DISSERTATION

Cytokine Profile in Inflammatory Cardiomyopathy. Are there Differences in Cytokine Expression between Mild and Severe Courses?

Zytokinprofil bei entzündlicher Kardiomyopathie. Gibt es Unterschiede in der Zytokinexpression zwischen milden und schweren Verläufen?

zur Erlangung des akademischen Grades

Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät

Charité – Universitätsmedizin Berlin

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Datum der Promotion: 29.11.2024

Vorwort

Teilergebnisse der vorliegenden Arbeit wurden eingereicht in: *Suwalski P, Golpour A, Weiner III J, Musigk N, Balzer F, Giesa N, Amr A, Trebing J, Sedaghat F, Meder B. Cytokines as potential novel therapeutic targets in severe inflammatory cardiomyopathy. medRxiv. 2023:2023.07. 27.23293253.*

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Abbreviations

Deutsches Herzzentrum der Charité = DHZC

German Centre for Cardiovascular Research = DZHK

Idiopathic dilated cardiomyopathy= IDCM

Electronic medical records = EMR

Translational Registry for Cardiomyopathies = TORCH

Left ventricular ejection fraction = LVEF

Ribonucleic acid = RNA

Interleukin = IL

Dilated cardiomyopathy = DCM

Chimeric antigen receptor therapy = CAR

Cytokine release syndrome = CRS

Cytokine storm syndrome = CSS

Coronavirus SARS-CoV-2 = COVID-19

Cytotoxic T-lymphocyte–associated antigen 4 = CTLA4

Programmed death-1 = PD-1

Glutathione peroxidase 1 = Gpx1

Major histocompatibility complex = MHC

Human leukocyte antigen = HLA

Human heart α-myosin = hCAM

Coxsackievirus = CVB

Human immunodeficiency virus = HIV

Hepatitis C virus = HCV

Giant cell myocarditis = GCM

Necrotizing eosinophilic myocarditis = NEM

American Heart Association = AHA

American College of Cardiology Foundation = ACCF

European Society of Cardiology = ESC

Heart Failure Society of America = HFSA

Electrocardiography = ECG

Magnetic resonance imaging = MRI

Magnetocardiography = MCG

Intravenous immunoglobulin = IVIG

Immune checkpoint inhibitor = ICI

Human herpesvirus 6 = HHV-6

Angiotensin-converting enzyme 2 = ACE2

Parvovirus B19 = B19V

New York Heart Association = NYHA

Proximity Extension Assay = PEA

Normalized protein expression = NPX

Troponin T = hs-TnT

N-terminal pro B-type natriuretic peptide = NT-proBNP

Glomerular filtration rate = GFR

Body mass index = BMI

False discovery rate = FDR

Vascular Endothelial Growth Factor = VEGF

Low-density lipoprotein = LDL

Natural resistance-associated macrophage protein 1 = NRAMP1

Follistatin = FSTL

Growth differentiation factor 11 = GDF11

Cysteine-rich motor neuron 1 = CRIM-1

Plasminogen Activator, Urokinase Receptor = PLAUR

Signal transducer and activator of transcription 3 = STAT3

Zusammenfassung

Hintergrund: Bei der entzündlichen Kardiomyopathie handelt es sich um eine Entzündung des Herzmuskels, die mit einer Abnahme der Herzfunktion und einem ventrikulären Umbauprozess einhergeht. Die Myokarditis ist eine der häufigsten entzündlichen Kardiomyopathien. Bislang stellt ihre Therapie für Kliniker oft eine Herausforderung dar. Daher werden neue Therapien für Patienten benötigt, die auf die Standardtherapie nicht ansprechen. Zytokine sind über mehrere Signalwege an der Pathogenese entzündlicher Kardiomyopathien beteiligt. Eine gezielte Hemmung dieser Signalwege und die damit verbundene Unterdrückung der freigesetzten Zytokine könnte ein zukünftiger Therapieansatz sein.

Methoden und Population: In der Hauptstudie wurden Proben von Patienten mit bioptisch gesicherter entzündlicher Kardiomyopathie untersucht. Darüber hinaus wurden die Daten in einer Validierungskohorte (n=425) und in einer Verallgemeinerungskohorte (n=41) repliziert. Patienten, die eine Immunsuppression einschließlich Glukokortikoiden erhalten hatten, wurden von der Studie ausgeschlossen. Insgesamt wurden 104 Patienten vom Deutschen Herzzentrum der Charité (DHZC) in die Zytokinanalyse einbezogen. Von diesen hatten 63 Patienten eine bioptisch gesicherte entzündliche Kardiomyopathie (Ableitungskohorte). 41 Patienten hatten eine idiopathische dilatative Kardiomyopathie (IDCM) ohne Entzündungszeichen in der Biopsie. Die Patienten mit IDCM dienten als Verallgemeinerungskohorte. Zusätzlich wurden 425 Patienten aus dem DZHK, TORCH (Deutsches Zentrum für Herz-Kreislauf-Forschung e.V., Translationales Register für Kardiomyopathien) Netzwerk, bei denen eine entzündliche Kardiomyopathie mittels Biopsie oder Bildgebung bestätigt wurde, als Validierungskohorte verwendet. Die Studienpatienten wurden in zwei Gruppen eingeteilt: Patienten mit einem weniger schweren Verlauf, d. h. mit einer linksventrikulären Ejektionsfraktion (LVEF) von mehr als 35 %, und Patienten mit einem schwereren Verlauf, d. h. mit einer LVEF von 35 % oder weniger. Die Zytokinmessungen im Plasma der Patienten wurden mit dem Olink-384 "Inflammation Panel" durchgeführt.

Ergebnisse: Wir fanden insgesamt 77 Zytokine, die eine starke Assoziation mit einer reduzierten LVEF aufwiesen (False Discovery Rate, FDR<0,05). Einige dieser Zytokine wurden in der Literatur bereits als therapeutische Ziele vorgeschlagen, was die Plausibilität unserer Daten erhöht. Unsere Ergebnisse wurden durch eine zusätzliche Analyse elektronischer Krankenakten untermauert, wonach die Unterdrückung einiger dieser Zytokine tatsächlich eine kardioprotektive Wirkung haben könnte.

Schlussfolgerungen: Unsere Analysen ergaben 77 Zytokine, die signifikant mit einer reduzierten LVEF assoziiert waren. Unsere Ergebnisse wurden in einer unabhängigen Kohorte repliziert und validiert und waren auf IDCM verallgemeinerbar. Diese Zytokine

könnten in Zukunft als therapeutische Ziele dienen, insbesondere für Patienten, die auf die Standardtherapie nicht ansprechen.

Abstract

Background: Inflammatory cardiomyopathy consists of inflammation of the heart muscle, which is accompanied by a reduction in heart function and ventricular remodeling. Myocarditis is one of the most common inflammatory cardiomyopathies. To date, the therapy of inflammatory cardiomyopathies is often a challenge for clinicians. Hence, novel therapy options are needed for the patients who do not respond to standard therapy alone. Various cytokines are involved in the pathogenesis of inflammatory cardiomyopathies via several pathways. Targeted inhibition of these relevant signaling pathways and the associated suppression of the cytokines released could be a relevant future therapeutic approach.

Methods and population: In the main study, specimens from individuals with biopsy-proven inflammatory cardiomyopathy were investigated. In addition, data was replicated in a validation cohort (n=425) and in a generalizability cohort (n=41). Patients who had received immunosuppression including glucocorticoids were excluded from the study. 104 subjects from Deutsches Herzzentrum der Charité (DHZC) were considered for cytokine analysis. Among these patients, 63 patients had an inflammatory cardiomyopathy (derivation cohort) based on endomyocardial biopsy and 41 patients had an idiopathic dilated cardiomyopathy (IDCM) with no signs of inflammation in the biopsy. Patients with IDCM served as generalizability cohort. In addition, 425 patients from the DZHK- TORCH (German Center for Cardiovascular Research e.V., Translational Registry for Cardiomyopathies) network, who had confirmed inflammatory cardiomyopathy by biopsy or cardiac imaging, were used as a validation cohort. The patients in the study were further divided into two groups: Those with a less severe course, specified as individuals with a left ventricular ejection fraction (LVEF) greater than 35%, and those with a more severe course, characterized as individuals with an LVEF of 35% or lower. Cytokine measurements of the patients' plasma were performed using the Olink - 384 "Inflammation panel".

Results: We found a total of 77 cytokines that showed a strong association with reduced LVEF (false discovery rate, FDR<0.05). Some of these cytokines have been previously proposed as therapeutic targets in recent literature, which enhances the plausibility of our data. Our findings were further supported by additional analysis of

electronic medical records data (EMR), which revealed that suppression of some of these cytokines might indeed have a cardioprotective effect.

Conclusions: Our analyses demonstrated 77 cytokines that were significantly associated with decreased LVEF. Our results were replicated and validated in an independent cohort and were generalizable to IDCM. These cytokines may serve as therapeutic targets in the future in particular for patients who do not respond to standard therapy.

1. Introduction

Different subtypes of inflammatory cardiomyopathies, in particular myocarditis, are considered to be a leading etiology of sudden cardiac arrest in young adults. This is evident from autopsy studies in which myocarditis was found in 20% of cases(1, 2). To date, it is difficult to determine the exact prevalence and incidence of myocarditis. This is mainly because of the fact that the disease is not easy to diagnose(3). Therefore, it is important to provide diagnostic means that allow early identification of patients with myocarditis. Modern technologies including ribonucleic acid (RNA) sequencing and cytokine assays could help us characterize inflammation more precisely, while there is increasing debate about whether the diagnosis of myocarditis by histology only is sufficient(4-8). Many colleagues have previously discussed the role of cytokine analysis in patients with myocarditis and other inflammatory cardiomyopathies(9-11). The determination of cytokines in the plasma of patients with probable myocarditis could both facilitate the disease treatment in this group of patients and predict the course of myocarditis.

There are different guidelines for therapy management depending on which is the cause of the myocarditis or inflammatory cardiomyopathy(12). Therapy is not always successful and non-responders pose a major challenge in clinical practice. In most autoimmune cases, glucocorticoids can be administered(13). However, they have side effects and not all patients respond to glucocorticoid therapy(14, 15). Especially in virus-negative inflammatory cardiomyopathy, immunosuppressive therapy has been demonstrated to be beneficial(16). However, literature on this topic has been controversial(17-19). Overall, there continues to be a strong clinical need to develop novel drug regimens for individuals who do not improve sufficiently with standard medications including established immunosuppressive therapies or who have to discontinue such treatments due to side effects.

Many studies have evaluated the possible contribution of inflammation in the development of heart failure(20, 21). The blockade of certain pathways could be the target for novel therapies. These novel therapies could be used in patients who do not respond to standard therapies. Analysis of cytokines in patients with myocarditis or other non-ischemic cardiomyopathies may lead to insights that could facilitate therapy and at the same time support diagnosis in patients with myocarditis. The level of circulating cytokines could even be used to predict the course of myocarditis. The aim

of this study is to investigate how the level of circulating cytokines in the plasma of patients with inflammatory cardiomyopathies, including myocarditis, is related to the severity of the disease. In this study, there are 3 cohorts (derivation, validation, and generalizability cohort) divided in two subgroups. One subgroup comprises individuals with a more severe clinical course of inflammatory cardiomyopathy, characterized by a left ventricular ejection fraction ≤35% measured by echocardiography, and the other group includes individuals with mild inflammatory cardiomyopathy, characterized by those with a left ventricular ejection fraction >35%. The introduction of my thesis is structured by background on cytokines and inflammatory cardiomyopathies, followed by their characteristic symptoms, diagnosis, and treatment.

1.1 Background on Cytokines and Inflammatory Cardiomyopathies

Cytokines are increasingly regarded as mediators that are crucial for both normal and pathological processes in the body(22-24). Several cytokines, such as Interleukin (IL)-1 or tumor necrosis factor, have been found to be associated with myocarditis(25, 26). A study aimed at investigating the role of circulating proteins in acute myocarditis, dilated cardiomyopathy (DCM), and hypertrophic cardiomyopathy(11). Tumor necrosis factor was observed in high concentrations in myocarditis patients(11). Moreover, IL-1a was found in about one-fifth of myocarditis cases and macrophage colony-stimulating factor was increased in this group(11).

In an experimental approach in myocarditis, it was demonstrated that circulating tumor necrosis factor-a was increased in the initial phase of the disease(26). It was also shown that tumor necrosis factor-a delivery aggravated myocarditis, whereas its inhibitor prevented the development of myocarditis, suggesting that tumor necrosis factor-a may play a significant role in the pathogenesis of damage to the myocardium in viral and autoimmune myocarditis(26).

Nakano and colleagues attempted to address the role of cytokines in the pathogenesis of myocarditis by cytokine gene therapy using in vivo electroporation(27). The group suggested that blocking the IL-1 receptor in this way is effective in treating virus-induced myocarditis(27).

Chimeric antigen receptor therapy (CAR) is increasingly used used therapeutically in the field of oncology(28). However, a very important side effect, cytokine release

syndrome (CRS), must be considered(29). CRS may cause cardiac dysfunction, as well as vascular leak syndrome with peripheral and pulmonary edema(29). CRS was reported in a woman with endometrial carcinoma after CAR T-cell therapy(29). Biopsy confirmed the presence of myocarditis in this case, likely induced by immunotherapy. In addition to immune cell activation, cytokines such as IL-6 and IL-2 are released in such a reaction(29-31). Therefore, an important role of cytokines in the development of myocarditis should be considered, as in this case.

Lately, there have been reports of myocarditis in COVID-19(32-34). There are interesting parallels with COVID-19 and myocarditis regarding cytokines such as IL-6 and IL-1(35-38). Hyperinflammation characterized by cytokine storm syndrome (CSS) has been reported as one of the most common underlying causes of mortality in COVID-19 patients(39). There are some clinical studies that investigated the role of blocking IL-1 and IL-6 as a targeted therapy for COVID-19(40-42). However, the benefit of blocking these cytokines remains unclear(40-42). A randomized, placebo-controlled, blinded trial failed to demonstrate a survival benefit with IL-1β blockade(41), while Kyriazopoulou et al. support a survival benefit of IL-1 blockade(40).

Based on this evidence from the literature, we sought to investigate which cytokines are increased in inflammatory cardiomyopathy and how these differ in patients with a severe versus mild to moderate clinical course. We hypothesized that these cytokines may represent potential candidates for targeted cytokine inhibition.

1.2 Myocarditis

The diagnosis myocarditis describes an inflammation of the heart muscle, which may have various etiologies, most commonly viral infection(43). In particular, in adults under the age of 40 and young athletes, it has been frequently reported as cause of sudden death(2, 44). Unfortunately, the diagnosis of myocarditis is still hard to make and there are many patients who remain undiagnosed. Consequently, the assessment of the prevalence of myocarditis remains challenging(3).

1.2.1 Causes

In most cases, myocarditis is caused by viral infections(45). Other pathogens, toxic or hypersensitivity drug reactions, sarcoidosis, as well as genetic variations may also result in myocarditis(43). The symptoms that occur with this disease are variable and

include mild dyspnea or chest pain, cardiogenic shock, and death(46). Myocarditis may lead to DCM and heart failure(47, 48).

It is previously mentioned in the literature that a combined administration of ipilimumab and nivolumab can lead to a rare, potentially fatal immune reaction mediated by T cells(49). A study demonstrated that the concomitant administration of nivolumab and ipilimumab can lead to a higher frequency of unfavorable events(50). Ipilimumab is an anti–cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) antibody, and nivolumab is an anti–programmed cell death protein 1 (PD-1) antibody(49). CTLA-4 and PD-1 have immunoregulatory functions(51-53). Their inhibition may lead to an aggressive immune response and myocarditis(54-56). A systematic review and meta-analysis demonstrated that the second most common adverse event associated with the concomitant administration of anti-PD-1/CTLA-4 was myocarditis (25%)(57).

The causes of myocarditis vary and often there is an underlying genetic predisposition (58). Genetic alterations that may negatively affect regulation of the immune system may contribute to the development of myocarditis(59). Some genes, such as ActB, RPL5, B2M, HPRT1, and HMBS, have been previously studied as predictors of myocarditis(60), but further studies are necessary to confirm those findings. Furthermore, an association of MHC and, in particular, non-MHC genes with myocarditis has been previously described(58, 61). Moreover, alterations of genes such as ICOS and PD-1 as well as CD45 have been associated with myocarditis(62-66). Kulkarni, Ashok B., et al. successfully generated a TGF-β1 null mutant investigating the role of TGF-β in inflammatory response(67). In this study, 14 TGF-β1 null (-/-) mice aged between 10 to 21 days showed massive inflammatory lesions in many organs, particularly in the heart(67). In all examined TGF-β1 null (-/-) mice, atrial endocarditis and myocarditis could be detected, with additional pericarditis and atrial thrombosis in some cases(67). Whether a mutation in the TGF-β1 gene could also cause heart disease in humans is a question that requires further research. Another study demonstrated that the presence of glutathione peroxidase 1 (Gpx1) genes in mice may have an impact on the development of myocarditis(68). Based on these studies, an interesting question is whether variants in these genes of the human genome also represent a predisposition for myocarditis.

Some studies have previously reported that myocarditis may often co-occur with other autoimmune diseases, suggesting that autoimmune processes are indeed involved in

the pathogenesis of the disease(69-71). For some autoimmune diseases, an association with myocarditis has been previously mentioned(69-71). A common complication that may occur in patients with eosinophilic granulomatosis with polyangiitis is myocarditis(72, 73). Since any heart segment can be affected by eosinophilic myocarditis(73), a complete cardiac diagnostic workup is reasonable in patients who receive this diagnosis. Myocarditis may also be a manifestation of other autoimmune diseases such as Behcet's disease, sarcoidosis, Takayasu arteritis, systemic lupus erythematosus or systemic sclerosis(74-78). In Behcet's disease, myocarditis is rather uncommon, however it has been reported in some cases(74, 79, 80). Some case reports describe myocarditis in patients with Takayasu arteritis(75, 81, 82). Furthermore, post-mortem studies have previously shown a high incidence of myocarditis in patients with systemic lupus erythematosus, in some cases in more than 50% of this patient group in the past(69, 83).

MHC-HLA association with myocarditis and IDCM

There are 2 major categories of MHC molecules, with HLA A, B and C belonging to the first category and HLA D/DR, DQ and DP to the second category(84). The members of the first category are able to recognize antigens for cytotoxic T cells, whereas those of the second category have a regulatory function(84). The immune system appears to have a significant involvement in the progression of DCM, myocarditis and atherosclerosis(85, 86). Over the last few decades, some studies have demonstrated that various pathological conditions are the result of pathological autoimmune processes(84, 87).

There is already strong consensus that HLA antigens are associated with various cardiovascular diseases. For example, a high frequency of HLA-B7 has been linked to the development of DCM(84). Most available information in the context of IDCM relates to the association between the HLA system and susceptibility to cardiac inflammatory conditions(12). A substantial correlation of IDCM with MHC class II antigens has been demonstrated in several studies, and HLA-DR4 appears to be of great importance(88-91). Furthermore, HLA DQB1*0303 has been previously linked to myocarditis and cardiomyopathy(92), while a relevant relationship was reported between HLA-DQB1*0601 and cardiac sarcoidosis(93). Additionally, HLA-DQB1*0601 has been found to be involved in the pathogenesis of inflammatory cardiomyopathy; however, its role should be further investigated(92).

The immune system is able to distinguish between endogenous and foreign particles(94). The MHC molecules play an important role in this process, as they are marked by variations that are crucial for this differentiation(94). The selection of T cells depends on the MHC molecules, and errors may occur leading to defense mechanisms against self- and autoimmunity as a consequence(94). A link between MHC genes and autoimmunity as well as inflammatory conditions has been mentioned in the literature(95). For example, it has been shown that the presence of MHC II molecules is increased in mice with myocarditis compared to healthy mice(96). In the past, it has been demonstrated based on myocarditis biopsies that upregulation of MHC II is potentially involved in the development of myocarditis(97). In addition, a link between MHC and diabetes as well as rheumatoid arthritis was previously mentioned(98, 99). The range of diseases in which MHC has a relevance in pathophysiology is broad and also includes other diseases such as celiac disease, multiple sclerosis, autoimmune thyroid diseases and systemic lupus erythematosus(100-103). For celiac disease, a prevalence of 5.7% has been reported in patients with IDCM(104, 105). In addition, a high prevalence of DQ2 was found in patients with celiac disease and cardiomyopathy, while a higher frequency of DQ8 was observed in myocarditis with elevated anti-tissue transglutaminase antibodies(106). A possible association of anti-tissue transglutaminase in human cardiomyocyte apoptosis has been previously reported(107).

HLA A3 appears to have an impact on the course of myocarditis and has been associated with moderate to poor clinical outcomes in Coxsackie B virus myocarditis(108). A study demonstrated that the human molecule HLA-DQ8 by itself may induce autoimmune myocarditis in three different lines of transgenic, diabetic MHCII deficient ($mII^{-/-}$) mice without obesity(109). The expression of HLA-DR11 may have a protective role in patients with IDCM, as lower expression of HLA-DR11 was found in a Mexican IDCM cohort(91). All these findings in the literature suggest that HLA genes may play a key role in the pathogenesis of inflammatory cardiomyopathy and that HLA analysis in patients could provide new insights into the various pathological processes behind this disease. At the same time, this could open up new therapeutic approaches for this group of patients.

1.2.2 Pathophysiology

The components and mechanisms that lead to the occurrence of myocarditis have been studied primarily in experiments(110). The immune system is triggered by certain factors that lead to a reaction that, over several stages, results in the development of myocarditis or, in some cases, even DCM(111, 112). Myocarditis is defined as an inflammation of the heart muscle(46). The mechanisms of this disease are host immune system dysregulation and viral triggers such as Coxsackievirus (CVB)(113, 114), Adenovirus(115), Human immunodeficiency (HIV)(116) and Hepatitis C Virus (HCV)(117). Pathophysiology is induced by viral proliferation in a susceptible host, triggering an immune response of the host(111). Conceptually, the disease can be classified into three phases as follows: (1) acute, (2) subacute and (3) chronic(111).

In the acute phase, which lasts only a few days, the virus spreads in the blood and/or lymphatic system, replicates, and penetrates the target cells in the heart(43). Subsequently, the immune system is activated by several signaling pathways that regulate the activation of immune cells(43, 118, 119). The virus invades via its receptor and triggers signaling pathways(118, 119). The first immune cells at the site of infection are natural killer cells and macrophages(120). Other immune cells involved in the immune reactions are neutrophils and dendritic cells(121).

The initial response after viral infection is determined by components of the innate immune system(43). During the second phase, innate immunity initiates inflammatory reactions(43). TLR4 appears to be involved in the development and pathogenesis of the disease, as patients and mice with myocarditis had increased expression of this protein(122, 123). The expression of TLR4 has been found to be higher in males compared to females(122).

Through various signals, the innate immune system triggers activation of the adaptive immune system and, among others, leads to an activation of T and B cells(43). The T cells are able to identify foreign particles, leading subsequently to an increased production of T cells after alerting the immune system(43). T-killer cells are activated, the cause (e.g. parts of the viral envelope protein) is then addressed, and the parts infected by virus as well as the virus are combated(43). Sometimes, even parts of the heart muscle resembling the virus are mistakenly attacked, which leads to autoimmunity(43). After activation of T cells, B cells are also stimulated and support

the immune defense with the synthesis of antibodies for the elimination of antigens(43). These processes of the adapted immune system may lead to subacute to chronic inflammation, which may be accompanied by cardiac muscle remodeling up to necrosis(43). Thus, suppression of the adaptive immune system may play an important role in the treatment of myocarditis.

In the third phase, if the inflammatory reaction is not suppressed, the heart remodels with a structural and functional change, leading to DCM(43). The immune system's inflammatory response can trigger the release of cytokines, which in turn activate matrix metalloproteases(124). These enzymes participate in the degradation of the interstitial collagen and elastin scaffold in the heart, contributing to the inflammatory process(124). Some of these enzymes, among them the urokinase-type plasminogen activator, play a role in the dilatation and inflammation of the heart(125). Moreover, pathological fibrosis can be caused by cytokines, with IL-1b, IL-17 and transforming growth factor having an important contribution(43, 126). As a consequence, DCM ensues, characterized by concurrent systolic and diastolic dysfunction, leading to the development of heart failure(43). It has been previously demonstrated that the intake of type 1 interferons could influence both viral clearance and change of cardiac structure(125). In addition, angiotensin modulators and β blockers are considered to be beneficial in treating an enlarged heart after myocarditis due to their effect on cardiac remodeling(43).

1.2.3 Classification

Myocarditis can be differentiated according to cause, disease stage, histology, immunohistology, as well as pathological and clinical criteria(43). The disease can be divided into an infectious and a non-infectious/autoimmune form(121). In Europe and North America, CVB3 and adenoviruses are most common in myocarditis(127). Kühl et al. found persistent viral genome in about 70% of DCM cases, indicating that DCM is indeed preceded by subclinical viral myocarditis(128). The different classifications indicate that there are various factors involved in the development of the disease. Histopathological classification of myocarditis using the Dallas Criteria involves, in part, describing the activity of the disease based on the amount of infiltration, myocardial damage and fibrosis(129). In the following, the histological classification of myocarditis is discussed in particular. Histologically, myocarditis can be classified

into various forms, including lymphocytic, giant cell, granulomatous and eosinophilic myocarditis(43).

a. Lymphocytic Myocarditis

Symptoms of lymphocytic myocarditis are heterogeneous, and this group includes from asymptomatic patients to patients with abrupt heart failure(130). Patient biopsy samples reveal myocyte damage and accumulation of lymphocytes(131). Biopsy is important to differentiate lymphocytic myocarditis from giant cell myocarditis (GCM) and necrotizing eosinophilic myocarditis (NEM). This differentiation is also important for therapy, because while immunosuppressants have a positive effect in GCM and NEM myocarditis, their role in lymphocytic myocarditis is debatable(132-135).

b. Giant Cell Myocarditis (GCM)

GCM is a variant of myocarditis marked by an aggressive course and the existence of enlarged polynuclear cells in cardiac infiltrates(136). The outlook for this disease is poor, and although drug suppression of the immune system can prolong survival, long-term survival in the absence of transplantation remains limited(133, 137). GCM is considered to be the result of autoimmune mechanisms(138). While lymphocytic myocarditis generally carries a favorable prognosis(139), both GCM and NEM myocarditis are characterized by a poor prognosis and outcome(133).

c. Necrotizing Eosinophilic Myocarditis (NEM)

Among the various forms of myocarditis, NEM is the most severe and develops in a short time, leading to diffuse eosinophilic accumulation and pervasive necrosis(140). The prognosis in this group of patients is not bright and high-dose immunosuppressive therapy is recommended(134). NEM differs from eosinophilic myocarditis, which occurs after an overreaction to drugs(141), by its sudden onset, unfavorable outcome and exclusive cardiac manifestation(138). Elevated body temperature, cutaneous abnormalities, and increased eosinophil laboratory parameters can be indicative of hypersensitivity myocarditis(138). Eosinophilic myocarditis may also be observed in coexistence with neoplastic conditions or parasitic diseases(138).

1.2.4 Diagnosis

Based on the current guidelines of the American Heart Association (AHA), the American College of Cardiology Foundation (ACCF), the European Society of Cardiology (ESC) and the Heart Failure Society of America (HFSA), it is advisable to initially rule out other pathological cardiac conditions when there is a suspected diagnosis of myocarditis(142-144).

If a clinical suspicion of acute myocarditis is to be confirmed, serum biomarkers must first be determined(43). If troponin and creatine kinase MB concentrations are elevated, myocarditis should also be considered as a differential diagnosis(145, 146). At the same time, inflammatory parameters may be elevated in myocarditis(43). Due to their low specificity, however, they have a low diagnostic value(43). Finally, in acute myocarditis, the evaluation of anti-cardiac antibodies can serve as a valuable tool to gauge the risk of mortality or the necessity for transplantation(147).

In the majority of cases of myocarditis, electrocardiography (ECG) measurements reveal unspecific alterations such as sinus tachycardia, ST wave irregularities, repolarization changes, or less commonly, a disturbance in the conduction of electrical activity with block formation(148). ECG changes in acute myocarditis, such as widened QRS, Q-waves, a prolonged QT interval and high-degree atrioventricular block suggest a less favorable prognosis(149, 150). PR depression with ST elevation is more suggestive of inflammation and especially perimyocarditis than myocardial infarction(150).

Echocardiography plays a crucial role in evaluating acute myocarditis by providing the capability to rule out underlying primary valvular or congenital conditions, as well as pericardial stenosis(43). The assessment of cardiac contractility may be helpful in confirming or predicting the prognosis of acute myocarditis(151). In addition, in echocardiography, cardiac wall thickening and spherical deformation of the ventricle may be found(43). A reduction in right ventricular function also serves as an indicator of a critical condition, possibly leading to fatality or the requirement for a heart transplant(152). Echocardiography can also be used to differentiate between acute and fulminant forms of myocarditis, because in the latter form, a greater recovery in ventricular function is observed by echocardiography(135). Thus, echocardiography may also be helpful in classifying the disease.

Endomyocardial biopsy (EMB) is widely regarded as gold standard for the diagnosis of myocarditis(43). It allows histological or immunohistological detection of the marked inflammatory infiltrates typically associated with myocarditis(43). However, it is essential to reserve EMB for cases where it can provide valuable prognostic and therapeutic insights(43). According to the joint scientific statement of AHA/ACCF/ESC, EMB is indicated in certain conditions(137). The statement recommends the use of EMB in patients with heart failure and a regular or enlarged ventricle, showing clinical signs that persist for less than 2 weeks. In addition, EMB is suggested for heart failure patients who have an enlarged ventricle with symptoms lasting 2 to 12 weeks, new cardiac arrhythmias, or for those who do not improve under therapy(137). Both left ventricular biopsy and right ventricular biopsy are equally safe(153). In individuals with persistent DCM who demonstrate a favorable response to immunosuppressive therapy, specific criteria based on EMB can be employed to identify them(43). These criteria include the presence of inflammation as detected in the immunohistological examination and viral genomes missing in PCR(43).

In addition to invasive methods of diagnosing myocarditis (EMB), magnetic resonance imaging (MRI) has emerged as the noninvasive gold standard for diagnosing myocarditis(154) and is recommended by ESC and the AHA(8, 155). Both functional and structural changes can be detected with the help of cardiac MRI(156). The Lake Louise Criteria, consisting of an assessment of tissue damage, swelling, edema, and perfusion, can be used to make a diagnosis of myocarditis(157). Since the different structures in the body are displayed differently in cardiac MRI, evaluation of tissue structure and potential damage is a major strength of MRI(158). In 2018, the diagnostic accuracy and sensitivity of cardiac MRI were improved(159, 160). Using various mapping techniques and sequences, better results can be achieved(159). However, the diagnostic accuracy of cardiac MRI in patients with chronic myocarditis disease is lower(161).

Magnetocardiography (MCG) is a novel promising instrument in the field of cardiology, particularly for the diagnosis and, potentially, therapy monitoring of conditions like inflammatory cardiomyopathies and amyloidosis(162). MCG is an instrument for recording the electric magnetic field of the heart generated by its activity(163). In cases such as inflammatory cardiomyopathies or amyloidosis, the electromagnetic field of the heart may exhibit variations compared to that of healthy individuals(162). In some

cases of inflammatory cardiomyopathy and amyloidosis, the response to therapy could be monitored by MCG(162, 164). However, the exact role for MCG in patients with inflammatory cardiomyopathy and amyloidosis remains to be investigated.

1.2.5 Treatment

It remains uncertain if and how to treat oligosymptomatic patients with myocarditis(43). It has been described in the literature that these patients may have poor outcomes if left untreated(165). However, it is not easy to identify this group of patients because the clinical symptoms are not always distinct(166). When ventricular function remains within the normal range, the most suitable therapeutic approach is to schedule a follow-up clinical evaluation of the patient after a period of 1 to 2 weeks(43). During this follow-up, it is crucial to assess the patient for any new symptoms related to heart failure or changes in ECG(43). Current guidelines recommend the administration of an angiotensin-converting enzyme inhibitor or an angiotensin receptor antagonist and potentially a β-blocker for patients with reduced pump function(142-144).

Management of acute myocarditis should be individualized and differs from case to case(43). In patients with DCM and decreased cardiac output, treatment should include neurohormonal blockade, as in chronic heart failure(43). Data on the effects of captopril and candesartan in patients are limited; however, there are observations suggesting potential benefits of these drugs in mouse models of myocarditis(167, 168). Most of the patients improve under standard heart failure treatment(43). In patients with severely reduced cardiac output, the use of inotropics should also be considered(169). In some cases, where the risk of cardiac rhythm abnormalities is elevated, patients should be provided with an implantable cardioverter-defibrillator(170). In patients with a mild course, administration of nonsteroidal anti-inflammatory drugs may be sufficient against thoracic pain; however, their role in the treatment of myocarditis is still controversial(171-175). Recently, it has been reported that their administration is not associated with any unwanted effects(172). Furthermore, in addition to drug treatment, individuals diagnosed with acute myocarditis are advised not to exercise for the initial 6 months until their ventricular function has fully recovered(176, 177).

The treatment of myocarditis depends on the cause that led to the onset of the disease and clinical presentation of the patient. Immunosuppression is not a standard part of myocarditis therapy, although it should be used in some forms such as GCM or

eosinophilic myocarditis(43). The results of a trial conducted to investigate the effect of immunosuppression in myocarditis patients were neutral(178). At the same time, there is controversial evidence about the effect of intravenous immunoglobulin (IVIG) in myocarditis (179). In eosinophilic, sarcoidosis or GCM(43, 180) as well as in immune checkpoint inhibitor- (ICI) associated myocarditis(181), steroid administration is recommended. Immunosuppression should also be considered in patients who do not respond to a therapy, and in whom neither histology nor PCR can detect viral replication(182). A detailed description of the therapy of myocarditis is also partly given in section "inflammatory cardiomyopathy".

1.3 Inflammatory Cardiomyopathy

1.3.1 Definition, Causes and Clinical Symptoms

Inflammatory cardiomyopathy is an inflammation of the heart muscle (myocarditis) with loss of heart function and ventricular remodeling(183). The prognosis of the disease in complicated cases therefore remains poor(12). The gaps in knowledge need to be urgently addressed to reduce mortality and the number of heart transplants associated with this disease.

Variable viruses are involved in the development of inflammatory cardiomyopathy(12). The spectrum of viruses that may contribute to inflammatory cardiomyopathy is growing and comprises parvovirus B19 (B19V), lymphotropic viruses of the Herpesviridae family (such as human herpesvirus 6 (HHV-6), Epstein-Barr virus, and human cytomegalovirus), human immunodeficiency virus (HIV), hepatitis C virus (HCV), influenza A virus, and influenza B virus(12). In addition, members of the Coronaviridae family can indirectly contribute to myocarditis through cytokine-mediated or autoimmune mechanisms, in a way comparable to influenza A and B(45).

The delay in diagnosis of myocarditis can lead to inflammatory cardiomyopathy(12). Hence, it is very important to determine the time of onset of cardiac symptoms in patients and to distinguish inflammatory cardiomyopathy from myocarditis(12). Patients with acute myocarditis are hemodynamically less stable than those with inflammatory cardiomyopathy because in the latter there is a slow cardiac remodeling and enlargement of the ventricles recompensing the systolic dysfunction(184, 185). Moreover, left ventricular dilatation and the concomitant presence of a moderate

troponin increase unrelated to the extent of LVEF impairment should be regarded as indicative of inflammatory cardiomyopathy rather than myocarditis(184).

1.3.2 Treatment of Inflammatory Cardiomyopathies

Based on the myocardial biopsy, a distinction is made between virus-negative and virus-positive myocarditis(12). In the latter case, it is also important to differentiate between virus-induced and virus-associated myocarditis(12). This classification is essential for a targeted therapeutic strategy.

In cases of chronic inflammatory cardiomyopathy without viral presence, the use of prednisone and azathioprine for suppression of the immune system may have the potential to improve heart performance(186, 187). It has been previously demonstrated that the use of immunosuppressive therapy can be beneficial in non-viral inflammatory cardiomyopathy(19). Recently, the long-term cardiovascular benefit of immunosuppressive therapy in this group of patients has also been demonstrated(16). However, the results of an observational study showed that steroid therapy was unsuccessful in 53 % of patients with inflammatory cardiomyopathy(188).

Eosinophilic myocarditis is induced, among others, by systemic diseases such as eosinophilic granulomatosis with polyangiitis or hypereosinophilic syndrome (189). Eosinophilic myocarditis should be treated symptomatically and immunosuppressively (190). In addition, the administration of immunoglobulins has proven to be effective in this group of patients; at the same time, anticoagulants may also influence the development of the disease(191, 192). Immunosuppressive therapy is indicated in GCM(133, 193). In ICI-myocarditis, the causative agent, specifically ICI, should be withdrawn and high-dose intravenous corticosteroids should be administered (194). However, the positive effect of immunosuppressive therapy is not evident in all non-viral inflammatory cardiomyopathies, and further studies are needed to determine the exact effect and role of immunosuppressive therapy.

The simultaneous administration of steroids and cyclosporine or mycophenolate mofetil or treatment with immune adsorption followed by IVIG are further therapy alternatives(195-197). The administration of mycophenolate mofetil in the setting of non-viral inflammatory cardiomyopathy has been investigated only in small studies and specific cases(198-200). It is now being investigated in patients with virus-negative myocarditis in the first prospective multicenter study in myocarditis - the TRINITIY-

DHZK26-study, in which my mentor Dr. Heidecker is the principal investigator of our center and which I have followed closely myself. In individuals with autoimmune disorders, the elimination of circulating antibodies could be of great importance(201). Pilot studies have shown a reduction in myocardial inflammation when combining immunoadsorption and IVIG(202). The safety of immunoadsorption has been demonstrated in patients with IDCM(203).

It is essential to differentiate between inflammatory cardiomyopathy triggered by a virus and inflammatory cardiomyopathy associated with a virus(12). In particular, there are two distinct categories of viruses: those with the ability to directly infect the heart, known as cardiotropic and vasculotropic viruses, and those that exert their effect indirectly (lymphotropic viruses)(12). Some viruses are capable of causing negative inotropy and indirectly damaging the heart by structurally aligning their proteins and carbohydrates with those of their host and triggering a high release of cytokines(12).

Myocarditis due to viral infections can be attributed to adenoviruses, enteroviruses, and other viruses(45). However, in cases of B19V myocarditis detection, it is not always clear whether the virus merely coincidentally coexists with or directly triggers the myocarditis(12). The administration of interferons is also a potentially significant approach to the treatment of inflammatory cardiomyopathies, as it has already proved successful in virus-positive patients(125, 204, 205). The positive effect of interferon-a in viral myocarditis has been previously reported in the literature(205). Moreover, the protective role of IFN-γ in chronic viral myocarditis was demonstrated in mice(206). A phase II study showed that IFN-b-1b therapy can achieve viral elimination or reduction in patients with viral cardiomyopathy, induced by adenoviruses and enteroviruses. However, there was no effect on the elimination of viral DNA in individuals diagnosed with myocarditis caused by B19V after IFN-b-1b therapy(204). Patients with B19V myocarditis who have a high viral load and severe clinical symptoms may benefit from IVIG therapy(12). However, patients with B19V viral DNA in the biopsy without myocardial inflammation should not receive a therapy(8). Further treatments, including cidofovir and brincidofovir, flavonoid compounds, and hydroxyurea, are presently under examination for myocarditis associated with B19V (207).

Myocarditis associated with HIV, HCV, or influenza infection warrants treatment with antiviral agents(12). Specifically, HIV-associated myocarditis can be managed with antiretroviral therapy(208), HCV-associated myocarditis typically involves a

combination therapy with antiviral agents(209), and individuals suffering from myocarditis associated with influenza may benefit from neuraminidase inhibitors(210). NT-pro BNP is an important marker for patients with HCV myocarditis and reduced cardiac function(211). The treatment approaches for COVID-19-associated myocarditis are still undergoing investigation and encompass agents such as chloroquine, hydroxychloroquine, camostat mesylate, remdesivir, and umifenovir(212, 213). Additionally, protease inhibitors, RNA polymerase inhibitors, and anticytokine agents are being explored(214, 215).

1.3.3 Innovative Therapeutic Concepts

Inhibition of the immune response at various levels, and thereby breaking the release of targeted cytokines, could successfully prevent the development of myocarditis. A systemic approach would allow the development of targeted and personalized therapies that could inhibit these components that lead to the immune response in inflammatory cardiomyopathies. This has been previously attempted by many colleagues and also in our work(11, 27).

Novel therapies currently under investigation include soluble anti-CAR antibodies(216), antagonists of IL-1 receptor(217), anti-IL-17 antibodies(217), cell-based therapies(218), aldosterone antagonists(219), cannabidiol(220) and antagomirs(221), and modulation of the gut microbiome(222). The role of NLRP3 inflammasome activation in the development of myocarditis has been highlighted in the literature(223). There has also been a recommendation for studies on patients with monoclonal antibodies against IL-17(224, 225). In addition, mechanical circulatory support systems can be an option in patients with fulminant myocarditis(226, 227).

Regulatory T cells have the task of maintaining immune homeostasis(228). They suppress the development of effector T cells, especially Th17 cells, which have a pro-inflammatory effect through the production of IL-17(229, 230). An imbalance between Treg cells and Th17 cells in favor of Th17 cells plays an important role in various inflammatory and autoimmune diseases(231). Increasing the production of Th17 cells, and thus the ratio of Treg cells to Th17 cells, could enhance the function of Treg cells and reduce inflammation, e.g., in the context of myocarditis or other inflammatory cardiomyopathies. New approaches show that the number of Treg cells can be elevated, for example, by the administration of Treg cells(218) or IL2 agonists(232) as well as by the administration of mesenchymal cells(233).

1.4 Idiopathic Dilated Cardiomyopathy (IDCM)

IDCM is defined as a primary myocardial disease whose origin is not yet known and which is marked by left ventricular dilatation and cardiac systolic dysfunction(234). Sex (men have a 2.5 times higher risk compared to women), nationality (blacks are more susceptible compared to whites), alcohol consumption and hypertension seem to be related to IDCM(235-237). About half of the familial DCM cases are associated with genetic alterations. The actual incidence of IDCM is unknown, as many asymptomatic cases go undetected(238). The disease is associated with a poor prognosis(239, 240).

IDCM is morphologically characterized by a dilatation of both ventricles and hypertrophy(241, 242). However, it is difficult to detect hypertrophy due to the dilatation of the ventricles(243). Pathologically, thrombi can be detected(241). Moreover, morphological changes in the mitral and tricuspid valves can be found(243). Microscopically, significant myocyte enlargement and degeneration, fibrosis and infiltrates can be seen(244).

The median age of affected patients is 35 years(245, 246) and in most cases, advanced heart failure is the earliest symptom(246-248). Most patients report decreased exercise capacity, shortness of breath, and in some cases even chest pain and palpitations(243, 248, 249). Peripheral edema can be detected in approximately one third of patients(248). Ventricular arrhythmias and sudden cardiac arrest may also occur(250). At the same time, asymptomatic cases are possible(246-248). In patients with severely reduced LVEF, implantable cardioverter-defibrillator or cardiac resynchronization therapy could be beneficial in an effort to decrease the risk of sudden cardiac death and improve heart function(251, 252).

1.4.1 Pathogenesis of IDCM

The occurrence of IDCM is influenced by several key factors, including genetic elements and predisposition in the family, infectious causes (for example, in the context of myocarditis), toxic causes, hormonal influences (peripartum cardiomyopathy), and immunological abnormalities(243, 253).

Family and Genetic Aspects of IDCM

IDCM with familial components has a higher frequency than was previously thought (253, 254). Obtaining a thorough history is critical to differentiate between familial or

non-familial IDCM(254). It has been previously demonstrated that in one-fifth of affected patients, at least one close family member has manifestations of reduced cardiac output and cardiac dilation(254). Moreover, it has been reported that genetic abnormalities may be present in more than one-third of patients with IDCM(255). In most families, the disease is inherited in an autosomal dominant manner(254). However, there are also cases with autosomal recessive(256), X-linked recessive(257) and mitochondrial inheritance in addition to autosomal dominant inheritance(258). A recent study was able to examine the prevalence of familial IDCM using a model: IDCM was found in approximately one-third of close first-degree family members and in more than half of the entire family(259). This shows that family screening in IDCM patients is important so as to identify and treat these subjects early, before the disease progresses to an advanced stage.

The MHC genes serve as a bridge connecting innate and adaptive immunity(260). The occurrence of familial IDCM has been previously related to MHC genes, namely HLADR4(261). The frequency of HLADR4 was found to be higher in patients with familial IDCM(261). This was also demonstrated by other colleagues who found a correlation between IDCM and DQw4 as well(88).

1.4.2 IDCM and Viral Myocarditis

IDCM is often regarded as a consequence of viral myocarditis; however, when assessing the initial clinical presentations, it is challenging to differentiate between these two conditions(262). The precise mechanisms underlying virus-related myocardial damage remain unclear. MHC molecules play a relevant role in myocarditis and IDCM(58, 88, 263).

In IDCM and myocarditis, genetic variants play a significant role with regard to the phenotype, as they may affect the immune system and induce an inflammatory response in the myocardium(58, 264). Consequently, it is not surprising that these two conditions often co-occur with other autoimmune diseases(265).

It has been suggested in the literature that some patients with IDCM represent an advanced late stage of myocarditis caused by CVB(266). There are reports of patients with myocardial inflammation and IDCM with evidence of enteroviral infection(267-270). In addition to enteroviruses, B19V and HHV-6 viruses have been detected in patients with IDCM, supporting the hypothesis that viral persistence in the myocardium

may lead to the occurrence of IDCM(271). However, it is unclear whether viral myocarditis and, more precisely, viral residuals are closely related to IDCM, because enteroviruses, B19V and HHV-6, have also been found in control cases(272-275).

1.4.3 Cardiac Transplantation and IDCM

DCM is the primary cause of heart transplantation in the majority of cases(276, 277). Heart transplantation can lead to an overall increase in the patient's quality of life(276). However, due to the limited availability of donor organs, heart transplantation is not an option for all patients (278, 279). In particular, in familial DCM, affected individuals often remain undiagnosed until an advanced stage, resulting in a higher likelihood of requiring transplantation. Thus, family screening of affected individuals is of great importance in order to minimize the number of transplants needed. Heart transplantation is particularly reasonable for patients under 60 years of age with ongoing complications of disease(243).

2 Materials and Methods

2.1 Population

a. Derivation Cohort and Generalizability Cohort

Our derivation and generalizability cohorts included patients treated at Charité Campus Benjamin Franklin between 2014 and 2021 from whom serum samples and endomyocardial biopsies were systematically collected. All patients were recruited from the Deutsches Herzzentrum der Charité (DHZC). The samples were obtained at the same time as the endomyocardial biopsies and stored in a freezer at -80 °C. Based on the endomyocardial biopsy, we classified the patients from our center into 2 subgroups: 1) Patients with inflammatory cardiomyopathy (study cohort, N=63), and 2) patients with IDCM (generalizability cohort, N=41). The generalizability cohort was used to assess the external validity/generalizability of the study's findings. Patients with amyloidosis, glucocorticoids or immunosuppression were ruled out for the analyses. Statistical analyses encompassed data from 529 patients.

b. Validation Cohort

We also used a validation cohort to perform validation of our results. The samples from the patients used for the validation cohort were collected as part of the TORCH network of the German Centre for Cardiovascular Research (DZHK). Since 2014, these have

been obtained from 11 distinct centers throughout Germany, stored in a biobank and then transported at -80 °C. For our validation cohort, we only received patients with inflammatory cardiomyopathy. These patients were diagnosed by biopsy, clinical findings or cardiac MRI. All patients with immunosuppression as well as patients with missing important data, for example LVEF, were excluded. 425 patients were included in the analyses. For cytokine analysis by Olink (Olink Bioscience, Uppsala, Sweden), EDTA samples were of great importance to ensure sample homogeneity. Therefore, we included only patients from whom samples were available in EDTA tubes.

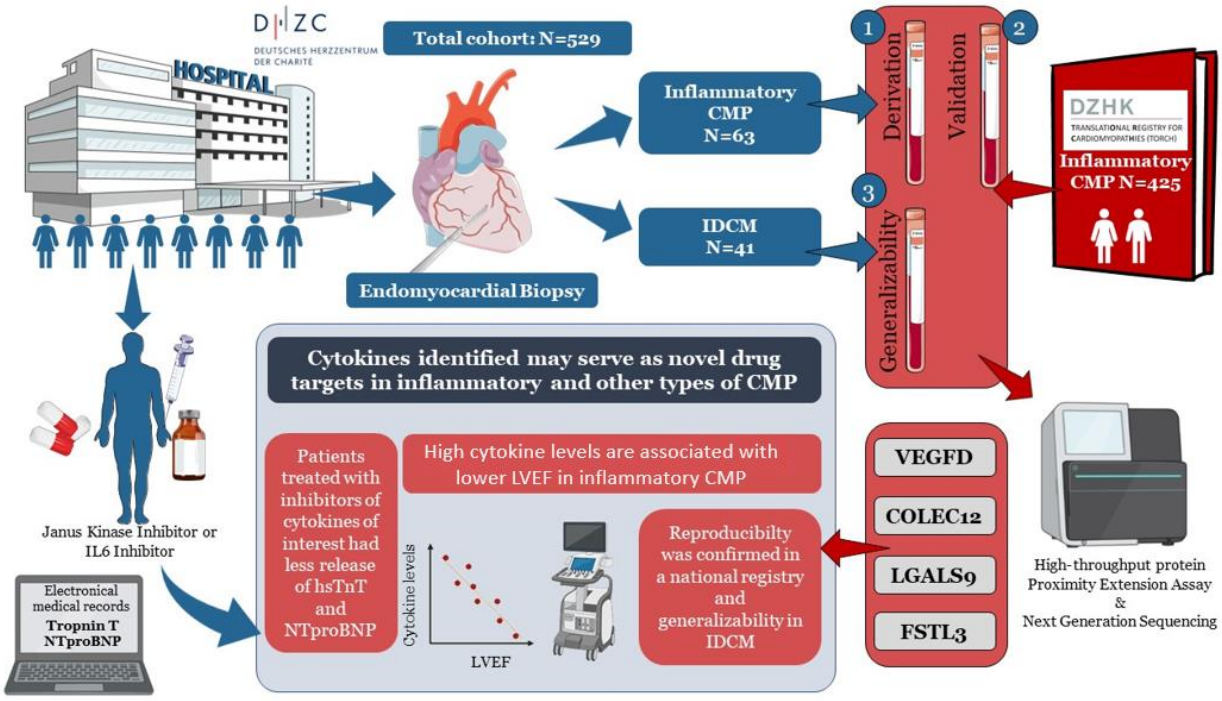


Figure 1: *Graphical abstract.*

Patients included in the analyses were finally divided into the following groups: Derivation Cohort (patients with inflammatory cardiomyopathy, N=63), Generalizability Cohort (patients with IDCM, N=41), and Validation Cohort (patients with inflammatory cardiomyopathy, N=425). Published in our preprint manuscript(280).

2.2 Cytokine Analysis

The company Olink carried out cytokine analyses using Proximity Extension Assay (PEA) technology. The samples used in the analyses were EDTA blood from patients.

The samples were systematically transported at -80 °C using dry ice to maintain their stability during shipment. The analysis was conducted following the manufacturer's instructions for the PEA technology. PEA technology uses pairs of oligonucleotide-labeled antibodies that bind to specific protein targets, including cytokines (please see https://olink.com/our-platform/our-pea-technology/). Once the two antibodies bind to neighboring epitopes on an identical protein target, their oligonucleotides are strung together and can be elongated by a DNA polymerase to build a DNA amplicon. To quantify this signal, real-time PCR or NGS can be used, enabling simultaneous detection and quantification of numerous biomarkers in a unique sample. The technology has several advantages, including high sensitivity, specificity, and dynamic range, as well as the ability to analyze multiple analytes in parallel with minimal sample requirements and without compromising data quality(281).

For the cytokine analyses described here, the Explore 384 - "Inflammation" subpanel from Olink was selected. This subpanel is part of the larger Explore 3072 panel, which is designed for high-throughput protein biomarker analysis in a wide range of research areas. The Explore 384 - "Inflammation" subpanel specifically targets a range of cytokines and other inflammation-related proteins relevant to various inflammatory diseases and conditions. A comprehensive list of cytokines featured in the subpanel can be accessed in the supplemental material (Supplemental File 1, "Olink 384 Inflammation list").

The creation of sample libraries, quality assessment, and data analysis were all conducted using a standardized process developed by Olink. Additional information about the standardized process used by Olink can be found on their website at https://www.olink.com.

Data generated by Olink's cytokine analyses can be used for multivariate statistical analysis, which involves examining the relationships between multiple variables (in this case, the expression levels of multiple cytokines) and identifying patterns or correlations among them. The results are displayed as normalized protein expression (NPX), enabling comparisons between samples within the same assay. However, it is important to note that NPX does not offer absolute quantification of protein levels. Log-transformed values are used for cytokines to obtain a better normal distribution.

2.3 Statistical Analysis

To assess the relationship between cytokine levels and LVEF, we performed a broad analysis considering covariates (such as patients' sex and cohort) and applying robust statistical methods for correction and analysis. We used a linear regression model in R to assess the effect of cytokine concentration on LVEF. In this model, LVEF was the response variable and cytokine levels (NPX) served as predictor variables. The false discovery rate (FDR) was tested with the Benjamini-Hochberg method. The two data collections from Olink (the data for DHZC samples and the data for DZHK samples) were merged with the olink_normalization option from the OlinkAnalyze package (v. 3.4.1). For the normalization of the data sets, we used 16 identical bridging samples in the two different batches. We corrected P values for multiple testing using the Benjamini-Hochberg method.

2.4 Hypothesis Verification by Evaluation of Electronic Medical Records (EMR)

To test the causality of our findings, we examined whether patients who received cytokine inhibitors had less cardiac damage. The degree of cardiac damage was determined by the concentration of the cardiac enzymes troponin T (hs-TnT) and N-terminal pro B-type natriuretic peptide (NT-proBNP). All necessary data for this further testing of our hypothesis were obtained from electronic medical records (EMR) in three rheumatology divisions of Charité Universitätsmedizin Berlin, covering the period from January 2018 to May 2023. In this study, we firstly identified specific cytokines that had a significant association with lower LVEF. To test our hypothesis, we focused only on patients who underwent treatment with antagonists of the proteins identified or cytokine inhibitors targeting the molecules that are activated downstream by these proteins. We were able to obtain data for patients who received IL-6 or Janus kinase inhibitors. These underwent our analysis. We excluded patients for analyses if the levels of hs-TnT and NT-proBNP were not available. Patients who had not been administered cytokine inhibitors served as reference group.

For the group with the cytokine inhibitors, the median of all laboratory results following the start of cytokine inhibitor treatment was computed, while for the reference group without cytokine inhibitors, the median value of all available laboratory results was computed. The median of relevant laboratory values was compared among different groups using a Kruskal-Wallis test.

To form comparable reference groups, we used the nearest-match method and formed a control group for each cytokine in a ratio of 1:4. To find the best controls to match the cytokine inhibitor group, cardiac, rheumatologic, renal, and autoimmune disorders were considered. Since low renal function may result in higher plasma concentrations of hs-TnT and NT-proBNP, participants with a reduced kidney function (glomerular filtration rate, GFR, <30 ml/min/m²) were not included in the analysis to avoid misinterpretation. Boxplot method(282) was used to detect and exclude all outliers. A linear model was created to test our hypothesis.

2.5 Ethics Committee

Written informed consent was obtained from all patients in this study. The ethics committee of DHZC Universitätsmedizin Berlin (EA4/056/20, EA1/187/22) and the ethics committee of Medizinische Fakultät Heidelberg (S-344/2014) have reviewed and approved this study.

Every subject from the TORCH registry provided a written consent. The study received approval from the ethics committees of all involved centers. An additional blood sample was obtained from every subject.

2.6 Data Sharing Statement

Access to an anonymized dataset is restricted to academic scientists who have signed a data sharing agreement.

3 Results

3.1 Demographic Data

The groups showed no significant differences in terms of age, sex, body mass index (BMI), renal function and LVEF. Study participants had a mean age between 49 and 54 years old. The median age in the validation cohort was 49 and in the derivation and generalizability cohorts it was 54. The proportion of men in the study ranged from 68.3% to 71.8%. Participants were predominantly overweight, with a BMI between 26-27 kg/m². The study population had normal renal function, since the median blood

creatinine levels of the patients were between 0.93 and 0.94 mg/dl, which is in the normal reference range. The median LVEF measured was between 42% and 45%. As mentioned previously, all cohorts were divided into two subgroups based on LVEF: Patients with a significant reduced LVEF (≤ 35%) and patients with minor to intermediate LVEF (>35%) reduction. Among patients, between 36.5% and 41.5% were classified in the first group with a severe reduction in left ventricular function.

	Derivation/Study cohort (n=63)	**Validation cohort (n=425)**	**Generalizability cohort (n=41)**
Cardiomyopathy Type	Inflammatory Cardiomyopathy	Inflammatory Cardiomyopathy	Idiopathic Dilated Cardiomyopathy
Registry	DHZC	DZHK	DHZC
Female sex (n, %)	19 (30.2%)	120 (28.2%)	13 (31.7%)
Age in years (median, IQR)	54 (36 - 62)	49 (37 - 58)	54 (48 - 65)
BMI in kg/m2 (median, IQR)	27 (25 - 29)	26 (24 - 30)	27 (24 - 32)
LVEF ≤ 35% (n, %)	23 (36.5%)	169 (39.8%)	17 (41.5%)
LVEF (median, IQR)	45 (31 - 58)	42 (30 - 55)	42 (30 - 58)
Creatinine in mg/dl (median, IQR)	0.93 (0.83 - 1.1)	0.93 (0.82 - 1.1)	0.94 0 (85 - 1.1)

Table 1: *Demographic data.*

Presentation of demographic data for the 3 cohorts (derivation, validation, and generalization cohort). Published in our preprint manuscript(280).

3.2 Cytokine Analysis - Derivation Cohort

In this study, we sought to evaluate the hypothesis that high concentrations of cytokines are related to a more severe course of inflammatory cardiomyopathy. To test this hypothesis, a linear regression model was performed.

In the derivation cohort (DHZC, n=63), 5 cytokines were identified that were significantly related to LVEF after correction for multiple testing (FDR<0.05, see Table 2 and Supplemental File 2 (“Full Results”)), suggesting that elevated levels of these cytokines may lead to lower LVEF in patients with inflammatory cardiomyopathy.

Cytokine	Estimate	r2	P value	FDR
VEGFD	-15.418	0.421638	5.47E-08	2.01E-05
CLSTN2	-12.3104	0.315359	1.01E-05	0.003696
FSTL3	-14.615	0.313893	1.08E-05	0.00394
KRT19	-8.64163	0.31284	1.13E-05	0.004121
CRIM1	-20.57	0.287229	3.54E-05	0.012901
COLEC12	-14.5942	0.244778	0.000219	0.079625
CHRDL1	-13.612	0.228297	0.000436	0.15771
LAIR1	-10.3162	0.222985	0.000542	0.195762
PRSS8	-12.4807	0.22271	0.000548	0.197437
LY6D	-11.7192	0.217577	0.000677	0.243021

Table 2: *Top 10 cytokines in the derivation cohort.*

These cytokines were inversely associated with LVEF. The higher the level of these cytokines, the lower the LVEF. Published in our preprint manuscript(280).

3.3 Normalization/Bridging of the Data

Samples for this study were obtained in two batches and normalization of the data was required. The first batch included the samples of the derivation and generalizability cohort, while the second batch comprised the samples of the validation cohort. The OlinkAnalyze package, version 3.1.0, was used to normalize the data according to the recommendations of the manufacturer. Figures 2 and 3 show the results.

To verify that the derivation and validation cohorts did not differ in any subgroups, a principal component analysis was carried out. The two data sets showed no relevant differences and were thus comparable (Figures 4 and 5).

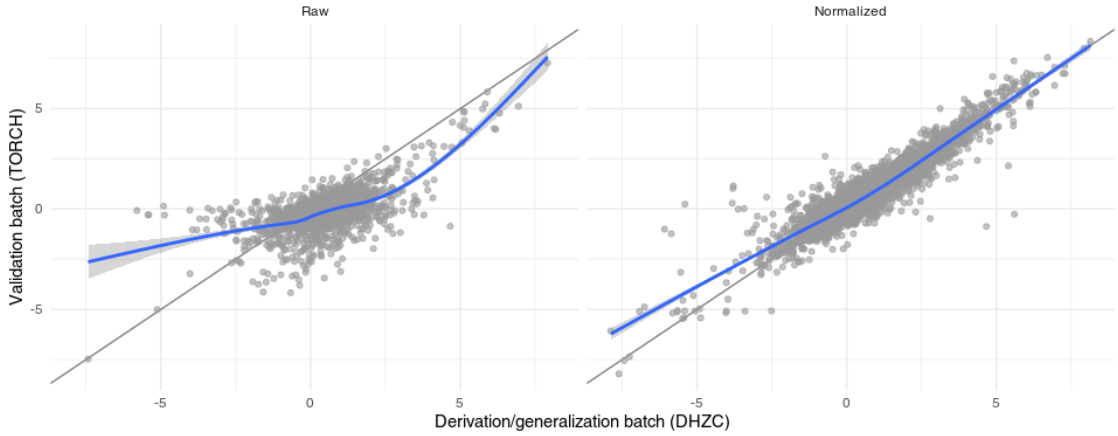


Figure 2: *Normalization/Bridging of the data.*

The raw data are presented on the left and the normalized data on the right. This figure shows NPX values of all protein assays for the 16 bridging samples from the first (X-axis, derivation and generalization cohorts) and second batch (Y-axis, validation cohort). Each protein assay is represented with a dot. The blue line demonstrates a fitted LOESS model and overall, there were 5 samples, 368 assays and 1840 total measurements. Published in our preprint manuscript(280).

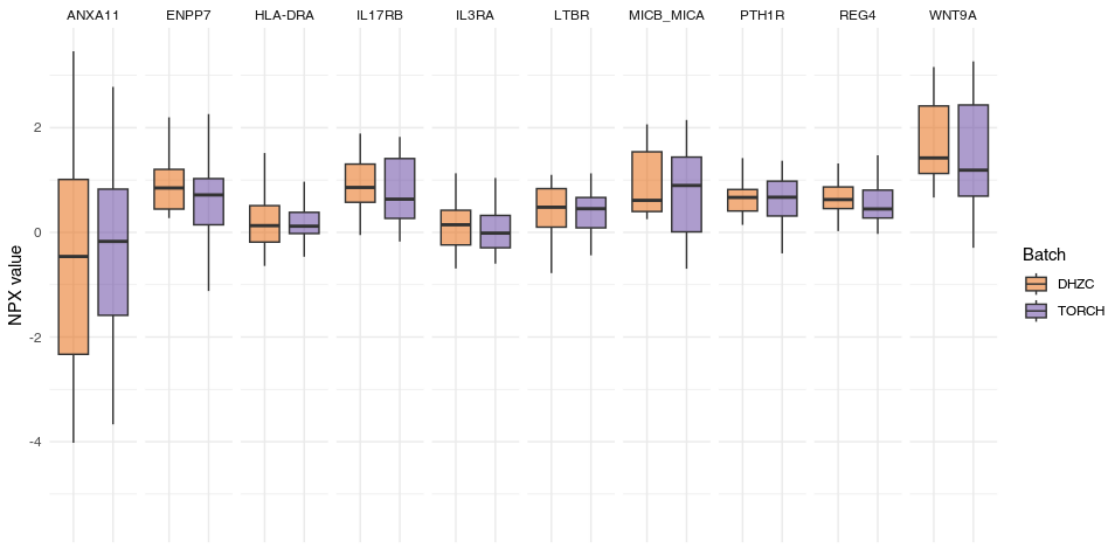


Figure 3: *Comparison of protein assays.*

Comparison of ten randomly chosen protein assays across the different data sets and different normalizations for the 16 bridging samples. In total, 16 unique samples and 420 measurements are shown. Published in our preprint manuscript(280).

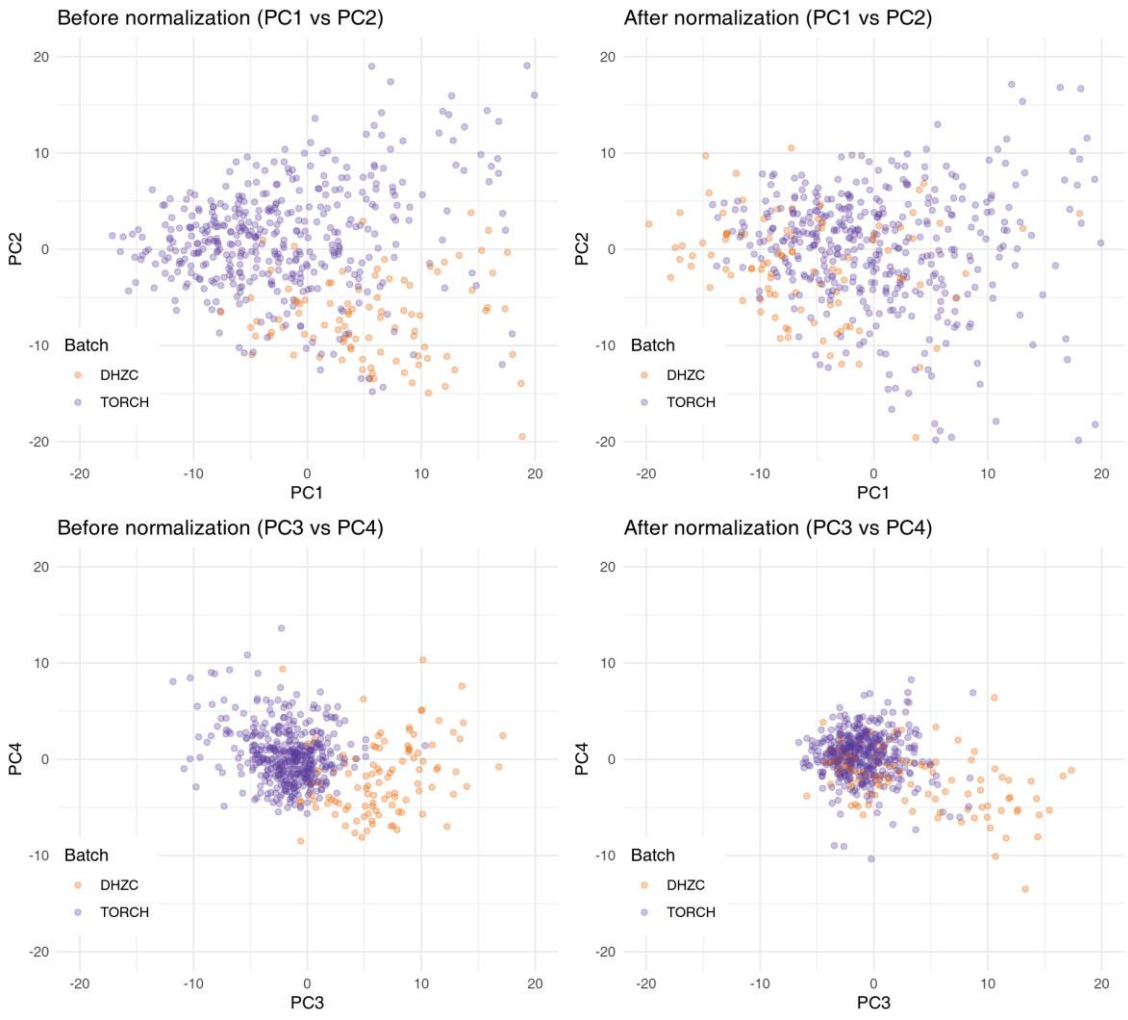


Figure 4: *Principal component analysis.*

The different batches are marked with different colors. A principal component analysis of the 529 measurements before normalization is shown on the left and after normalization on the right. While the top rows present the first two principal components, the bottom rows present the third and fourth principal components. Published in our preprint manuscript(280).

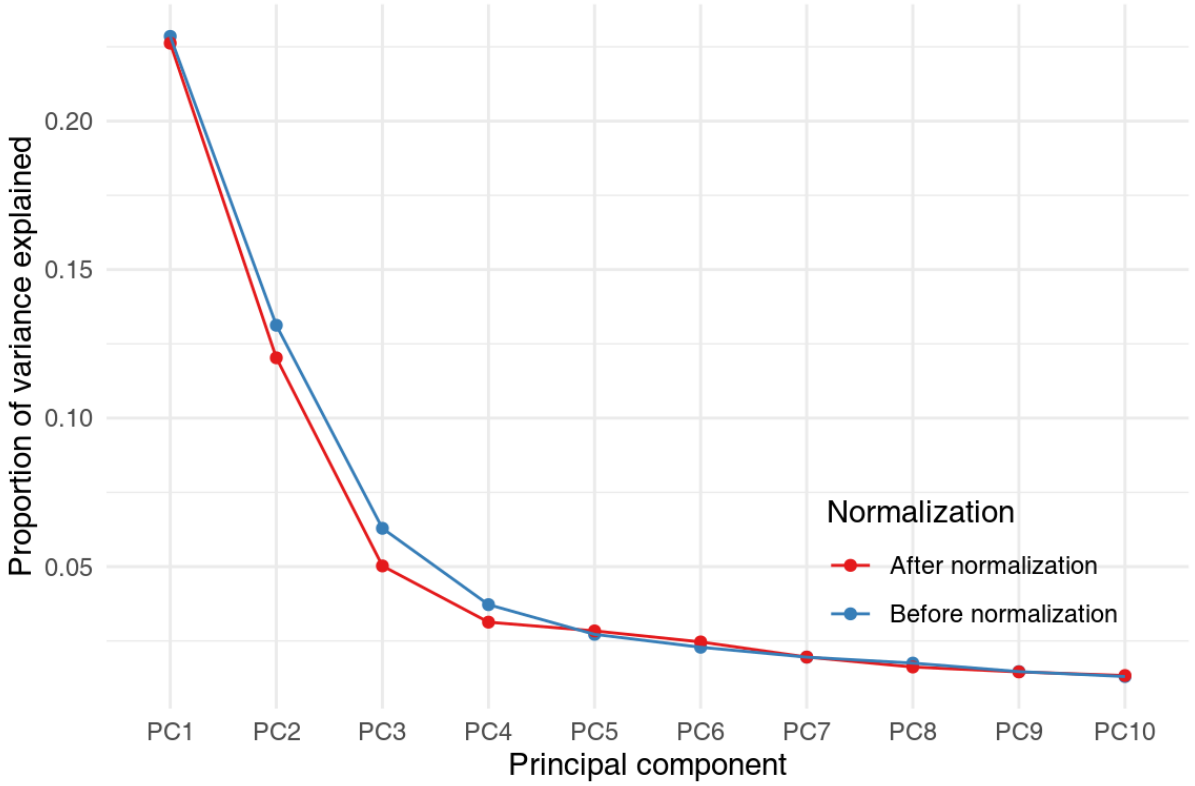


Figure 5: *Scree plot.*

Scree plot of the principal component analysis of the 529 measurements before normalization illustrated in red and after normalization in blue. The magnitude of variance explained by each principal component is shown in the y-axis. The x-axis displays the principal components. Published in our preprint manuscript(280).

3.4 Cytokine Analysis - Validation Cohort

After normalization of our data, cytokine analysis was performed for the validation cohort. For this purpose, a linear regression model was constructed and the relationship of elevated concentrations of cytokines to reduced LVEF was reexamined in the validation cohort. The results revealed 77 cytokines, including the 10 most

significant cytokines already found in the derivation cohort. This replication verified that the relationship of these cytokines to decreased LVEF was highly significant in patients with inflammatory cardiomyopathies (FDR<0.05; Table 3; see Supplemental File 2 ("Full Results")).

Cytokine	**Estimate**	**r2**	**P value**	**FDR**
COLEC12	-12.1741	0.164675	4.46E-16	1.64E-13
PLAUR	-11.1866	0.163439	6.12E-16	2.24E-13
CCL3	-7.14421	0.152285	1.04E-14	3.81E-12
LILRB4	-9.01034	0.151545	1.25E-14	4.58E-12
CXCL17	-5.54511	0.149364	2.18E-14	7.92E-12
AGRN	-10.9161	0.148426	2.75E-14	1E-11
WNT9A	-9.78385	0.141907	1.41E-13	5.11E-11
CD4	-11.4165	0.137435	4.31E-13	1.56E-10
FABP1	-4.20844	0.136129	5.96E-13	2.15E-10
LAIR1	-7.74702	0.133987	1.01E-12	3.64E-10
LGALS9	-8.96591	0.123841	1.24E-11	4.44E-09
CRIM1	-12.4333	0.122375	1.78E-11	6.35E-09
TNFSF13	-10.0253	0.121528	2.19E-11	7.79E-09
FSTL3	-7.4228	0.113642	1.5E-10	5.33E-08
CCL7	-4.43945	0.109818	3.8E-10	1.35E-07
TGFA	-7.94094	0.109175	4.44E-10	1.57E-07
CLSTN2	-6.20831	0.107495	6.67E-10	2.35E-07
HGF	-4.49271	0.104511	1.37E-09	4.82E-07
ANGPTL4	-6.68144	0.104232	1.47E-09	5.14E-07
SPON1	-7.40211	0.103125	1.92E-09	6.69E-07
IL17D	-9.61421	0.101249	3.01E-09	1.05E-06
LRRN1	7.205829	0.101132	3.1E-09	1.08E-06
LY6D	-7.43169	0.098996	5.18E-09	1.79E-06
PON3	11.39412	0.095766	1.13E-08	3.88E-06
HLA-E	-11.4467	0.095722	1.14E-08	3.91E-06
SPINK4	-4.40881	0.095501	1.2E-08	4.11E-06

Cytokine	Estimate	r2	P value	FDR
LGALS4	-5.27358	0.09436	1.58E-08	5.39E-06
IL6	-2.99671	0.093813	1.8E-08	6.13E-06
NPPC	-4.98094	0.093211	2.08E-08	7.06E-06
CHRDL1	-7.91593	0.092942	2.21E-08	7.51E-06
TREM2	-4.77254	0.091431	3.18E-08	1.07E-05
CXCL10	-4.17866	0.088631	6.21E-08	2.09E-05
EPO	-3.74666	0.087718	7.72E-08	2.59E-05
CXCL8	-4.01822	0.087691	7.77E-08	2.6E-05
MATN2	-9.39644	0.086945	9.28E-08	3.1E-05
CXCL14	-4.49222	0.08555	1.29E-07	4.31E-05
GAL	4.65288	0.082948	2.4E-07	7.98E-05
TFF2	-4.06551	0.081606	3.31E-07	0.000109
PREB	-10.3113	0.081157	3.68E-07	0.000121
SULT2A1	-4.04533	0.078762	6.5E-07	0.000214
ENPP7	-3.32201	0.077855	8.06E-07	0.000264
IL15	-6.78804	0.077687	8.39E-07	0.000274
DNER	9.589636	0.077289	9.22E-07	0.0003
CXCL9	-3.22193	0.074958	1.6E-06	0.000521
IL4R	-6.07854	0.074123	1.95E-06	0.000633
REG4	-4.97668	0.073114	2.48E-06	0.000801
BTN3A2	-6.09672	0.071531	3.61E-06	0.001162
FST	-5.18705	0.071502	3.63E-06	0.001166
EGLN1	-3.95482	0.07108	4.01E-06	0.001284
TNF	-5.94492	0.070676	4.42E-06	0.001409
IL1RN	-3.4451	0.070089	5.08E-06	0.001614
NTF3	-5.07255	0.069915	5.29E-06	0.001677
TNFRSF13B	-6.8624	0.069553	5.76E-06	0.001821
CXADR	-4.82297	0.068877	6.76E-06	0.00213
CD276	-5.47858	0.068871	6.77E-06	0.00213
TNFRSF11A	-4.2661	0.06851	7.38E-06	0.002308
SMOC2	-5.99596	0.068308	7.73E-06	0.002413
CCL25	-4.11859	0.067723	8.88E-06	0.002763

Cytokine	Estimate	r2	P value	FDR
NFASC	-7.98799	0.067721	8.89E-06	0.002763
CCL28	-3.05271	0.067265	9.9E-06	0.003059
SIGLEC10	-5.64472	0.066844	1.09E-05	0.003368
PRSS8	-5.97756	0.066519	1.18E-05	0.003626
LAMA4	-6.9675	0.066442	1.2E-05	0.00368
PRELP	-8.7097	0.065305	1.57E-05	0.004801
ENPP5	5.817113	0.063325	2.51E-05	0.007643
CCL21	-4.3005	0.063229	2.57E-05	0.007793
TNFRSF4	-4.64774	0.061676	3.71E-05	0.011215
HLA-DRA	-5.76163	0.061424	3.94E-05	0.011865
CCL23	-5.004	0.061069	4.29E-05	0.012863
FASLG	4.476295	0.060662	4.72E-05	0.014115
TPP1	-5.9269	0.059997	5.53E-05	0.016466
CCL11	-4.03204	0.059247	6.6E-05	0.019599
CCL13	-2.60901	0.057756	9.39E-05	0.0278
KRT19	-3.53618	0.057209	0.000107	0.031541
LIFR	-6.6545	0.05658	0.000124	0.036485
ITM2A	-4.51045	0.056553	0.000125	0.036589
CKAP4	-5.15816	0.056407	0.000129	0.037749

Table 3: *Validation of the results.*

An inverse association between the concentration of specific cytokines and LVEF was evident in the validation cohort as was the case in the derivation cohort. There were 72 significant cytokines detected (FDR < 0.05). Estimated value, the estimated coefficient from the linear regression model; r^2, coefficient of determination (r^2) value for the model; P value, raw p value for the model; FDR, false discovery rate calculated with Benjamini-Hochberg correction. Published in our preprint manuscript(280).

Cytokine	Estimate	r2	p value	FDR
COLEC12	-12.17	0.1647	4.5e-16	1.6e-13
LAIR1	-7.747	0.134	1.0e-12	3.6e-10

Cytokine	Estimate	r2	p value	FDR
CRIM1	-12.43	0.1224	1.8e-11	6.3e-09
FSTL3	-7.423	0.1136	1.5e-10	5.3e-08
CLSTN2	-6.208	0.1075	6.7e-10	2.3e-07
LY6D	-7.432	0.099	5.2e-09	1.8e-06
CHRDL1	-7.916	0.09294	2.2e-08	7.5e-06
PRSS8	-5.978	0.06652	1.2e-05	0.0036
KRT19	-3.536	0.05721	0.00011	0.0315
VEGFD	-3.324	0.04488	0.00202	0.5383

Table 4: *Reproduction of the results.*

The results of the analyses in the derivation cohort with the 10 top significant proteins could also be reproduced in the validation cohort. Higher levels of these cytokines were associated with lower LVEF. Published in our preprint manuscript(280).

3.5 Generalizability Testing of the Findings

In the previous steps, cytokines were detected that showed a significant association with LVEF. Subsequently, the generalizability of these cytokines to patients with IDCM (DHZC=41) was examined with a Welch two-tailed t test, comparing IDCM patients with severely reduced LVEF to patients with mildly to moderately reduced LVEF. The results were not significant in the IDCM patients following correction for multiple testing; however, there was a similar trend to the derivation and validation cohorts. More specifically, high levels of the key cytokines from the other two cohorts were also related to reduced LVEF in IDCM patients. At the same time, a significant relationship was found between the outcomes of the derivation and generalizability cohorts (Pearson's correlation coefficient=0.29). Supplemental File 2 shows a detailed overview of the values for all analyzed cytokines.

3.6 Association between LVEF and Sex

In this part, it was examined whether there is an association between LVEF and sex in the derivation and validation cohorts.

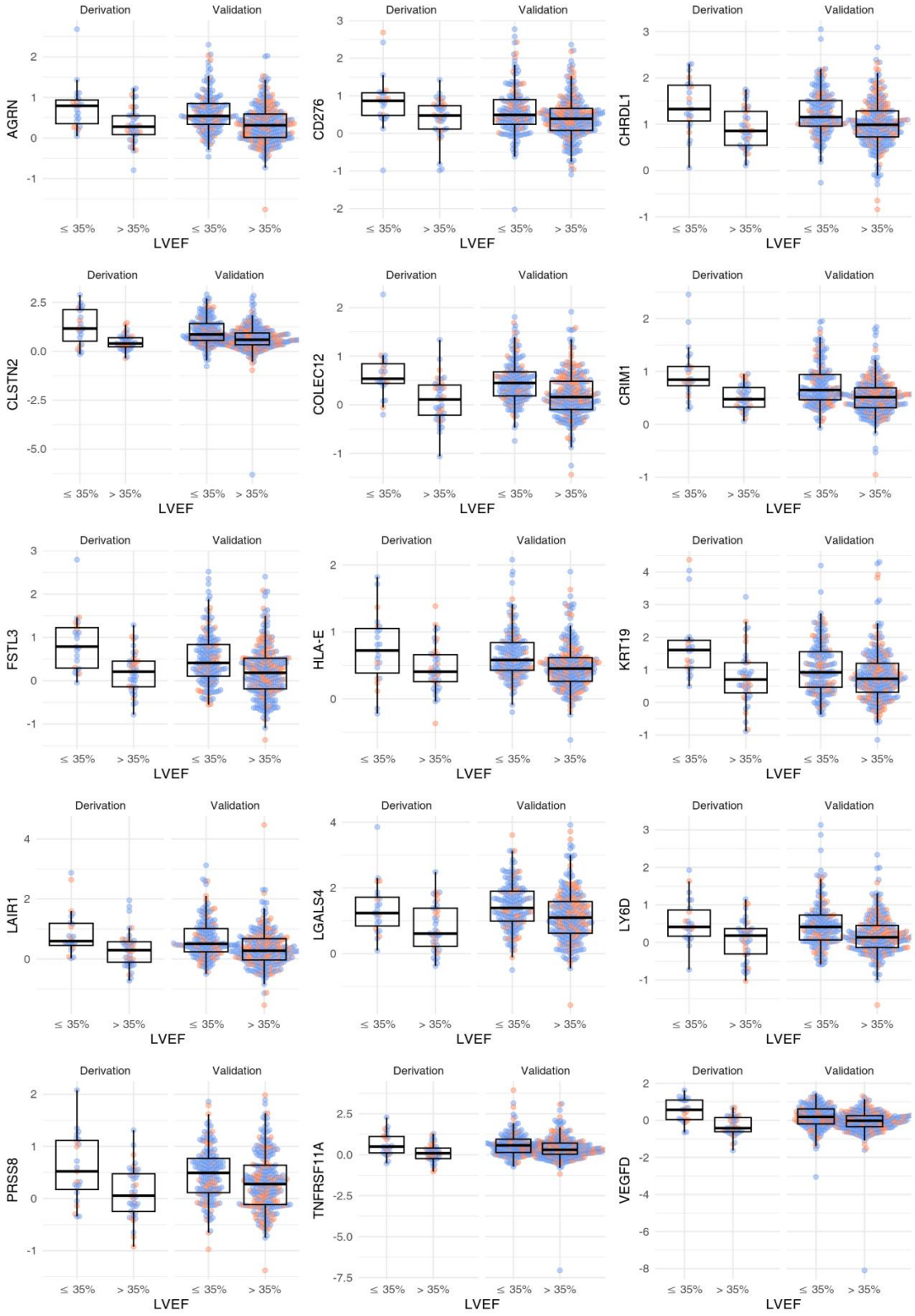
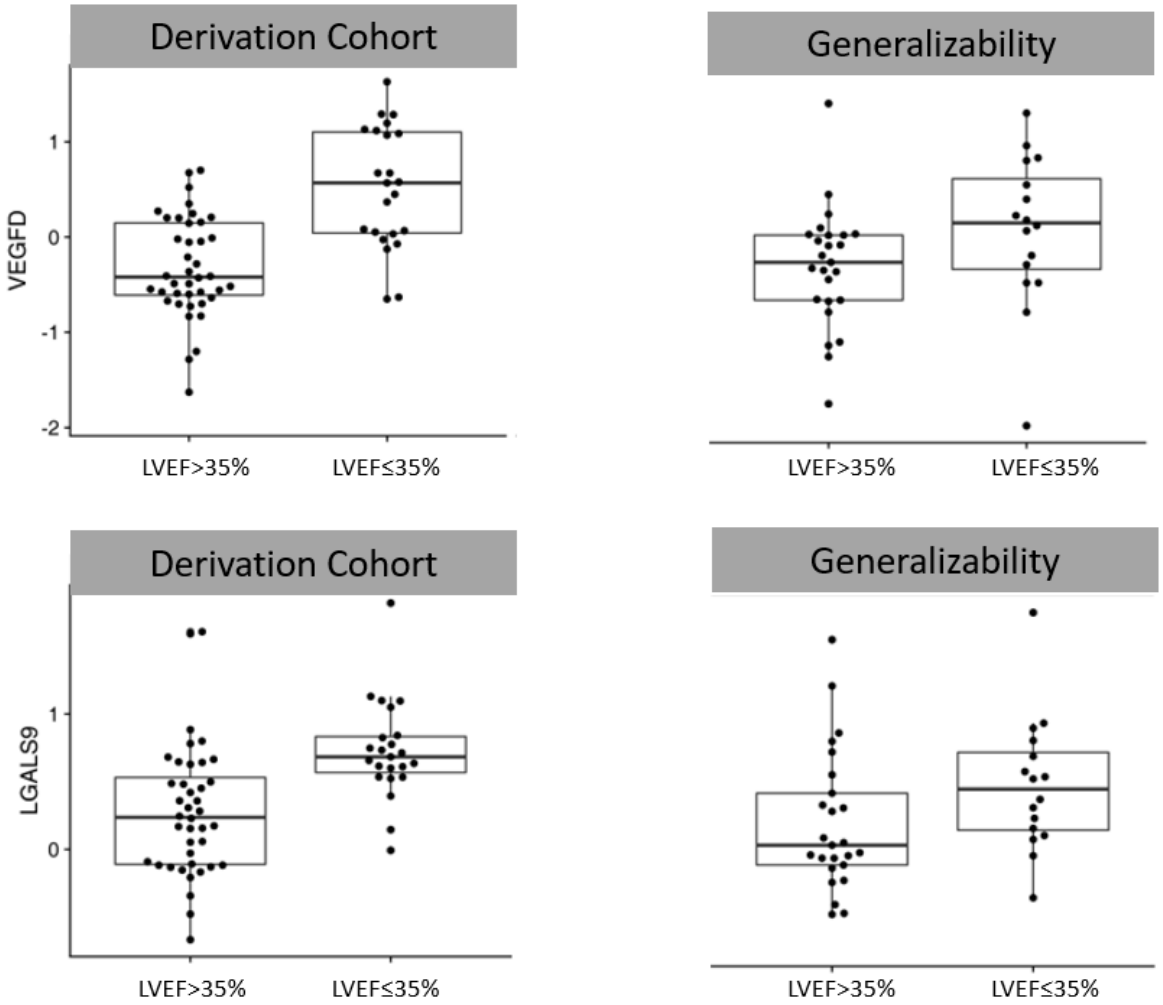


***Figure 6**: Association between LVEF and sex.*

The relative cytokine concentration in the different cohorts is illustrated in this figure. We investigated whether there is a correlation between heart function and sex in the derivation and validation cohorts (n=488). In each cohort, patients were divided into two subgroups based on LVEF, with males represented in blue and females in red. There was no correlation between LVEF and sex. However, all cytokines at high concentrations showed a significant association with low LVEF (FDR < 0.001). Published in our preprint manuscript(280).

3.7 Top 4 Significant Cytokines when Combining Derivation and Generalizability Cohorts



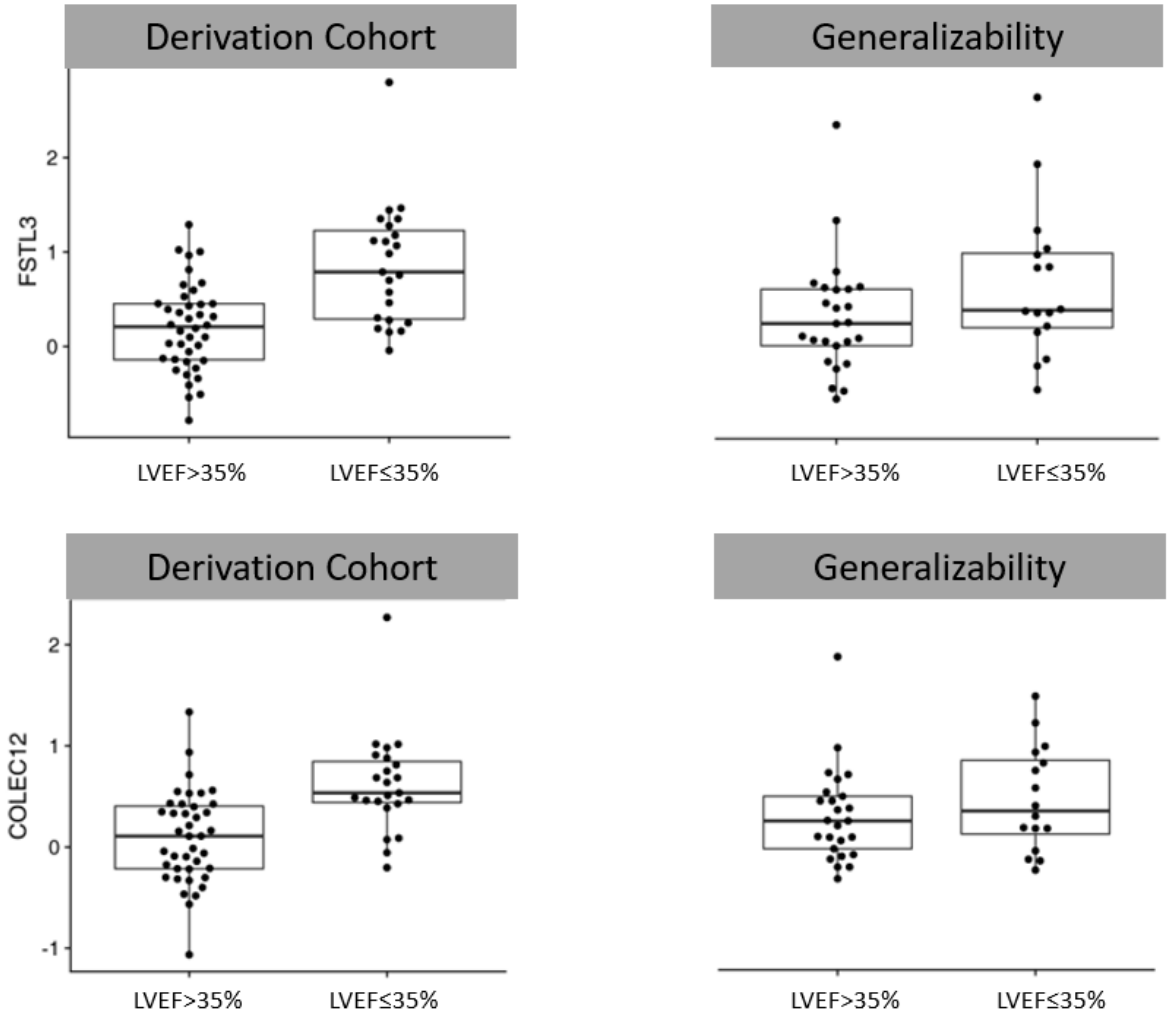


Figure 7: *Top proteins of derivation and generalizability cohorts.*

Top 4 significant proteins when combining derivation and generalizability cohorts. The box plots show that the median concentration of these cytokines is greater in patients with lower LVEF than in patients with higher LVEF.

3.8 Post Hoc Analysis - Derivation and Validation Cohort

Here, we analyzed all cytokines from the derivation and validation cohorts collectively, compared them with each other, and examined their capacity to differentiate between mildly and severely reduced LVEF. Sex and origin data sets were determined as covariates and a total of 77 cytokines exhibited statistical significance after adjusting for the increased risk of false positives due to multiple comparisons and statistical tests. Table 5 shows the values of the top cytokines and a detailed overview is given in the Supplemental File 2 (“Full Results”).

Cytokine	Estimate	r2	p value	FDR
COLEC12	-12.58	0.1762	< 2e-16	< 2e-16
PLAUR	-11.25	0.163	< 2e-16	4.2e-15
AGRN	-11.2	0.1549	< 2e-16	4.6e-14
WNT9A	-10.03	0.1492	6.5e-16	2.4e-13
LAIR1	-8.063	0.144	2.9e-15	1.0e-12
LILRB4	-8.768	0.1439	3.0e-15	1.1e-12
FABP1	-4.191	0.1416	5.8e-15	2.1e-12
CRIM1	-13.45	0.1403	8.4e-15	3.0e-12
CCL3	-6.865	0.1389	1.3e-14	4.5e-12
FSTL3	-8.187	0.1324	8.1e-14	2.9e-11

Table 5: *Top significant cytokines in derivation and validation cohorts.*

Top significant cytokines when combining derivation and validation cohorts. High levels of these cytokines are associated with a lower LVEF. Published in our preprint manuscript(280).

3.9 EMR Data Assessment

To test our hypothesis, we conducted a review of EMR from three rheumatology departments at Charité Universitätsmedizin Berlin. We specifically screened for patients who had received treatment with the cytokine inhibitors under investigation. Of the 18,566 patient records screened, hs-TnT and/or NT-proBNP levels were provided for only 8,767 patients and of these, 348 patients were treated with the two

cytokine inhibitors selected for the analysis (IL-6 inhibitors and Janus kinase inhibitors). Our analysis focused exclusively on this subset of patients, as for the remaining cytokines, either there were no cytokine inhibitors currently being applied in practice or only a few patients were treated with the specific cytokine inhibitor, which was not sufficient for a meaningful statistical analysis.

Among the group of 348 patients who underwent treatment with both cytokine inhibitors, the eligibility for analysis was limited to just 190 individuals as measurements of hs-TnT and/or NT-proBNP were absent for the remaining patients following inhibitor administration. There were 159 patients included in the final analysis after filtering out the statistical outliers.

3.9.1 NT-proBNP Reduction under Treatment with Cytokine Inhibitors

Both therapy with IL-6 antagonists (n=69) and therapy with Janus kinase inhibitors (n=90) significantly decreased ($p < 0.001$) the median NT-proBNP level by 136 ng/L and 170.5 ng/L, respectively (Figure 8).

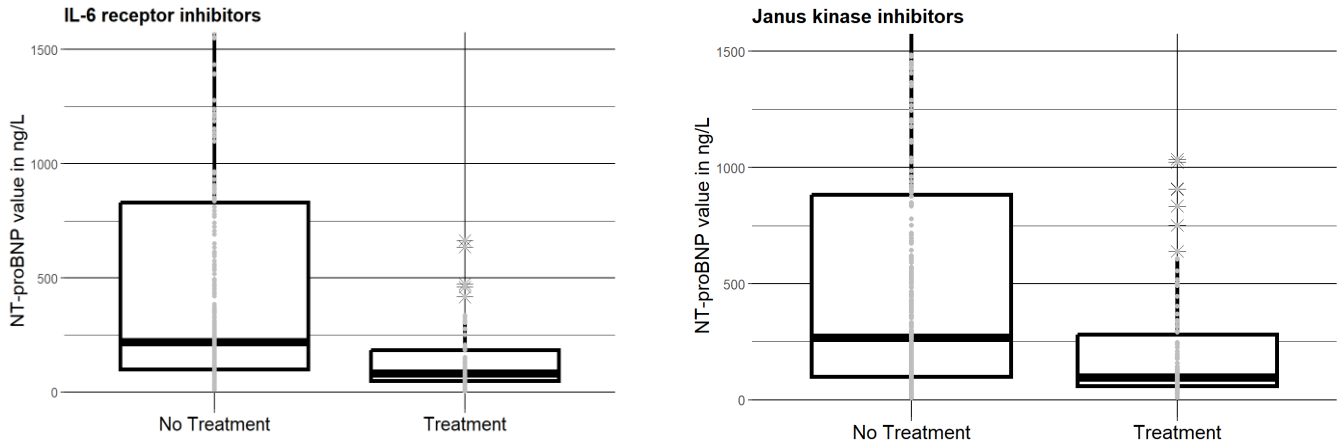


Figure 8: *NT-proBNP levels (ng/L).*

Lower NT-proBNP levels (ng/L) in patients taking IL-6 receptor inhibitors or Janus kinase inhibitors compared with matched controls. Published in our preprint manuscript(280).

3.9.2 hs-TnT Reduction under Treatment with Janus Kinase Inhibitors

In patients treated with Janus kinase inhibitors, a reduction of 9 ng/L in median hs-TnT levels was observed ($p = 0.001$).

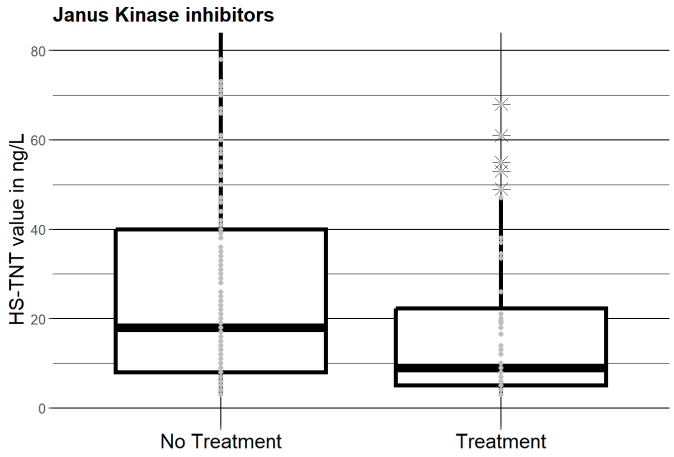


Figure 9: *hs-TnT levels (ng/L).*

Lower hs-TnT levels (ng/L) in patients taking Janus kinase inhibitors compared with matched controls. Published in our preprint manuscript(280).

4. Discussion

The mechanisms leading to myocarditis are still not entirely understood to date. Therefore, the treatment of myocarditis and other inflammatory cardiomyopathies remains challenging. In our study, we demonstrated that certain proteins are expressed more strongly in individuals with a severe course of inflammatory cardiomyopathy than in those with a mild course. These cytokines could be an effective therapeutic target in myocarditis and provide the stimulus for drug development.

For some of the cytokines identified in this study, an association with cardiac injury has been previously mentioned in the literature (see “Overview of some of the top proteins identified”). Inhibition of cytokines has been successfully used in various autoimmune diseases(283, 284) as well as in other diseases such as sepsis(285), COVID-19(286, 287), and in cytokine storm after CAR-T cell therapy(288). In many cases of ICI myocarditis(289, 290) and COVID-19 associated cardiomyopathy(291), the use of cytokine inhibitors has been successful. This literature highlights the potential and importance of further research in this area.

To address the research question of our project, we used three cohorts: a derivation cohort, a validation cohort, and a generalizability cohort. In these 3 different cohorts, there were no significant differences related to sex, age, BMI, renal function, and LVEF. Our initial step involved identifying cytokines that are elevated in patients with a worse

course of inflammatory cardiomyopathy (EF ≤ 35%), starting with our derivation cohort (n=63). We subsequently validated these findings in a separate validation cohort. Furthermore, our results extended to patients with IDCM, confirming the robustness and high power of our study, a validation that was reinforced by a post-hoc analysis. Our hypothesis was also validated after a review of patients' data from our EMR system, which revealed that when treated with some of the cytokines or with cytokines that block the signaling pathways of the cytokines we identified, a cardioprotective role was exhibited in patients. Additionally, existing studies in the literature indicate the positive cardioprotective role of several of these cytokines (please see "Overview of some of the top proteins identified").

The study's participant demographics align with the existing literature on inflammatory cardiomyopathy. Approximately one-third of the study cohort consisted of women, aligning with a sex distribution observed in prior research(12, 292-295). The median age of the participants was between 49 and 54, which is in the range reported by previous studies of inflammatory cardiomyopathies(295). Additionally, our study population exhibited a relatively high median BMI, which is consistent with findings in the existing literature concerning patients with inflammatory cardiomyopathy(294, 296). Thus, it could be suggested that proinflammatory effects of adipose tissue may have a role in the development of inflammatory cardiomyopathies(294).

Many patients in this study had angiotensin-receptor blockers in their medication history – in particular the group with a lower ejection fraction according to ESC guidelines(297). Angiotensin receptor blockers have anti-inflammatory properties(219, 298). Thus, the effect of the observed cytokines on LVEF may have been even more significant than found, as it might have been masked by this drug class. It has been previously reported in the literature that Angiotensin II receptor blockers can reduce hypertension-induced vascular inflammation in peripheral organs(299).

Our hypothesis that certain cytokines are associated with lower LVEF was confirmed by our investigations. Our findings may enable personalized medicine in patients with inflammatory cardiomyopathy while improving treatment in this patient population. The therapy of myocarditis is often challenging. A considerable amount of patients do not respond sufficiently to current state-of-the art therapy. In particular, those patients unresponsive to current treatment may benefit from the administration of cytokine blockers or blockade of their receptors and signaling pathways. Elimination of these

cytokines by immunoadsorption or -filtration may also be an option. Additionally, if drugs could be developed that would be better tolerated by patients and have fewer side effects than glucocorticoids, then these might also be used in patients who experience severe side effects under glucocorticoid therapy.

4.1 Overview of Some of the Most Relevant Proteins Identified

Vascular Endothelial Growth Factor (VEGF)

VEGF is a glycoprotein that stimulates angiogenesis, the formation of new blood vessels, and is involved in various pathophysiological processes, including the development of cardiovascular diseases(300-303). Most of the cells found in the heart are cardiomyocytes and these present the target receptors for VEGF(303). While VEGF-1 promotes morphogenesis, contractility and wound healing by activating cardiomyocytes, it is simultaneously secreted by cardiomyocytes in response to inflammation, mechanical stress or cytokine stimulation and, at high levels, is associated with poorer prognosis and more severe outcomes in patients with cardiovascular diseases(303).

VEGF has been previously associated with various cardiovascular diseases, especially myocardial infarction and atherosclerosis, which are the most recognized cardiovascular diseases worldwide(303, 304). Ischemic heart disease is especially manifested by the occurrence of myocardial infarction(305). Experiments in rat models suggest a positive role of VEGF-A serum levels in myocardial remodeling and angiogenesis(306-308). Similarly, in myocardial infarction, high VEGF levels have been found to be associated with higher levels of other inflammatory markers, including IL-6, suggesting that high blood VEGF levels are potentially a sign of ongoing inflammatory activity(309). In our study, we also showed that high VEGF levels are related to high IL-6 levels, with VEGF being a negative prognostic marker in patients with a severe form of inflammatory cardiomyopathy.

Therapeutic angiogenesis is currently being considered critically and cautiously as a new therapeutic strategy for myocardial infarction(310, 311). Whether angiogenesis is advantageous or detrimental in inflammatory conditions is still unclear(312). It is important to conduct new studies that examine the effect of therapeutic angiogenesis in patients with inflammatory cardiomyopathies. A study published in 2013

demonstrated that prolyl hydroxylase domain protein (PHD) suppression might lead to cardiac improvement in murine models of autoimmune myocarditis(313). This improvement is achieved by stimulating neovascularization, which subsequently decreases myocardial hypoxia and enhances heart function in myocarditis(313).

In our study, we demonstrated that high concentrations of VEGF are related to severe inflammatory cardiomyopathy. It remains to be investigated whether highly specific VEGF antagonists may be beneficial in inflammatory cardiomyopathy resistant to all other therapies or whether therapeutic angiogenesis is rather the key to the therapy of inflammatory cardiomyopathies. This calls for new studies specifically addressing this question.

IL-6

Our analyses have revealed that there is a link between IL-6 and the severity of inflammatory cardiomyopathy. IL-6 is an important protein of innate immunity involved in numerous physiological processes, including host defense, immune cell regulation, proliferation and differentiation(314). IL-6 can be both protective and damaging(315). If the acute response of IL-6 is only short-term, it acts protectively and limits damage to host cells(315). If the acute response is prolonged and becomes chronic, IL-6 is pathogenic to the host(314, 315).

There are several studies looking at the role of IL-6 in heart disease. Increased levels of IL-6 have been associated with an adverse prognosis in congestive heart failure as a result of IDCM(316). In addition, it has been reported that healthy men with elevated serum IL-6 levels are more likely to experience a cardiac infarction(317). Furthermore, the role of IL-6 in cardiac inflammation has been previously studied in mice(318). In this study, IL-6 +/+ mice developed severe myocarditis, whereas the prevalence of myocarditis and the severity of the disease were decreased in IL-6 -/- mice(318). These results are in agreement with the findings of our work.

High concentrations of IL-6 have also been observed in patients with COVID-19(315, 319). Severe clinical course has been reported in the context of cytokine storm(320). Cytokine storm may also lead to fulminant myocarditis. In COVID-19 infection, fulminant myocarditis has been described(321). IL-6 and proinflammatory cytokines, in general, regulate the inflammatory process and immune response, playing an

important role in viral myocarditis(322). The work of Toru Tanak et al. demonstrates that IL-6 is involved in the progression of viral myocarditis by inhibiting pathogen elimination and enhancing viral-induced damage, among other factors(322).

Collectin-12 (COLEC12)

One of the most important functions of this protein is its role as a scavenger receptor(323, 324). Scavenger receptors are responsible for the recognition and removal of cellular debris, pathogens, and other foreign bodies(325, 326). In addition, this protein is involved in the uptake and depletion of low-density lipoprotein (LDL) (324, 327). In summary, the COLEC12 gene encodes a member of the collectin family with multiple functions related to host defense and the clearance of oxidized LDL. Its role in these processes makes it an important component of the immune system and cardiovascular health(326).

There is limited information in the literature on the role of COLEC12 in patients with inflammatory cardiomyopathies. COLEC12 has been reported to be present in macrophages(328), and because the latter play an important role in the immune system(329) and are involved in the development of inflammatory cardiomyopathy(330), their precise role should be further investigated(328).

Follistatin (FSTL) 3

FST, the allied proteins FST-like-1 and FSTL3 are known to regulate the activity of TGF-β family members, such as activins(331, 332). While follistatins are involved in various pathways in the human body, encompassing those related to cell regeneration, growth, and inflammatory immune responses, their role in the heart is still not entirely understood and remains an active area of research(331).

Recent studies have shed light on the possible role of follistatins in the heart. An association between follistatins and cardiac development, cardiac fibrosis, cardiac hypertrophy, and cardiac regeneration has been previously reported(333). Lara-Pezzi, Enrique, et al. demonstrated increased expression of FSTL1 and FSTL3 in heart failure using real-time PCR(334). FSTL1 and FSTL3 levels normalized after recovery. They further demonstrated a relationship between FSTL3 concentrations and parameters

important for predicting disease outcome(334). In their work, Heidecker et al. indicated that a low concentration of Growth differentiation factor 11 (GDF11) or a high concentration of its inhibitor FSTL3 is associated with unfavorable cardiac outcomes in subjects with stable coronary disease(335). The results of another study suggested that FSTL3 can modulate hypertrophy and myocyte growth by modifying the Smad signaling pathway when stimulated by stress conditions(336). However, it is important to note that the role of follistatins in the heart is complex and multifaceted, and further research is needed to completely understand their mechanisms of action and their roles in cardiac physiology and pathology. Additional studies, including genetic and molecular approaches, as well as clinical investigations, are required to uncover the precise roles of follistatins in the heart and their potential implications for cardiovascular health and disease.

Cysteine-rich motor neuron 1 (CRIM-1)

CRIM-1 is a protein presented in multiple cell types including vascular endothelial cells(337). It has been demonstrated to serve a significant function in angiogenesis, the phenomenon involving the creation of new blood vessels from pre-existing ones(337, 338). The release of VEGF from podocytes to endothelial cells in the glomerulus of the kidney is mediated and controlled by CRIM-1(339, 340). In patients with chronic heart failure, high CRIM-1 levels were found in particular in those with advanced cardiac disease(341). Additionally, in our cohort of patients with inflammatory cardiomyopathy and lower ejection fraction, we demonstrated significantly higher CRIM-1 levels. However, further studies on the exact role of CRIM-1 in the development of heart disease are needed.

Plasminogen Activator, Urokinase Receptor (PLAUR)

It has been previously reported that soluble urokinase plasminogen activator receptor could serve as a biomarker for systemic chronic inflammation(342). PLAUR, a membrane-bound three-domain receptor, is primarily present on immune cells(343). The soluble form of PLAUR can be measured in plasma, urine, and saliva as well as in other body fluids when PLAUR is released from these cells in the context of inflammation(344-346). High soluble PLAUR levels have been reported to be related

to inflammatory, infectious, or malignant processes, as well as renal dysfunction(347-351). In the Danish MONICA10 (Monitoring Trends and Determinants of Cardiovascular Disease) study, an association was found between high levels of soluble PLAUR and the onset of cardiovascular disease(350, 352). In 2014, Borné et al. reported a potential association between soluble PLAUR and elevated NT-proBNP concentrations and, thus, an association with more frequent heart failure(353).

AGRN

Agrin is a large proteoglycan protein that plays a critical role in the formation and maintenance of the neuromuscular junction(354). It is produced and secreted by motor neurons and is responsible for initiating the clustering of acetylcholine receptors on the postsynaptic membrane of the muscle fiber(354, 355). Agrin binds to receptors located on the muscle cell surface(356, 357). Bassat et al. found in their study that agrin has a significant role in heart regeneration in mammals and that the extracellular matrix can contribute to cardiac repair(358). However, there is limited information on the exact role of agrin in the development of cardiac diseases such as inflammatory cardiomyopathy(358).

In a study in 2014, it was demonstrated that RAW264.7 macrophages express agrin and that there was a 15-fold increase of Il-10 induced by agrin, which then contributes to the suppression of inflammation(359). In addition, the concentration of signal transducer and activator of transcription 3 (STAT3) was increased fourfold(359). STAT3 is able to control the anti-inflammatory activity under the effect of agrin(359). Studies have reported that agrin plays a role in T cell receptor signaling and activation, among other functions(360-362). However, knowledge about the exact role of agrin in T-cell function and the immune system is incomplete.

WNT9A

Wnt genes are responsible for coding highly conserved, lipid-modified glycoproteins with a regulatory role in various developmental processes(363). However, their exact function is still unclear. Furthermore, WNTa causes early proliferation of hematopoietic

stem and progenitor cells(364, 365). Understanding of the exact role of WNT9A in the development of inflammatory cardiomyopathies still requires further research.

Wnt proteins have been demonstrated to fulfill various functions in the process of cardiac differentiation and development(366). Wnt proteins are growth factors that operate both during embryonic development and in mature organisms(367). They exert their influence by overseeing a wide range of cellular activities, including gene transcription and the control of processes such as cell proliferation, migration, polarity, and division(367). Not surprisingly, Wnt signaling is also implicated in the process of cardiac formation(366).

4.2 Strengths and Limitations

One limitation of our study is that the cytokine levels of patients may have been affected by the intake of certain drugs and by certain comorbidities. To reduce that risk, we excluded patients who received immunosuppressive treatment such as corticosteroids. In addition, the number of subjects in the generalizability cohort was small and served primarily an exploratory approach. Larger studies are needed to evaluate the generalizability of our results to other cardiomyopathies. Since we also included samples from patients who presented to our clinic many years ago, some clinical variables were missing and could not be retrieved at the time of the study.

Despite these limitations, our work revealed clinically relevant data, as we condected the largest clinical study to perform comprehensive cytokine analysis in inflammatory cardiomyopathy. Since many of the identified cytokines in patients with severe inflammatory cardiomyopathy are biologically plausible, our study offers many potential novel therapeutic targets that we will be investigating further.

5. Conclusions

The release of cytokines plays an important role in the development of inflammatory cardiomyopathies. The course of inflammatory cardiomyopathies may be attenuated by targeting specific pathways that play a significant role in inflammatory cardiomyopathies. Our results show that the cytokine profile of patients with a severe clinical course is different from that of patients with a mild course. We demonstrated

that certain proteins are significantly increased in patients with severe inflammatory cardiomyopathy as compared to those with mild to moderate forms. For these cytokines, targeted drugs may be developed in the future to block the downstream pathways and thus the progression of the disease.

Summary:

- Cytokine analysis of the derivation cohort revealed 77 cytokines related to a lower LVEF in patients with inflammatory cardiomyopathy.
- Our results were reproduced in an external validation cohort, which is evidence of the robustness of the data. Furthermore, the results were generalizable to patients with IDCM.
- The results of this work are plausible as several of the identified proteins have been previously described in the literature as potential therapeutic targets in inflammatory cardiomyopathy.
- Cytokines identified in this study may be novel therapeutic targets in the treatment of inflammatory cardiomyopathies.
- Our findings could provide the foundationes for the development of personalized medicine in patients with inflammatory cardiomyopathies and improve treatment in this patient population.

Future Directions:

- Further studies should be conducted to investigate the effect of inhibitors of these cytokines on the clinical course of inflammatory cardiomyopathy.
- To further evaluate the robustness of our findings and generalizability, it is essential to replicate the results in larger cohorts including other types of cardiomyopathy.
- To gain a deeper understanding of how the cytokines identified in this study may contribute to the reduction in LVEF, future investigations are necessary to evaluate potential causality and pathomechanisms.

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Eidesstattliche Versicherung

„Ich, Ainoosh Golpour, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „ Cytokine Profile in Inflammatory Cardiomyopathy. Are there Differences in Cytokine Expression between Mild and Severe Courses? (Zytokinprofil bei entzündlicher Kardiomyopathie. Gibt es Unterschiede in der Zytokinexpression zwischen milden und schweren Verläufen?)“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren/innen beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) werden von mir verantwortet.

Ich versichere ferner, dass ich die in Zusammenarbeit mit anderen Personen generierten Daten, Datenauswertungen und Schlussfolgerungen korrekt gekennzeichnet und meinen eigenen Beitrag sowie die Beiträge anderer Personen korrekt kenntlich gemacht habe (siehe Anteilserklärung). Texte oder Textteile, die gemeinsam mit anderen erstellt oder verwendet wurden, habe ich korrekt kenntlich gemacht.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit der Erstbetreuerin, angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass ich mich zur Einhaltung der Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis verpflichte.

Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§§156, 161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

Datum: _____ Unterschrift: _____

Lebenslauf

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht

Danksagung

An dieser Stelle möchte ich meinen Kolleginnen und Kollegen und allen anderen beteiligten Personen, die mich bei der Anfertigung dieser Dissertation unterstützt haben, einen großen Dank aussprechen.

Besonders danken möchte ich dabei meiner Mentorin PD Dr. med. Dr.med. univ. Bettina Heidecker für die ausgezeichnete Betreuung und die wertvolle Unterstützung bei der Umsetzung der gesamten Arbeit.

Nicht zuletzt gilt mein Dank meiner Familie, meinen Freundinnen und Freunden und meinem Partner für ihre Geduld sowie für ihre Ermutigungen während des Schreibens dieser Dissertation.

Komplette Publikationsliste

Originalarbeiten

Erstautor

1. Golpour, A., Patriki, D., Hanson, P. J., McManus, B., & Heidecker, B. (2021). Epidemiological impact of myocarditis. Journal of Clinical Medicine, 10(4), 603, impact factor: 4.96
2. Golpour, A., Bereswill, S., & Heimesaat, M. M. (2019). Antimicrobial and immune-modulatory effects of vitamin D provide promising antibiotics-independent approaches to tackle bacterial infections–lessons learnt from a literature survey. European Journal of Microbiology and Immunology, 9(3), 80-87, impact factor: 4.5
3. Golpour, A., Suwalski, P., Landmesser, U., & Heidecker, B. (2023). Case report: Magnetocardiography as a potential method of therapy monitoring in amyloidosis. Frontiers in Cardiovascular Medicine, 10.
4. Suwalski, P.*, Golpour, A.*, Weiner III, J., Musigk, N., Balzer, F., Giesa, N., ... & Heidecker, B. (2023). Cytokines as potential novel therapeutic targets in severe inflammatory cardiomyopathy. medRxiv, 2023-07. (*geteilte Erstautorschaft)

Koautor

1. Brala, D., Thevathasan, T., Grahl, S., Barrow, S., Violano, M., Bergs, H., Golpour, A., Suwalski, P., Poller, W., Skurk, C., Landmesser, U., Heidecker, B. Application of Magnetocardiography to Screen for Inflammatory Cardiomyopathy and Monitor Treatment Response. Journal of the American Heart Association, impact factor: 6.1.
2. Suwalski, P., Golpour, A., Musigk, N., Wilke, F., Landmesser, U., & Heidecker, B. (2023). Case report: Recurrence of inflammatory cardiomyopathy detected by magnetocardiography. Frontiers in Cardiovascular Medicine, 10.
3. Musigk N., Suwalski P., Golpour A. et al. The inflammatory spectrum of cardiomyopathies (under review).

Bescheinigung – Statistik

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20. Oktober 2023

Bestätigung für Ainoosh Golpour, geboren am .

Hiermit bestätige ich, dass die verwendeten statistischen Verfahren in der mir vorgelegten Dissertation (in der Fassung vom 19.10.2023) mit dem Titel:

Cytokine Profile of Patients with Inflammatory Cardiomyopathy. Are there Differences in Cytokine Composition and Levels between Patients with Mild and Severe Myocarditis?

korrekt angewendet wurden.

Dr. Ulrich Gauger