.11
GLS, %	**0.38**	0.01	**0.35**	0.02	**0.46**	< 0.01	-0.31	0.06
E/GLS, [× 10^2] cm/s	**-0.5**	< 0.01	**-0.51**	< 0.01	**-0.65**	< 0.0001	**0.46**	< 0.01

The bold font character means statistically significance ($p < 0.05$)

DT deceleration time, *E/A* ratio of early-diastolic LV inflow velocity (E) to atrial-systolic velocity (A), *E/e'* ratio of early-diastolic LV inflow velocity (E) to early-diastolic mitral annular velocity (e'), *E/GLS* ratio of early-diastolic LV inflow velocity (E) to global longitudinal strain (GLS), *GLS* global longitudinal strain, *LAVi* left atrium indexed volume, *LVEF* left ventricular ejection fraction, *PCWP* pulmonary capillary wedge pressure, *mPAP* mean pulmonary arterial pressure, *PVR* pulmonary vascular resistance, *TR* tricuspid regurgitation

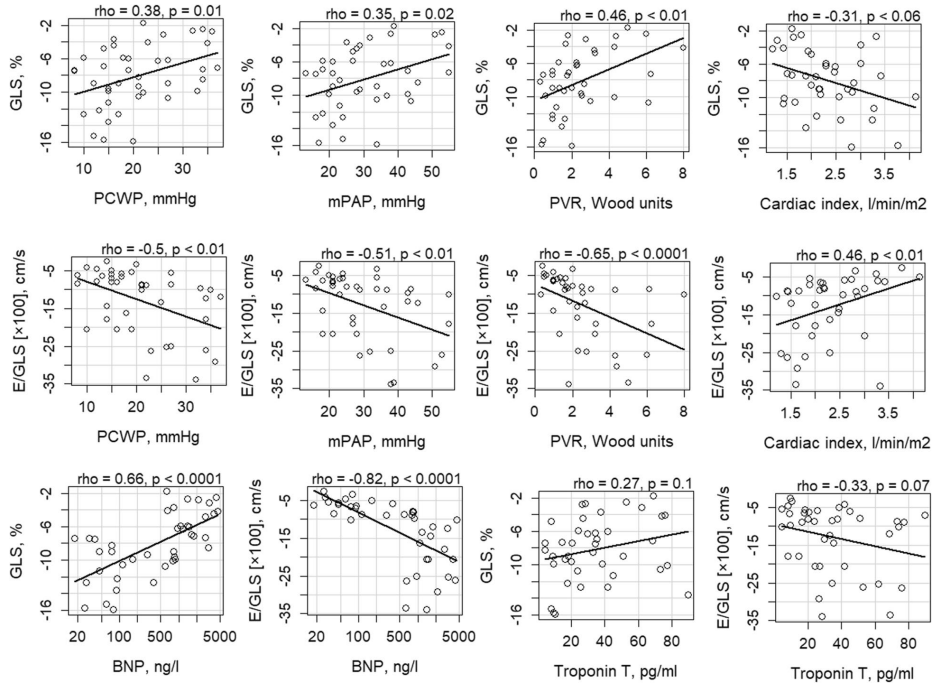

Fig. 1 Correlations between myocardial deformation parameters and invasive hemodynamic measures, and serum biomarkers. BNP – B type natriuretic peptide; E/GLS – ratio of early-diastolic LV inflow velocity (E) to global longitudinal strain (GLS); GLS – global longitudinal strain; LVEF – left ventricle ejection fraction; mPAP – mean pulmonary arterial pressure; PCWP – pulmonary capillary wedge pressure; PVR – pulmonary vascular resistance

Table 4 ROC analysis for identifying elevated hemodynamic parameters by echocardiographic markers

	AUC (95% CI)		
	PAWP ≥ 15 mmHg	**mPAP > 20 mmHg**	**PVR > 3 Wood units**
DT, ms	0.61 (0.42–0.79)	0.60 (0.37–0.81)	0.68 (0.49–0.86)
E/A	0.68 (0.49–0.86)	0.69 (0.45–0.90)	**0.85 (0.68–0.98)**
Average E/e'	0.62 (0.41–0.80)	0.53 (0.29–0.76)	0.67 (0.46–0.85)
TR velocity, m/s	0.68 (0.49–0.85)	0.66 (0.42–0.90)	0.51 (0.23–0.78)
LAVi, ml/m^2	0.68 (0.50–0.85)	**0.78 (0.60–0.95)**	**0.76 (0.57–0.92)**
LVEF, %	0.65 (0.43–0.83)	0.63 (0.41–0.85)	0.66 (0.47–0.84)
GLS, %	**0.74 (0.57–0.88)**	0.65 (0.45–0.86)	**0.78 (0.60–0.95)**
E/GLS, [× 10^2] cm/s	**0.76 (0.59–0.91)**	0.72 (0.49–0.92)	**0.84 (0.68–0.96)**

The bold font character means statistically significance ($p < 0.05$)

AUC area under the curve, *95% CI* 95% confidence interval, *DT* deceleration time, *E/A* ratio of early-diastolic LV inflow velocity (E) to atrial-systolic velocity (A), *E/e'* ratio of early-diastolic LV inflow velocity (E) to early-diastolic mitral annular velocity (e'), *E/GLS* ratio of early-diastolic LV inflow velocity (E) to global longitudinal strain (GLS), *GLS* global longitudinal strain, *LAVi* left atrium indexed volume, *PCWP* pulmonary capillary wedge pressure, *mPAP* mean pulmonary arterial pressure, *PVR* pulmonary vascular resistance, *TR* tricuspid regurgitation

poor clinical outcomes during the short- and long-term follow-ups (Fig. 2). The differences between the curves were not significant. Additionally, the analysis identified optimal cut-off values for the short- and long-term composite outcome measure: -5.34% (78% sensitivity, 91% specificity) and -5.96% (60% sensitivity, 100% specificity) for GLS, -10.12 cm/s (70 and 100% sensitivity, 72 and 81% specificity) for E/GLS, and 22.5% (100% sensitivity, 72% specificity) and -24.5% for LVEF (80% sensitivity, 81% specificity), respectively.

To obtain additional information for risk stratification, we performed survival analysis with GLS, E/GLS and LVEF. The survival curve estimations demonstrated that patients with GLS values above the cut-off or E/GLS and LVEF below it had significantly lower event-free survival rates during both short- and long-term follow-up ($p < 0.001$) (Fig. 3). The graph shows that more than half of the patients with GLS above the cut-off value experienced adverse cardiac events during the first year of follow-up. In addition, univariate Cox analysis demonstrated that GLS values above cut-offs indicated 18- (HR 18.52; 95% CI 3.79–90.41, $p < 0.01$) and 12-fold (HR 12.47; 95% CI 4.6–33.82, < 0.0001) higher risk of poor clinical outcomes at one and five years, respectively.

Given that LVEF is a well-established prognostic marker, a subgroup survival analysis was performed to evaluate whether GLS has an additional predictive value in patients stratified by LVEF. In patients with LVEF values above the cut-off, GLS did not stratify the risk, while all patients had GLS values below the cut-off. However, for patients with LVEF below the cut-off value, the risk was further stratified by GLS (Fig. 4). Patients with GLS above the cut-off value (short-term GLS > -5.34% or long-term > -5.96%) had significantly increased events rates within the severely reduced LVEF group.

We then performed univariate Cox proportional-hazards model analysis to evaluate the prognostic significance of GLS and E/GLS as continuous variables. All baseline variables from Tables 1 and 2 were enrolled in univariate Cox regression analysis. The analysis showed that arterial blood pressure, severe right ventricle systolic dysfunction and cardiac index were associated only with short-term clinical outcomes (Table 5). While GLS, E/GLS, LVEF, BNP, Troponin T cardiac pressures and right ventricle enlargement were associated with adverse cardiac events during short- and long-term follow-up (Tables 5 and 6). GLS increase by 1% was associated with 55% and 41% higher risk of adverse cardiac events during short- and long-term follow-up, respectively.

The significant univariate predictors were enrolled in multivariate Cox regression analysis, which was performed using stepwise backward elimination method. GLS showed significant association with the occurrence of adverse cardiac events during long-term follow-up (adjusted HR 1.25 (95% CI 1.01–1.55); $p = 0.04$), even after adjusting for univariate outcome predictors (Table 6). However, E/GLS were not significantly associated with clinical outcomes after adjusting for other univariate predictors.

Discussion

This study evaluates the association between myocardial deformation parameters and invasively assessed cardiac pressures and PVR as well as the prognostic value of both GLS and E/GLS for predicting clinical outcomes in NIDCM patients. The main findings are that:

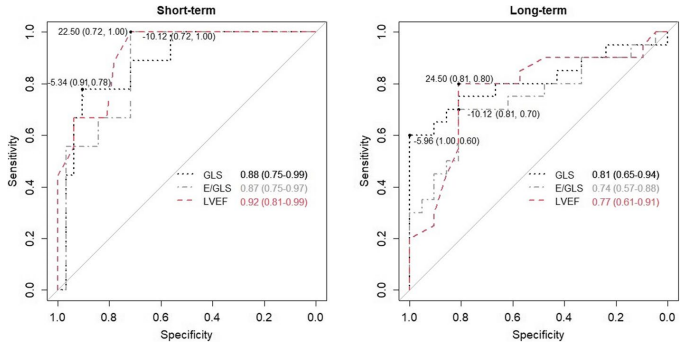

Fig. 2 ROC analysis of GLS, E/GLS and LVEF identified high-risk patients for an adverse cardiac event. E/GLS – ratio of early-diastolic LV inflow velocity (E) to global longitudinal strain (GLS); GLS – global longitudinal strain; LVEF – left ventricle ejection fraction

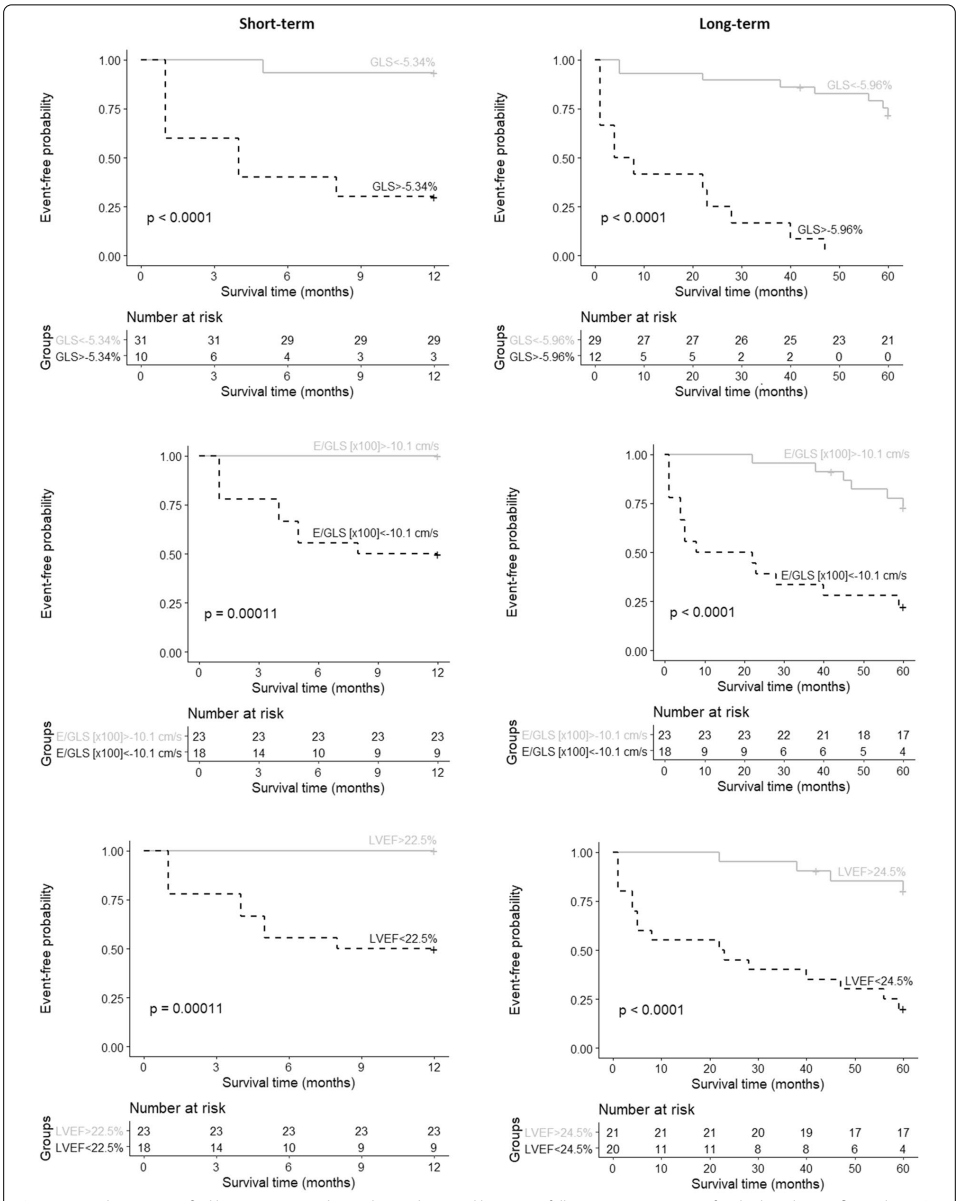

Fig. 3 Survival curves stratified by GLS, E/GLS and LVEF during short- and long-term follow-ups. E/GLS – ratio of early-diastolic LV inflow velocity (E) to global longitudinal strain (GLS); GLS – global longitudinal strain; LVEF – left ventricle ejection fraction

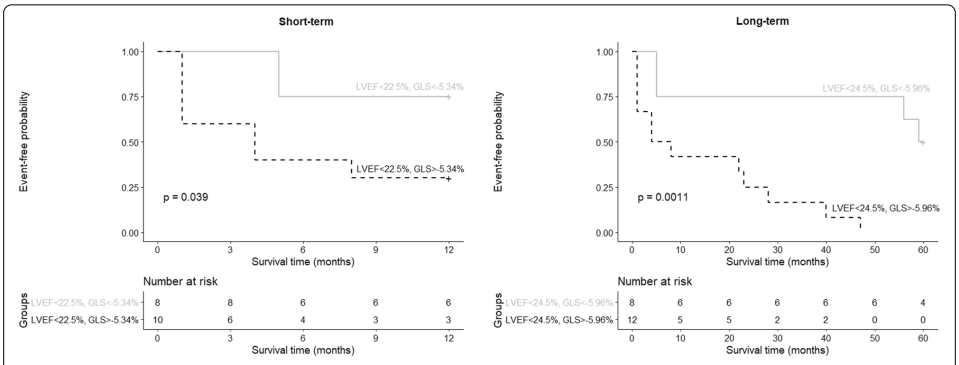

Fig. 4 Composite adverse cardiac events probability according to GLS within severely reduced LVEF patients. GLS – global longitudinal strain; LVEF – left ventricle ejection fraction

1) GLS and E/GLS correlate with PCWP, mPAP, PVR, and can predict elevated cardiac pressures
2) GLS and E/GLS are significantly associated with poor clinical outcome (both short- and long-term) in a well-defined NI-DCM cohort;
3) GLS > -5.34 and > -5.96% predicts adverse clinical events during one-year and five-year follow-ups, respectively. In the subgroup of patients with severely reduced LVEF, GLS values above the cut-off have an additional predictive value.

Echocardiographic evaluation of LV filling pressure is essential for HFrEF patients. Guidelines recommend a multiparametric echocardiographic approach for the evaluation [30]. Nevertheless, each parameter has limitations and may provide inconsistent results. In addition, their association with invasive LV filling pressures varies across studies [12, 13, 31]. For these reasons, there is a need for additional, non-invasive parameters for LV filling pressure evaluation. Recently, Hayashi et al. [22] in the study of 77 patients (39% had HFrEF) have demonstrated a correlation between GLS and time relaxation constant (tau). However, they did not evaluate the association between GLS and LV filling pressure. They have proposed a strain-based index E/GLS and demonstrated a correlation between E/GLS and LV mean diastolic

Table 5 Results of Cox regression analysis for predictors of adverse cardiac events during short-term follow-up

	Univariate		Multivariate	
	HR (95% CI)	***p***	**HR (95% CI)**	***p***
Systolic BP, mm Hg	0.95 (0.9–0.99)	0.03		
Diastolic BP, mm Hg	0.93 (0.86–0.998)	0.04		
BNP, ng/l	1.001 (1–1.001)	< 0.0001	1.001 (1.0–1.0014)	< 0.01
Troponin T, pg/ml	1.004 (1–1.008)	0.03	1.007 (1.002–1.01)	< 0.01
mPAP, mmHg	1.09 (1.03–1.16)	< 0.01		
PCWP, mmHg	1.16 (1.06–1.28)	< 0.01		
PVR, Wood units	1.53 (1.13–2.08)	< 0.01		
Cardiac index, l/min/m^2	0.16 (0.04–0.77)	0.02		
Severely impaired RV systolic function	5.29 (1.32–21.14)	0.02		
RV end-diastolic diameter, cm	2.44 (1.08–5.52)	0.03		
LVEF, %	0.8 (0.69–0.92)	< 0.01	0.84 (0.72–0.99)	0.04
LV GLS, %	1.55 (1.19–2.03)	< 0.001		
E/GLS [× 10^2], cm/s	0.96 (0.93–0.995)	0.02		

BNP B type natriuretic peptide, *BP* Blood pressure, *CI* confidence interval, *E/GLS* ratio of early-diastolic LV inflow velocity (E) to global longitudinal strain (GLS), *GLS* global longitudinal strain, *HR* hazard ratio, *LVEF* left ventricular ejection fraction, *mPAP* mean pulmonary arterial pressure, *PCWP* pulmonary capillary wedge pressure, *PVR* pulmonary vascular resistance, *RV* right ventricle

Table 6 Results of Cox regression analysis for predictors of adverse cardiac events during long-term follow-up

	Univariate		Multivariate	
	HR (95% CI)	***p***	**HR (95% CI)**	***p***
BNP, ng/l	1.001 (1–1.001)	< 0.0001	1.001 (1.0 -1.001)	0.02
Troponin T, pg/ml	1.004 (1.001–1.008)	0.02	1.004 (1.0–1.009)	0.04
mPAP, mmHg	1.1 (1.04–1.16)	0.002		
PCWP, mmHg	1.18 (1.05–1.13)	0.007		
PVR, Wood units	1.28 (1.03–1.59)	0.03		
RV end-diastolic diameter, cm	2.45 (1.24–4.87)	0.01		
LVEF, %	0.89 (0.83–0.96)	< 0.01		
LV GLS, %	1.41 (1.18–1.68)	< 0.0001	1.25 (1.01–1.55)	0.04
E/GLS [$\times 10^2$], cm/s	0.96 (0.93–0.98)	< 0.001		

BNP B type natriuretic peptide, *CI* confidence interval, *E/GLS* ratio of early-diastolic LV inflow velocity (E) to global longitudinal strain (GLS), *GLS* global longitudinal strain, *HR* hazard ratio, *LVEF* left ventricular ejection fraction, *mPAP* mean pulmonary arterial pressure, *PCWP* pulmonary capillary wedge pressure, *PVR* pulmonary vascular resistance, *RV* right ventricle

pressure. Romano et al. evaluated 78 patients with various etiology of HFrEF. They have demonstrated that four-chamber longitudinal strain was a predictor of elevated PCWP [32]. In agreement with this study, we have also estimated PCWP as a surrogate parameter for LV filling pressure. PCWP approximates the left atrial pressure, which, in turn, approximates LV end-diastolic pressure in the absence of pulmonary vein and mitral valve stenosis. In our study, GLS significantly, albeit weakly, correlated with PCWP. Furthermore, PCWP correlated with strain-based index E/GLS, and the correlation was stronger than the one between GLS and PCWP. We also found that GLS and E/GLS identified patients with elevated PCWP. These associations were also supported by strong correlations between myocardial deformation parameters and BNP, which secretion increases as a response to myocardial wall stretch due to pressure or volume overload.

Elevated mPAP and increased PVR are the consequence of long-lasting abnormal LV filling pressure in our cohort. The backward transmission of elevated LV filling pressure might also explain GLS and E/GLS correlation not only with PCWP, but also with mPAP and PVR. E/GLS correlated more strongly with these parameters than did GLS. Interestingly, the strongest correlation was between PVR and E/GLS. The ROC curve analysis demonstrated that both E/GLS and GLS could identify patients with PVR > 3 Wood units. This PVR value differentiates two distinct hemodynamic phenotypes of post-capillary pulmonary hypertension: isolated post-capillary (≤ 3 Wood units) hypertension from combined post-capillary and pre-capillary pulmonary hypertension (> 3 Wood units). This distinction is essential for accurate prognostication and treatment decision-making [33].

Prior studies have demonstrated that GLS has a significant predictive value in heart failure patients [4–6, 34], in various cardiovascular pathologies [35–42], and even in the general population [43]. To our knowledge, this is the first study to investigate the predictive potential of GLS and E/GLS ratio in patients with chronic heart failure due to NI-DCM. Our study showed that both GLS and E/GLS are predictors of poor short- and long-term outcomes in a well-defined cohort of patients with NI-DCM. Furthermore, GLS remained a significant long-term predictor when added to a model with other prognostic parameters.

Guidelines define a GLS ≤ –20% as a normal value in healthy subjects [26]. A recent meta-analysis reports that a GLS > -16% indicates significant myocardial dysfunction [44]. There are no defined GLS cut-off values for risk estimation in different cardiac pathologies, although these would be useful for prognostication, management, and future studies. Motoki et al. [4] included 194 patients with various etiology chronic heart failure. They identified a GLS cut-off value of -6.95% to be a predictor of poor clinical outcomes during a five-year follow-up. We identified the cut-off value of GLS -5.96% of adverse cardiac events during a five-year follow-up. The worse GLS cut-off value in our study compared to theirs might be due to the severity of heart failure in our cohort (NYHA III-IV functional class 88% versus 39%) and a higher event rate (49% versus 40%). Sengelov et al. [5] included 1065 patients with various origin HFrEF and found a mortality rate of 16.7% during the median follow-up of 40 months. They identified a GLS cut-off value of -5.9% to be a useful predictor of increased mortality in patients with severely reduced LV systolic function (LVEF < 22%). Our study's identified cut-off value is in line with their study, despite differences in heart failure etiology, follow-up duration, and event rate. It is important to note that our cut-off value had 100% specificity, supported by

Kaplan–Meier analysis: all patients with GLS > -5.96% experienced adverse cardiac events before the end of follow-up. In contrast to previous studies, we evaluated not only long-term but also short-term clinical outcomes. Our study's cut-off value (GLS > -5.34%) predicted short-term clinical outcome, with more than half of the patients experiencing cardiac events during the first year of follow-up. These cut-offs might be useful for identifying patients with advanced heart failure.

Limitations and clinical implications

Our study's main limitation is the small sample size, which might explain why we did not find any significant differences between AUCs of echocardiographic parameters in the ROC analysis. In addition, echocardiography was not performed simultaneously with a right heart catheterization. This might lead to differences in loading conditions, which in turn might reflect a weak to absent correlation between several echocardiographic and invasively measured parameters.

Despite the limitations, the study demonstrated that E/GLS might be an additional parameter for LV filling pressure assessment, while E/GLS correlated with PCWP stronger than most conventional echocardiographic parameters and GLS. E/GLS might improve LV diastolic function evaluation and might aid in clinical scenarios where conventional echocardiographic parameters' usage is limited, i.e., mitral valve pathology, advanced heart failure. However, a larger study is needed to estimate in which clinical scenarios E/GLS would be beneficial.

To our best knowledge, none of the previous studies evaluated E/GLS prognostic value. However, GLS seems to be a superior predictor than E/GLS, as it was an independent predictor for long-term clinical outcomes and further stratified patients with severely reduced LV systolic function. The use of GLS to identify high-risk NI-DCM patients could lead to changes in follow-up intensity, the timing for device therapy, or prioritization on the heart transplantation list.

Conclusion

GLS and E/GLS correlate with invasive hemodynamics parameters and identify patients with elevated PCWP and high PVR. GLS and E/GLS predict short- and long-term adverse cardiac events in patients with NI-DCM. Worsening GLS is associated with incremental risk of long-term adverse cardiac events and might be used to identify high-risk patients.

Abbreviations

ACE-I: Angiotensin-converting enzyme inhibitor; ARB: Angiotensin II receptor blocker; BNP: B type natriuretic peptide; BP: Blood pressure; DT: Deceleration time; E/A: Ratio of early-diastolic LV inflow velocity (E) to atrial-systolic velocity (A); E/e': Ratio of early-diastolic LV inflow velocity (E) to early-diastolic mitral annular velocity (e'); E/GLS: Ratio of early-diastolic LV inflow velocity (E) to global longitudinal strain (GLS); eGFR: Estimated glomerular filtration rate; GLS: Global longitudinal strain; LAVi: Left atrial indexed volume; LV: Left ventricle; LVEDD: Left ventricular end-diastolic diameter; LVEF: Left ventricular ejection fraction; mPAP: Mean pulmonary arterial pressure; MRA: Mineralocorticoid receptor antagonist; NYHA: New York Heart Association; NI-DCM: Non-ischemic dilated cardiomyopathy; PCWP: Pulmonary capillary wedge pressure; PVR: Pulmonary vascular resistance; TR: Tricuspid regurgitation.

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Authors' contributions

IK: conceptualization, formal analysis, data curation, writing – the initial draft preparation, visualization; GB: investigation, writing – reviewing and editing; VB: data curation, writing – reviewing and editing; JČ: conceptualization, writing – reviewing and editing; VVM: investigation, writing – reviewing and editing; KR: investigation, writing – reviewing and editing; VG: methodology, investigation, supervision, project administration, funding acquisition, writing – reviewing and editing. All the authors have read and approved the manuscript version submitted.

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Availability of data and materials

The datasets during and/or analyzed during the current study available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Ethical approval was obtained from the local Lithuanian Bioethics Committee (license numbers 158200–09-382-I03; 158200–382-PP1-23; and 158200–17-891–413). Written informed consent was obtained from every participant.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]Department of Pathology, Forensic Medicine and Pharmacology, Institute of Biomedical Science, Faculty of Medicine, Vilnius University, M. K. Čiurlionio 21, 03101 Vilnius, Lithuania. [2]Clinic of Cardiac and Vascular Diseases, Institute of Clinical Medicine, Faculty of Medicine, Vilnius University, M. K. Čiurlionio 21, 03101 Vilnius, Lithuania. [3]Center of Cardiology and Angiology, Vilnius University Hospital Santaros Klinikos, Santariškių 2, 08661 Vilnius, Lithuania.

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Vilniaus universiteto leidykla
Saulėtekio al. 9, III rūmai, LT-10222 Vilnius
El. p. info@leidykla.vu.lt, www.leidykla.vu.lt
Tiražas 20 egz.