Aus der Medizinischen Klinik mit Schwerpunkt Kardiologie Campus Virchow Klinikum

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DISSERTATION

Differentiated infection pathology and gene profile analysis of myocardial tissue for extended diagnostics of patients with unexplained heart failure

Differenzierte infektionspathologische und Genprofilanalysen im Myokardgewebe zur Erweiterung der Diagnostik von Patienten mit unklarer Herzinsuffizienz

zur Erlangung des akademischen Grades

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Table 1. List of abbreviations and acronyms

Abbreviation or acronym	Description
(LV)EF	(left ventricular) ejection fraction
ACE2	angiotensin converting enzyme 2
B19V	parvovirus B19
CCL	chemokine (C-C motif) ligand
CCR	chemokine receptor
CD	cluster of differentiation
CM	cardiomyopathies
COVID-19	coronavirus disease 2019
CPT1	carnitine palmitoyltransferase
CS	cardiac sarcoidosis
CVB-3	Coxsackievirus B3
DCM	dilated cardiomyopathy
DCMi	dilated cardiomyopathy with inflammation
E-gene	envelope small membrane protein-gene
EMB	endomyocardial biopsy
GCM	giant cell myocarditis
HHV6	human herpesvirus 6
IFNγ	interferon gamma
IGCM	idiopathic giant cell myocarditis
IL	interleukin
IVIg	intravenous immunoglobulin
NGS	next generation sequencing
NS1	B19V Non-structural protein 1
PBMCs	peripheral blood mononuclear cells
RdRP-gene	RNA-dependent RNA polymerase gene
ROC-AUC	receiver operating characteristic-area under curve
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2

T_h cell	T helper cell
TLR	toll-like receptor
VP1/2	B19V capsid protein 1/2

II. Abstract

Myocarditis and dilated cardiomyopathy represent acute or chronic disorders of the myocardium in most cases caused by myocardial virus infection with or without concomitant inflammation that are associated with poor prognosis without specific treatment. Only by applying endomyocardial biopsy (EMB) it is possible to establish a pathophysiological-proven diagnosis as a prerequisite for causal therapy. Since EMB is limited in sample size, a substantial sampling error due to focal disease processes impairs its sensitivity and predestine EMB for molecular diagnostics. The project focused on development and optimization of PCR-based diagnostic methods to detect (i) fulminant forms of myocarditis and to identify cardiotropic viral pathogens, such as (ii) parvovirus B19 (B19V) and (iii) severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) as potential causes of myocarditis.

(i) In order to improve diagnosis of idiopathic giant cell myocarditis (IGCM), a rare but fatal entity of myocarditis, a gene expression profiling test was developed. A panel of marker genes was revealed to distinguish IGCM from other fulminant forms of myocarditis. Screening of a large cohort demonstrated that 54.4% of IGCM patients would have been missed by solely conventional histopathological analysis.

(ii) To improve diagnostics of molecular virology, a PCR assay was established to distinguish an active infection of the myocardium with B19V from clinical irrelevant latent infection by detection of viral transcripts. By investigating a cohort of n = 576 patients that underwent EMB, B19V DNA was detected in 70% of samples, while a subgroup of 38.5% of these was characterized by viral transcriptional activity. Precise and sensitive diagnostics of active B19V will help to identify patients which will benefit from antiviral treatment and will contribute to the development of novel therapy approaches.

(iii) Using PCR, it was possible to identify SARS-CoV-2 as a potential cardiotropic pathogen in EMB samples. Presence of SARS-CoV-2 RNA could be confirmed in 5 of 104 patients. Infection was accompanied by myocardial inflammation and histopathological analysis demonstrated that rupture of vessel walls of capillaries led to local ischemia and secondary damage of cardiomyocytes. A mild to highly acute clinical course of the disease was observed and cardiac complications occurred directly after primary infection, after latency, or in the absence of pulmonary symptoms. Thus, screening for SARS-CoV-2 should be considered as a routine method of EMB based diagnostics.

Successful application during routine diagnostics highlights the clinical significance of findings and underscore the need to further develop and improve molecular diagnostics of EMB samples in order to initiate targeted and effective treatment strategies without delay.

III. Zusammenfassung

Die Myokarditis und dilatative Kardiomyopathie sind eine akute und chronische Erkrankung des Myokardiums, welche häufig durch eine myokardiale Virusinfektion mit oder ohne begleitende Entzündung verursacht werden und ohne spezifische Therapie mit einer schlechten Prognose assoziiert sind. Die Untersuchung mittels Endomyokardbiospie (EMB) liefert eine pathophysiologisch-begründete Diagnose als Grundlage einer kausalen Therapie. Da die entnommene Materialmenge der EMB limitiert ist und zudem Krankheitsprozesse fokal auftreten können, führt dies zu einem Verlust an Sensitivität und prädestiniert die EMB für eine molekulardiagnostische Analyse. Im Fokus des Projektes stand die Entwicklung und Optimierung PCR-basierter Methoden zum Nachweis (i) fulminanter Formen der Myokarditis und kardiotroper viraler Pathogene, wie (ii) Parvovirus B19 (B19V) und (iii) severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) als potentielle Auslöser einer Myokarditis.

(i) Um die Diagnose der Riesenzellmyokarditis (IGCM), einer seltenen aber mit schlechter Prognose verbundenen Form der Myokarditis, zu optimieren, wurde ein Genexpressions-Test entwickelt. Hierbei konnte ein Panel an Markergenen identifiziert werden, welche die Differentialdiagnostik der IGCM ermöglichten. Das Screening einer großen Kohorte zeigte, dass 54,4% aller IGCM-Patienten nicht identifiziert worden wären, insofern ausschließlich konventionelle histopathologische Diagnostik eingesetzt worden wäre.

(ii) Es wurde ein PCR Assay entwickelt, um anhand des Nachweises von viralen Transkripten eine aktive Infektion des Myokardiums mit B19V von einer klinisch irrelevanten latenten Infektion zu unterscheiden. In einer Kohorte von n = 576 Patienten konnten B19V DNA in 70% aller Patienten nachgewiesen werden, während 38,5% dieser Patienten durch eine aktive Infektion charakterisiert waren. Dies ermöglicht die Identifikation von Patienten, die von einer antiviralen Therapie profitieren werden und wird dazu beitragen, neue Behandlungsstrategien zu entwickeln.

(iii) Mittels PCR konnte SARS-CoV-2-RNA als potentielles kardiotropes Pathogen in EMB Proben von 5 von 104 Patienten nachgewiesen werden. Die Infektion war begleitet von einer intramyokardialen Entzündung. Die histopathologische Auswertung zeigte Rupturen der

Kapillarwände, welche zu lokaler Ischämie und sekundären Schäden an den Kardiomyozyten führte. Milde bis hoch-akute klinische Verläufe der Krankheit wurden beobachtet, wobei kardiale Symptome unmittelbar nach Primärinfektion, nach einer Latenzzeit oder ohne pulmonale Symptomatik auftraten. Daraus folgernd sollte der Nachweis von SARS-CoV-2 als Routinetest in der EMB Diagnostik durchgeführt werden.

Die erfolgreiche Anwendung in der Routinediagnostik konnte die klinische Signifikanz der Ergebnisse zeigen und bekräftigte die Notwendigkeit zur Entwicklung und Optimierung molekulardiagnostischer Methoden zur Analyse der EMB um so rasch eine gezielte und effektive Behandlung einzuleiten.

1 Introduction

1.1 Myocarditis: etiology and course of the disease

According to the American Heart Association scientific statement published in 2006, cardiomyopathies (CM) are defined as a heterogeneous group of diseases of the myocardium associated with mechanical and/or electrical dysfunction leading to failure of myocardial performance [74, 90]. CM must be distinguished from cardiac pathologies that are a direct consequence of cardiovascular diseases, such as systemic hypertension, valvular, coronary artery disease and congenital heart diseases resulting in secondary, ischemic damage of the heart [74, 90]. CM defines acute and chronic disorders of the heart caused by virus infection or other external or internal triggers, such as genetic factors, and in many cases, the underlying cause of disease remains unknown [74, 77]. By definition, primary CM that predominantly involve the heart, are segregated into genetic CM (e.g., hypertrophic CM), multifactorial CM (e.g., dilated CM), or acquired CM (e.g., inflammatory CM). Different forms of CM are classified by anatomical, functional, histopathological or molecular parameters. Therefore, the designations “cardiomyopathy” and “inflammatory cardiomyopathy” define both, a histological and functional diagnosis [18].

The heart muscle (myocardium), which is localized between the inner (endocardium) and outer layer (epicardium) of the heart wall, is composed of cardiomyocytes that form a functional syncytium and are connected by intercalated discs [20]. Myofibrils within cardiomyocytes are organized into sarcomeres and are responsible for contraction of the heart [20]. Cardiomyocytes are embedded in an extracellular matrix that mainly consists of collagen fibers [20]. Myocarditis is defined as an inflammatory disease of the heart muscle [25]. The Dallas criteria were the first

formal histological criteria to standardize diagnostic of myocarditis [98]. In brief, diagnosis of myocarditis was defined as histological evidence of inflammatory infiltrates within the myocardium that causes myocyte degeneration and necrosis and is of non-ischemic origin [98]. Acute myocarditis, which is defined by an histological evidence of myocytolysis, may heal or it may progress towards a chronic form presenting with or without inflammation [10]. Diagnosis of borderline myocarditis is characterized by a less pronounced inflammation without evidence of myocyte damage or necrosis according to Dallas criteria [7]. In contrast to myocarditis, dilated cardiomyopathy (DCM) or dilated cardiomyopathy with inflammation (DCMi) both represent chronic forms of the disease. DCM and DCMi are clinical diagnoses based on functional characterization of the heart. During DCM or DCMi, the left ventricle or both ventricles are affected by an impaired contraction capacity that is not caused by abnormal loading conditions or coronary artery disease [10]. Approximately 10 – 20 % of patients suffering from acute myocarditis progress towards a chronic stage and develop DCM or DCMi, as it was revealed by a long term follow up study [1].

The global prevalence of myocarditis is estimated to occur at approximately 22 of 100,000 patients and is estimated to account to 10 % of all cases of sudden death [36, 102]. The clinical presentation of myocarditis is widespread ranging from asymptomatic to life-threatening symptoms and [36]. Clinical symptoms last from a few weeks to several months and patients may present with fatigue, decreased exercise tolerance, palpitations, precordial chest pain, and syncope. Immunosuppressive therapy with prednisone and azathioprine is approved for virus-negative myocarditis and leads to clinical improvement [33].

The etiology of myocarditis often remains unclear and might represent multifactorial processes, but viral pathogens are suggested to be the most potential trigger of the disease in developed countries [18, 91]. In general, causative agents of myocarditis can be classified into infectious pathogens or non-infective external triggers. In contrast to rarely detected bacterial or fungal infections, cardiotropic viruses, such as coxsackievirus (CVB-3), echovirus, adenovirus, human herpesvirus 6 (HHV-6), and cytomegalovirus are the major causes of myocarditis [10, 24]. Besides the aforementioned viral species, hepatitis E, influenza virus, and human immunodeficiency virus are associated with myocarditis, however, evidence for cardiotropism of these viruses has not been established yet [18, 25, 81]. Among the most frequently detected bacterial pathogens that were identified as causative agents of myocarditis, are Streptococcus, Diphtheria, and Trypanosoma *cruzii* [24]. Non-infective causes of myocarditis comprise systemic diseases, drugs, and toxins [10]. Autoimmune diseases, such as systemic lupus

erythematosus, as well as immunomodulatory therapy were identified as possible triggers of myocarditis [13, 73, 79]. Frequently, the causative agent of myocarditis remains unknown [18]. Endomyocardial biopsy (EMB) which is obtained either from the left or right ventricle by le represents the best suited method to establish diagnosis of myocarditis. However, since EMB is performed infrequently due to its invasive procedure, the true incidence of myocarditis might be underestimated [25]. Only by analysis of EMB, it will be possible to determine and quantify the exact types of immune cells involved in inflammatory processes. Furthermore, EMB can also be used to quantify presence of viral nucleic acids including sequencing and subtype analysis and is able to differentiate between latent or active viral infection.

Idiopathic giant cell myocarditis (IGCM), as a rarely diagnosed entity, represents the most aggressive form of an active myocarditis and despite advances in science and surgery, prognosis for IGCM is poor [6, 10]. A fulminant form of IGCM requires the patient to be put on mechanical circulatory support and heart transplantation represents the only long-term survival option for patients affected by IGCM [6]. Multicenter studies report a 5-year transplant-free survival rate no better than 10% - 42% for IGCM [31]. Furthermore, progression of IGCM into chronic forms is considered to occur in more than 80% of all IGCM cases [31]. There is a high medical need to detect IGCM as early as possible in order to avoid ongoing myocardial tissue damage, resulting in a need of transplantation or cardiac death [6]. Histopathological, IGCM presents with myocyte necrosis that is associated with multifocal or diffuse inflammatory cell infiltrates composed of T-lymphocytes, multinucleated giant cells, plasma cells, eosinophils, and occasional neutrophils [10]. $CD68^+$ macrophages represent the cell type which is predominantly involved in giant cell formation [78]. Giant cells are often found in the surrounding border area of a site of active inflammation [78]. Due to its fast progression, particularly cardiotropic viral pathogens are supposed to trigger IGCM, however, the natural course of the disease remains to be elucidated [18, 82]. Poor prognosis and a significant sampling error of EMB to detect IGCM underscore the high medical need to improve diagnosis of this entity of myocarditis [95].

1.2 Viral myocarditis

Viral myocarditis is defined by histological criteria of myocarditis and presence of viral genomes in EMB samples [18]. A model of three phases is proposed to explain development of viral myocarditis and a possible progression of the disease into its chronic forms (
Figure ***1***) [82].

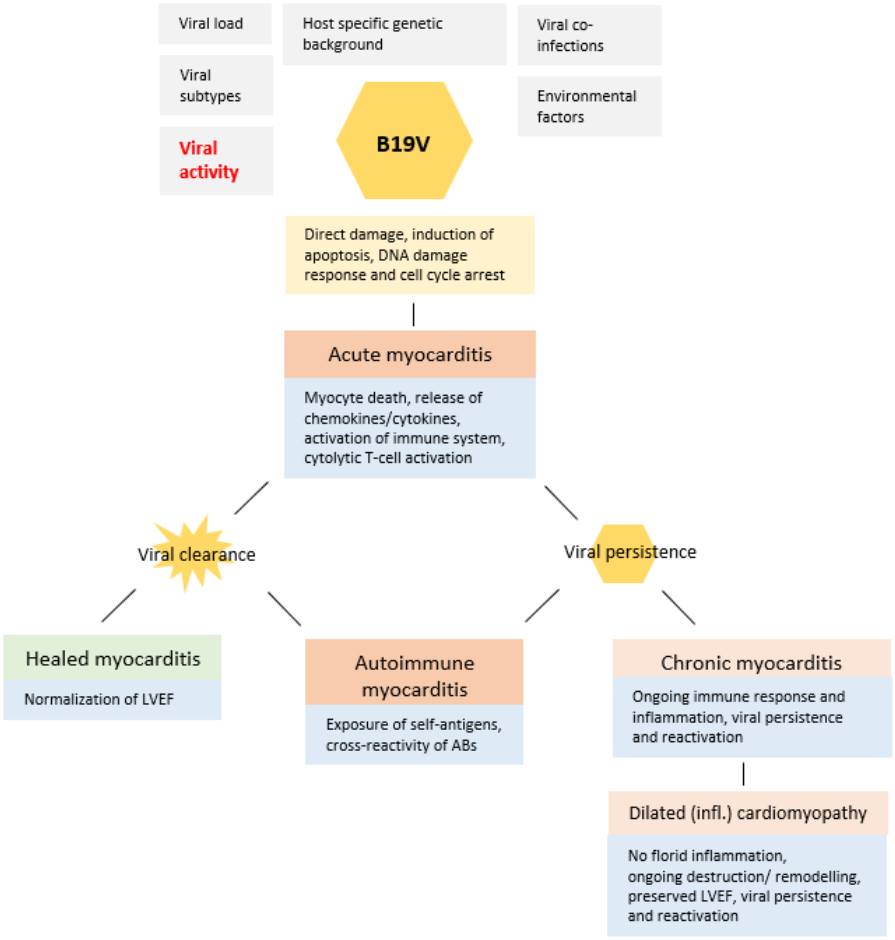

Figure 1. Schematic presentation of a proposed model of B19V-induced viral myocarditis and progression towards its chronic forms (Scheme adopted from Pollack et al., 2015) [82].

The initial phase is characterized by infection of cells of the myocardium and cardiac dysfunction is a consequence of virus-induced cellular damage and activation of the innate immune response. Cardiac damage and presence and replication of viral pathogens elicits adaptive immune response. Cytokine and chemokine release leads to inflammatory processes within the heart during the second, acute phase of myocarditis. Necrosis and myocytolysis, representing hallmarks of active myocarditis, are a consequence of T- cell mediated clearance of infected cells. The third phase is characterized by recovery from myocarditis or progression into chronic forms. Elimination of the pathogen may result in resolution of inflammation and may lead to healing processes and clinical improvement [18]. However, autoimmune reactions may be triggered due to release and exposure of self-antigens from damaged cardiomyocytes. As an example, autoantibodies against cardiac molecules were detected in 30 % of all patients following infection with CVB-3 [17]. An unbalanced immune response and persistence of pro-inflammatory cytokines after pathogen elimination, as well as incomplete clearance of pathogens are supposed to result in a chronic form of myocarditis [50]. Chronic inflammatory

myocarditis was defined as an onset of inflammation over three months after diagnosis of acute myocarditis [18]. The chronic stage is characterized by tissue remodeling, fibrosis, and loss of contractile function in accordance with reduced left ventricular ejection fraction (LVEF) [18, 82]. Interestingly, patients suffering from a fulminant form of myocarditis had a better prognosis when compared to patients suffering from an acute non-fulminant form of myocarditis which could be explained by a pronounced immune response leading to a complete viral clearance [75]. This may have prevented progression into DCM or DCMi and resulted in an increased transplant free survival time thus strongly supporting hypothesis of Caforio *et* al. [75]. However, the exact mechanism of progression from acute form of myocarditis towards a chronic form remain to be elucidated and are supposed to be multifactorial processes [75].

In accordance with advances in molecular biology, coxsackie-, echo-, and adenoviruses were the most commonly detected cardiotropic species in heart muscle tissue [25, 82]. Enteroviruses and adenoviruses are able to infect and replicate within cardiomyocytes by binding to the coxsackievirus and adenovirus receptor [82]. Cell lysis results in direct myocardial injury or cardiac damage via an unbalanced immune response. The majority of patients recovers after infection, however, viral persistence of entero- and adenoviruses is associated with a poor prognosis [61, 63]. In the recent past, Parvovirus B19 has become as the most frequently detected viral pathogen in EMB besides Epstein-Barr Virus and human herpesvirus 6 (HHV-6), what might represent an epidemiologic shift [14, 62]. The current project aimed to improve diagnostics of cardiac B19V infection thus molecular mechanism of pathogenicity and clinical significance were discussed in detail during publication 2 [80].

Immunosuppressive therapy is indicated for virus-negative chronic and active forms of myocarditis and giant cell myocarditis [33, 39, 40]. Treatment options of viral myocarditis are less well established. In particular, for therapy of cardiac B19V infection, large randomized trials of potential drugs are pending. During two phase II-studies, adeno- or enterovirus-induced myocarditis or viral persistence were successfully treated by interferon-beta-1B therapy [63, 93]. Similar beneficial effects of interferon-beta-1B were reported for B19V-induced myocarditis [93]. These results were confirmed when two cases of B19V-induced myocarditis in pediatric patients were treated successfully with interferon-beta-1B [21]. Controversial outcomes of intravenous immunoglobulin therapy were reported for treatment of B19V infection during two studies [30, 44]. Antiviral treatment with ganciclovir of chromosomally integrated HHV-6 has proven effective against viral reactivation associated with cardiac symptoms [59].

1.3 Gene profiling as a novel approach in molecular diagnostics

Gene profiling has become a breakthrough molecular method not only in cancer diagnostics. Only recently, commercially user-operated or end-to-end solutions that require sample transfer have become available to investigate cardiovascular diseases. Most products rely on non-invasive sample methods. When gene profile tests are available as kits, the test can be conducted directly by the user. Most gene profiling products available require extensive sample processing, for example the extraction of peripheral blood mononuclear cells (PBMCs) from blood samples or specific enrichment processes of analytes. In such cases, sample material is sent to the manufacturer and will be analyzed in-house. This method is preferred when sample preparation is an elaborate process or deviations in sample processing might lead to invalid results.

The majority of commercial cardiovascular-specific test platforms focus on risk assessment of inherited cardiovascular diseases, e.g., CardioGxOne™ (Admera health) and GeneSeq® (Integrated Genetics). Besides end-to-end solutions of gene profiling tests, a predesigned sequencing panel (TruSight™ CardioSequencing Kit) is available for Illumina® Next Generation Sequencing platforms that will detect mutations in 174 genes associated with inherited cardiovascular diseases [83].

In contrast, AlloMap® (CareDx®) is a commercially available gene profiling test for detection of allograft rejection after heart transplantation. This gene profile relies on non-invasive sampling and was developed in order to avoid EMB as an invasive method of rejection surveillance [28]. Briefly, AlloMap® requires the extraction of RNA from isolated PBMCs and analyzes the expression rate of marker genes in relation to one or several housekeeping, so called non-informative genes [28]. AlloMap® was initially developed by screening of EMBs for histopathological evidence of graft rejection and a parallel correlation of marker gene expression signatures within isolated PBMCs. To that end, EMBs were analyzed independently by six pathologists to assess level of graft rejection. Isolated PBMCs were then screened for expression of marker genes applying a microarray analysis of a panel of 7370 genes. From a training cohort of 107 patients, an 11-gene panel was identified which was able to indicate allograft rejection. Marker genes, which are used to identify rejection, code for T-cell/natural killer and $CD8^+$ T-cell activation markers and erythropoiesis markers (ALAS2, WDR40A, MIR). The identified marker genes were verified by investigating a validation cohort. As a result, AlloMap® is able to predict the probability of rejection solely from the blood cell gene expression signature [28]. First clinical studies (CARGO and CARGO II) evaluated the

performance of this test [27]. Since results of AlloMap® were optimized for detection of true-negative results (>95%), its predictive value (sensitivity) of detecting rejection events is low (9.1 -10.2% true positives) reflecting in low ROC-AUC values of 0.71 (2-6 months) and 0.67 (>6 months) post transplantation. However, the decision for these cut-off values was chosen consciously to avoid any false-positive results that might result in unnecessary catheterization and biopsy investigation with consequences for the patient [27].

Similar to AlloMap®, a gene profiling approach assessing rejection after heart transplantation has been discovered by NGS-based screening of PBMCs [23]. Although this profile was established in only a small cohort of 12 patients, it performed well in a validation cohort of 47 patients and was, useful to test for rejection after transplantation of other organs than heart [23]. To date, no commercial product exists that employs EMB sample material to diagnose cardiovascular diseases.

Only a limited number of research approaches investigated disease-specific marker gene expression in EMB samples. These will be discussed in regard to findings during publication 1. As an example, Heidecker *et* al. established a gene expression profile by transcriptome analysis of EMB samples which was able to differentiate lymphocytic myocarditis from DCM [45]. During this study, immune markers, such as genes from the Toll like receptor family, revealed a strong predictive power to detect myocarditis. Further research approaches to differentiate DCM from DCMi were investigated by others [5, 85, 107]. A study from Kittleson *et* al. identified disease-specific marker genes of IGCM when compared to transplantation donor hearts using microarray analysis [54]. However, each of these gene profiles relies on a limited patient cohort and clinical validation of these scientific approaches is pending. Besides gene expression profiling approaches, also circulating miRNAs have been shown to act as disease-specific molecular markers for DCM or DCMi to identify patients for whom EMB is indicated to clarify diagnosis [4].

1.4 Project aims

The present dissertation was performed within the GENPROVIC (Gene Profiling Test for Identification of Treatable Patients with Acute and Chronic Heart Failure) project. As a part of the European Research Area Network on Cardiovascular Diseases (ERA-CVD), GENPROVIC is funded by the European Union Framework Program for Research and Innovation 'Horizon 2020' and was carried out as a transnational project involving three European academic and clinical centers (IRCCS Lazzaro Spallanzan (National Institute for Infectious Diseases), Rome; Hospital Universitario Puerta de Hierro Majadahonda, Madrid; Charité University Hospital;

Berlin) and one diagnostic laboratory (Institute for Cardiac Diagnostics and Therapy, Berlin). GENPROVIC aimed to develop molecular diagnostic methods for investigation of EMB samples in order to improve diagnosis of fulminant forms or virus induced forms of myocarditis and will establish a rapid transfer of findings into clinical diagnostics.

Sensitive and specific diagnostic methods are a prerequisite to apply appropriate therapy strategies and to develop new treatment options. Non-invasive diagnostic methods, such as imaging techniques or testing of systemic parameters, have advanced in recent years, but in contrast to invasive EMB, still lack sensitivity and specificity to investigate diseases of the heart muscle. Since EMB must rely on small sample size, a sampling error due to focal disease processes was observed when conventional (immuno)histopathological analysis is applied. To remedy these shortcomings, development and optimization of precise molecular diagnostic methods as a prerequisite for causal therapy are of significant clinical importance.

An overview on the methodical workflow of GENPROVIC was depicted schematically in Figure **Figure *1***2 and a detailed description of each method is given in the respective publications comprising the cumulative dissertation (Figure 2). To summarize, EMB samples were analyzed by molecular and conventional (immune)histopathological methods using RNA and DNA extraction and staining of thin-cut tissue sections of EMBs. Results of both arms were then used to develop and improve molecular methods.

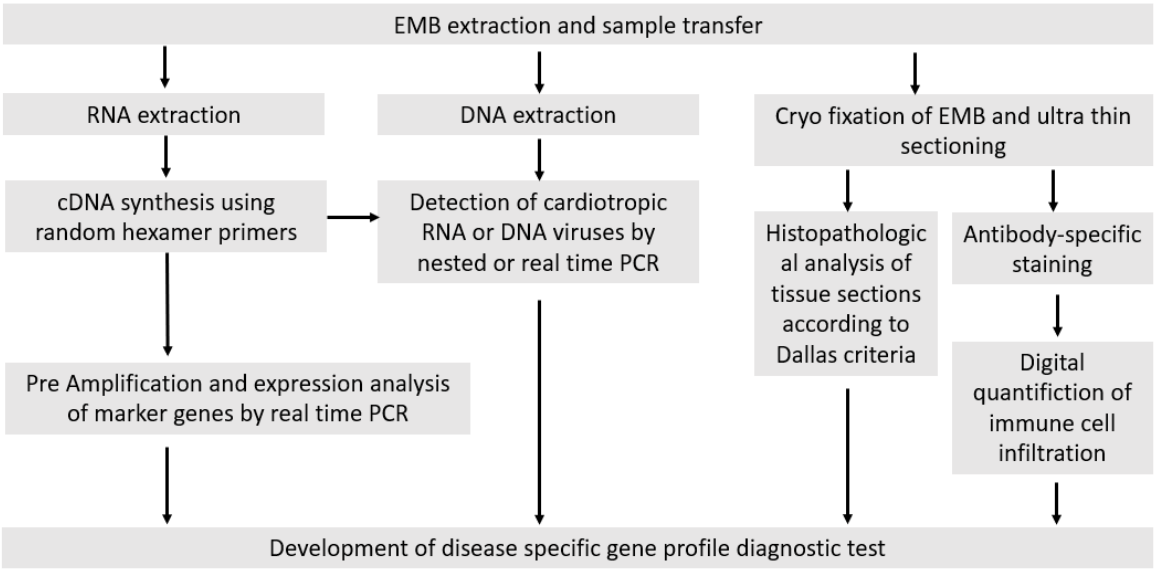

Figure 2. GENPROVIC workflow for gene profile development and molecular analysis of EMB samples
Schematic presentation of molecular and histopathological EMB analysis workflow for gene profile development. Sample preparation for gene expression analysis follows the same procedures as molecular virology analysis for RNA *viruses.* (self-developed scheme)

2 Results

2.1 Publication 1

Escher F, Pietsch H, Aleshcheva G, Wenzel P, Fruhwald F, Stumpf C, Westermann D, Bauersachs J, Enseleit F, Ruschitzka F, Nägele H, Laugwitz KL, Haake H, Frey N, Brachmann J, Huber K, Braun-Dullaeus RC, Bergmann MW, Strotmann J, Grönefeld G, Krülls-Münch J, Westenfeld R, Skurk C, Landmesser U, Pieske B, Gross UM, Morawietz L, Schultheiss HP.

Evaluation of Myocardial Gene Expression Profiling for Superior Diagnosis of Idiopathic Giant-Cell Myocarditis and Clinical Feasibility in a Large Cohort of Patients with Acute Cardiac Decompensation.

J Clin Med. 2020 Aug 19;9(9):2689. Doi: 10.3390/jcm9092689. PMID: 32825201; PMCID: PMC7563288.

Summary: Diagnosis of IGCM relies on histopathological detection of multinucleated giant cells in EMB samples and thus is subjected to sampling error due to focal processes of the disease and small sample size of EMB [95, 109]. Since prognosis of IGCM is poor, there is a high clinical need to improve diagnosis in order to subsequently initiate a specific therapy [31]. The present study aimed to establish a novel molecular diagnostic method to detect IGCM in EMB samples and evaluated its clinical significance in a large cohort of cardiac patients that underwent EMB.

The total patient cohort comprised samples from n = 427 patients including a group of 26 histopathological confirmed IGCM cases. In this group, a disease-specific gene expression pattern was revealed that allowed to discriminate IGCM patients from the control group with high sensitivity (96.2 %) and specificity (100 %). Gene expression level of 27 genes was shown to be differentially regulated among IGCM patients and control group and further analysis could establish a gene profile consisting of five marker genes (carnitine palmitoyltransferase I (CPT1), chemokine (C-C motif) ligand 20 (CCL20), C-C chemokine receptor type 5 (CCR5), chemokine receptor 6 (CCR6), Toll-like receptor 8 (TLR8)). The comparison between gene profile and histological and immunohistological results revealed a considerable sampling error through conventional analysis of EMB samples when the total cohort was tested by gene profiling. As the molecular results of the gene profile demonstrated, 31 of 57 (54.4%) patients positive for IGCM but without histopathological proof of giant cells would have received false-

negative diagnosis if only conventional analysis would have been applied. Successful therapy of IGCM, as the most aggressive form of myocarditis, however is only possible if immunosuppressive treatment is initiated early in the course of the disease to prevent ongoing myocardial tissue damage and progression into a chronic form. A significant higher number of immune infiltrates was observed, when histopathologically-diagnosed samples were compared to gene profile-diagnosed samples of IGCM. Thus, a mere quantification of immune cell infiltration by immunohistochemical analysis in EMBs did not prove to be an appropriate marker to identify IGCM. Immunosuppressive treatment of a subgroup of gene profile diagnosed and conventionally-diagnosed IGCM patients resulted in a significant clinical hemodynamic improvement after 6 months that was comparable between both groups treated.

Significance: Successful clinical application of gene profiling as a novel molecular diagnostic method to distinguish fulminant forms was demonstrated in a large cohort of patients with unexplained heart failure that underwent EMB. These data confirm the feasibility to apply gene profiling in routine diagnostics of EMB samples thus minimizing the sampling error and operator bias of pure conventional microscopic analysis of tissue sections. Sampling error of conventional EMB analysis could be shown for diagnostics of giant cells by histological criteria, as well as for quantification of inflammatory infiltrates. Furthermore, the study results demonstrated that prevalence of IGCM is underestimated. Molecular results from the present study may serve as a basis for the development and evaluation of non-invasive diagnostic tests.

2.2 Publication 2

Pietsch H, Escher F, Aleshcheva G, Lassner D, Bock CT, Schultheiss HP.

Detection of parvovirus mRNAs as markers for viral activity in endomyocardial biopsy-based diagnosis of patients with unexplained heart failure.

Sci Rep. 2020 Dec 18;10(1):22354. Doi: 10.1038/s41598-020-78597-4. PMID: 33339949; PMCID: PMC7749156.

Summary: Human parvovirus B19 (B19V) is the most frequently detected viral species in human heart tissue. Several studies indicate that, in contrast to latent infection of the myocardium with B19V, transcriptional activity as determined by presence of viral RNA, is associated with endothelial dysfunction and progression of viral myocarditis into a chronic form

of the disease [11, 57, 67, 88, 89]. However, diagnostics of B19V viral RNA in EMB is hampered by the detection limit of PCR reflecting the low expression rate of viral transcripts and the small sample size of EMBs. Thus, sensitive diagnostic methods are needed to improve diagnostics of active B19V infection of the heart muscle. The present study aimed to establish a diagnostic real time PCR method to characterize expression of viral capsid transcripts (VP1/2) and expression of non-structural protein 1 (NS1) transcripts in EMBs of patients with unexplained heart failure. In addition to already established protocol for VP1/2 detection, a RT-qPCR was designed to target B19V NS1 sequences and to further allow discrimination of B19V genotype 1 and 2 in a single reaction through genotype-specific probe design. In a cohort of n = 576 EMB samples, 70 % of patients tested positive for B19V genomes whereas 38.5 % of samples revealed an active B19V infection with viral transcripts being detectable. Differential expression of VP1/2 and NS1 transcripts was observed and viral B19V DNA load was higher when an active infection was detected. A significantly reduced left ventricular ejection fraction (LVEF) could be observed during the study comparing patients with a latent infection or virus free patients to those characterized by active viral transcription.

Significance: The study revealed that transcriptional activity of B19V in the heart muscle occurs only in a subset of persistently infected cardiac patients. RNA expression of both major viral transcripts, the VP1/2 and the NS1, must be considered for molecular diagnostics of B19V transcriptional activity, as otherwise, correct diagnosis is be missed. As a consequence of retrospective study design, a large number of EMB samples was available, however, clinical data was scarce. Nonetheless, a reduction of baseline LVEF indicated the clinical relevance of active B19V infection of the myocardium in contrast to latent infection or virus-free samples. It was not possible to correlate clinical effects to the differential expression of either NS1 or VP1/2 or both transcripts during this study. Further prospective studies should, as well, address the question of long-term pathogenicity of B19V active infection of the heart muscle. Only recently, novel treatment strategies against B19V infection of the myocardium were investigated and precise diagnosis of active infection will help to identify patients that will profit from antiviral treatment [67, 110].

2.3 Publication 3

Escher F, Pietsch H, Aleshcheva G, Bock T, Baumeier C, Elsaesser A, Wenzel P, Hamm C, Westenfeld R, Schultheiss M, Gross U, Morawietz L, Schultheiss HP.

Detection of viral SARS-CoV-2 genomes and histopathological changes in endomyocardial biopsies.

ESC Heart Fail. 2020 Oct;7(5):2440-2447. Doi: 10.1002/ehf2.12805. Epub 2020 Jun 12. PMID: 32529795; PMCID: PMC7307078.

Summary: Although experimental data on SARS-CoV-2 was scarce at that time, first clinical reports indicated that the virus affects other organs besides entering its main target cells, the pneumocytes of the lungs. SARS-CoV-2 viral genomes were detectable by PCR in tissue and body fluids, such as sputum, nasal swabs, feces, blood, and urine of infected individuals [103]. A wide distribution of angiotensin converting enzyme 2 (ACE2), which is acting as the primary SARS-CoV-2 receptor, theoretically allows infection of other organs, such as the kidneys, liver, and heart. In particular, cardiovascular diseases were identified as risk factors for a severe course of the disease and cardiovascular complications occur frequently during SARS-CoV-2 infection and progression of the disease [51, 72]. Cardiotropism of SARS-CoV-2 has not been demonstrated at the time the study was initiated, thus a possible detection of SARS-CoV-2 genomes in heart tissue might help to clarify the hypothesis of direct cardiac damage through SARS-CoV-2 infection.

EMBs of n = 104 patients suffering from unexplained heart failure or suspected myocarditis were analyzed retrospectively by immunohistochemistry, histopathological and molecular diagnostics. Patient samples were obtained from 3 February and 26 March 2020 from different German clinical centers. Molecular diagnostics of common cardiotropic viruses (enterovirus, adenovirus, parvovirus B19, and human herpesvirus 6) were performed and the diagnostic panel was extended to screen for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). To that end, a published PCR protocol to specifically amplify SARS-CoV-2 envelope small membrane protein (E)-gene and RNA-dependent RNA polymerase (RdRP)-gene sequences published by Corman *et* al. was adapted to small sample size of EMB nucleic acid extraction protocol and molecular work-up [26].

EMB samples of five of 104 patients (mean age: 57.90 ± 16.37 years; left ventricular ejection fraction: 33.7 ± 14.6 %, sex: n = 79 male/25 female) tested positive for the presence of SARS-

CoV-2 RNA. These five patients presented with heterogenous clinical symptoms which were observed either subsequently to clinical symptoms of pneumonia, with latency after pulmonary symptoms, or in the absence of clinical signs of pulmonary infection. Nasopharyngeal swab samples were not investigated for presence of SARS-CoV-2 RNA since COVID-19 was not suspected. The course of the disease ranged from mild to highly acute forms that required ICU monitoring of three patients (patients 1,3,5). Besides SARS-CoV-2 detection (Ct value: 36.66 ± 1.99), screening for other common cardiotropic viruses in EMB samples yielded negative results. An active myocarditis according to the Dallas criteria showing necrosis of myocytes was confirmed in patient 1. Patient 5 presented with a borderline type of myocarditis and the remaining patients were diagnosed with an inflammatory cardiomyopathy. Immunohistochemical analysis of EMB tissue sections demonstrated a pronounced inflammation in four of five patients (patients 1,3,4,5) that was characterized by an increased number of T-cells, macrophages, lymphocytes, and T-memory cells and furthermore demonstrated an elevated number of intercellular adhesion molecule ($CD54^{+}$). Histological evaluation revealed that inflammatory processes potentially led to rupture of capillary walls. Vacuoles in the neighboring myocardium indicated restricted metabolism and cellular stress that was caused by local ischemia. The histological findings were in accordance with an elevated increase in troponin level which was observed in each of the patients. Thus, myocardial complications during COVID-19 might result from impaired endothelial function and resulting local ischemia in these patients.

Significance: This study confirmed presence of SARS-CoV-2 viral genomes in the myocardium by PCR and thus was able to identify SARS-CoV-2 as a causal agent of cardiac complications during COVID-19. Although precise mechanism of virus induced cardiac damage remain to be investigated, histopathological analysis revealed that cardiac complications arose from impaired capillary function resembling those of systemic vasculitis. Although no specific treatment strategies exist to date, EMB diagnostics has proven its clinical usefulness to identify SARS-CoV-2 as the causal trigger of cardiac dysfunction. Since infection with SARS-CoV-2 might as well manifest with asymptomatic pulmonary primary infection, as it was also observed during the present study, PCR-based molecular screening for SARS-CoV-2 of EMB samples must be considered as a routine diagnostic method of patients with unexplained heart failure.

3 Discussion

Novel diagnostic methods for analysis of EMB samples are urgently needed to differentiate between different forms of fulminant myocarditis or virus induced entities of cardiomyopathies in order to apply specific and personalized treatment strategies [92]. Improved diagnostics will help to reveal the natural course of the disease and thus will allow to develop novel therapy options. Strict limitation in sample size predestines EMB samples for molecular diagnostics, such as PCR-based detection of pathogens or gene profile testing. Both of these methods were explored during the project and evaluated in a clinical setting.

3.1 Gene profiling as a novel approach in molecular diagnostics of EMB samples

During the present study, successful clinical application of gene expression profiling in a large cohort of heart failure patients that underwent EMB was demonstrated [35]. By using gene expression profiling it could be confirmed that conventional histopathological analysis of EMB to detect IGCM was hampered by a remarkable sampling error, thus correct diagnosis might have been missed [35]. Sampling error is a major drawback of EMB-based diagnostics questioning the clinical significance of this method [9, 109]. Correlation of EMB and complete heart pathology evaluation revealed that the formation of giant cells within the myocardium appears to be a focal process [95]. Sensitivity to detect IGCM by conventional EMB analysis was reported at 80 % [95]. In addition to focal processes of the disease, user experience and operator bias during histopathological analysis of EMB tissue sections were reported to affect correct diagnosis [94]. All of these aspects could be eliminated by the established gene profiling during our study. Despite its sampling error, EMB has proven superior for assessing myocardial inflammation when being compared to non-invasive cardiac magnetic resonance imaging, underscoring the diagnostic of invasive EMB [53].

In addition to giant cell formation, histopathological assessment of myocytolysis and immunohistological staining of inflammatory infiltrates, as well, reflected the sampling error of EMB analysis that might be caused by focal processes during IGCM. Except for perforin-positive cells, a significantly higher number of immune infiltrates was observed in tissue sections of histopathological diagnosed IGCM patients when being compared to those diagnosed by gene profiling [35]. Similar, active myocytolysis was observed more frequently when IGCM was diagnosed by histology [35]. Findings from the present study imply that in addition to giant cell formation, also inflammation and myocytolysis of the heart muscle represent focally distributed processes which require more sensitive detection methods. Results were in accordance with a study from Katzmann *et* al. reporting a sampling error of EMB but

higher sensitivity of immunohistochemical in contrast to mere histological analysis of EMB [53].

Previously published results from our group successfully demonstrated a first approach to apply gene expression profiling as a novel molecular method in EMB-based diagnostics of heart failure patients [64]. Clinical feasibility and significance of this method was shown by successful treatment of an IGCM patient [38]. Despite the studies from Lassner *et* al. comprised only a limited patient cohort, results could be confirmed during the current study [38, 64]. In contrast to the previous work, disease-specific regulation of genes coding for further Toll like receptor, except TLR8, was not observed during the present study and the panel of marker genes to identify IGCM could be limited to five genes [35, 64]. Besides work from Lassner *et* al., further scientific approaches successfully applied gene expression analysis for differential diagnostics of myocarditis and will be discussed in detail.

Heidecker *et* al. revealed a gene expression profile by transcriptome analysis of EMB samples which was able to differentiate lymphocytic myocarditis from DCM by using a panel of 13 marker genes [45]. During this study, immune markers, such as genes from the Toll like receptor family (TLR1, 2, and 7) and CD14 revealed a strong predictive power [45]. Results thus were in line with data from publication 1 also establishing immune response genes as a strong predictor [35]. Further research approaches to differentiate DCM from DCMi were investigated [5, 85, 107]. In accordance with the results from publication 1, Ruppert et al. were able to differentiate entities of DCM and DCMi only by gene expression profiling and found no correlation between molecular and histological results implying sampling error of conventional EMB analysis [85]. Ameling *et* al. identified a specific gene expression pattern in EMBs of DCM patients to predict response to immunoadsorption with subsequent immunoglobulin G substitution treatment. Genes of oxidative phosphorylation, mitochondrial dysfunction, hypertrophy, and ubiquitin–proteasome pathway revealed a significant regulation in either responders' or non-responders 'group. Although different research questions were addressed in both studies, IGCM specific gene profiling identified CPT1 as a mitochondrial marker gene during publication 1 [35]. Wittchen *et* al. were able to differentiate DCMi from DCM by gene profiling of EMB samples [107]. Marker genes of inflammation during DCM may potentially play a role in IGCM, but data from publication 1 could not support this hypothesis for genes coding for ADIPOR1, CCL14, STAT1, and IL17D [35, 107]. A study from Kittleson *et* al. was able to identify disease-specific marker genes of IGCM when compared to control hearts using microarray analysis [54]. The authors compared the

transcriptome of two cases of IGCM patients with the transcriptome of six unused organ transplant donor hearts by using microarray analysis [54]. Since heart tissue obtained during left ventricular assist device implantation was used, a large amount of sample size was available to establish the gene profiling test [54]. The study design did not represent a clinical feasible setting of EMB, despite results of the microarray analysis might be well-suited for becoming transferred into clinical diagnostics. In accordance with results from publication 1, predominantly upregulation of 115 differentially expressed genes was found in IGCM patients' samples [35, 54]. In addition, the majority of disease specific marker genes were involved in immune response regulation and T-cell activation as reported during publication 1 [35, 54]. Upregulation of genes specifically of the T_h1 pathway in IGCM patients indicates that autoimmune processes might be responsible for giant cell myocarditis [54].

Despite the discussed gene profile research approaches each of the studies yielded promising results, these relied on a limited patient cohort and thus might not be able to constitute a representative cross-section of patients. Further prospective clinical validation of these studies is pending.

In contrast to EMB-based screening, VanBuren *et* al. investigated gene expression patterns within peripheral blood cells of 72 heart failure patients, whereas 32 of those were non-ischemic patients, in comparison to 15 control patients and developed a risk stratification that was independent from NYHA class [99]. Low- and high-risk genes were identified that predominantly originated from the cluster of T cell receptor signaling [99]. The study did not aim to establish a differential diagnosis of cardiac diseases and clinical validation and feasibility as a prognostic marker still has to be achieved [99].

Since formation of giant cells in the myocardium is poorly understood, precise determination of molecular functions of marker genes identified in publication 1 remains speculative [35]. TLR8 as key marker gene, demonstrated the strongest predictive power [35]. Nevertheless, a combination of all marker genes was found superior [35]. TLR8, which is predominantly expressed in monocytes/macrophages and dendritic cells, is activated by presence of viral or bacterial RNA [22, 49]. Expression of TLR8 as a sign of activation of the innate immune response and antiviral response supports the hypothesis of virus-induced formation of giant cells during IGCM but may also be attributed to increased infiltration of macrophages [22, 82]. The fusion of macrophages to a multinucleated giant cell is stimulated by a pro-inflammatory cytokine pattern [25]. In particular, IL-4 and IL-13 activate M2 macrophages thus leading to formation of multinucleated giant cells as shown in a murine model [47]. Differential

expression of both genes could not be confirmed during publication 1 [35]. T_h1 pathway immune stimulation leads to aggregation of M1 activated macrophages by proinflammatory cytokines IL-12 and IFNγ [97]. Since these findings could not be confirmed during publication 1, one may speculate if the specific cytokine pattern responsible for initial triggering and formation of giant cells is a temporary processes [35]. A TLR7/8-mediated macrophage activated induction of multinucleated giant cells was described which supports our findings of TLR8 being up-regulated in patients harboring giant cells in the myocardium [32]. CCR6 regulates the migration and recruitment of dendritic and T cells during inflammatory and immunological responses and CCR5 is responsible for chemotaxis of macrophages [55]. In IGCM and active myocarditis, CCL20 elicits its effects on its target cells by binding and activating the chemokine receptor CCR6 [8]. CCR6 is predominantly expressed on T lymphocytes whereas its ligand CCL20 is found on the surface of macrophages, multinucleated giant cells, and epithelioid cells within sarcoid granuloma [37]. The importance of CCL20 and CCR6 as marker genes for IGCM was confirmed during publication 1 [35]. The possible molecular mechanisms that affect expression of mitochondrial marker gene CPT1 during IGCM require further investigation. Possibly, CPT1 which is involved in fatty-acid metabolism, may play a role during macrophage phagocytic function [19]. Down-regulation of CPT1 during IGCM, as observed in the present study, is associated with increased inflammatory signaling and loss of phagocytic functions of macrophages [19, 35]. To conclude, the molecular mechanism of giant cell formation in the heart are poorly understood and the precise function of identified marker genes of IGCM remains to be clarified. Hypothetically, identified gene regulatory pathways of marker genes might serve as potential therapeutic targets. Results from publication 1 and previous studies imply that rather autoimmune processes than acute viral infection may play a role in IGCM. Case reports associate IGCM with viral myocarditis, however by applying molecular virology, conclusive results could not be established yet when larger cohorts were investigated [29, 41, 46, 76]. Improved diagnosis will help to decipher the natural course of the disease.

3.2 B19V expression of viral RNAs as a marker of viral activity

Only EMB is able to establish evidence of viral infection of the myocardium and thus is a prerequisite to decide for adequate treatment strategy [25]. In particular, immunosuppressive therapy of inflammatory cardiomyopathy is contraindicated when adeno- or enteroviral infection of the myocardium was detected and was associated with an adverse outcome [40]. In accordance, immunosuppressive therapy can be considered for B19V-positive patients taking into consideration the viral load and the course of the disease when therapy is initiated while

presence of B19V RNA, as a sign of viral activity, is a contraindication for immunosuppressive therapy [18].

Presence of B19V DNA is a common finding in EMB samples of cardiac patients, as it was confirmed by presence of B19V DNA in 70% of all patients during publication 2 [80]. Similar results were also reported from other studies that investigated either EMB or myocardial autopsy samples of non-cardiac patients [30, 44, 60, 62, 87]. Clinical relevance of the myocardium with B19V is a still an issue since the majority of studies report no association of cardiac diseases with B19V infection [11, 42, 48, 56, 58, 70, 101]. Since consensus on diagnostics of B19V has not been established yet, protocols vary among different studies [2]. In particular, most studies did not assess presence of B19V viral RNA to discriminate an active infection from a clinical irrelevant latent infection [2]. Results from publication 2 clearly demonstrated that only a subgroup of B19V infected patients was characterized by an active viral infection of the myocardium [80]. Results were in line with previous studies investigating presence of B19V VP1/2 RNA in EMB samples and which were able to show a causal relationship between B19V viral activity and cardiac clinical symptoms [11, 57].

To date, studies investigating B19V infection of the myocardium, specifically with the aim to develop antiviral treatment approaches, are scarce. In a placebo-controlled randomized trial in a large cohort of B19V-associated chronic DCM patients, a potential beneficial effect of intravenous immunoglobulin (IVIg) therapy was analyzed. B19V positivity was determined by PCR in DNA extracted from EMB samples though viral transcriptional activity was not assessed [44]. A variety of clinical, molecular, and histopathological parameters were collected. Significant clinical improvement through IVIg therapy in addition to standard heart failure therapy after 6 months post therapy could not be confirmed [44]. One may speculate, if patients with active B19V might have benefitted from IVIg therapy despite study design did not allow to discriminate these patients. During a previous study, a significant decrease of B19V viral load and clinical improvement were shown after IVIg treatment of DCM patients [30]. In contrast to the aforementioned trial, cardiac B19V load was high in the total cohort [30]. As results of publication 2 demonstrated, B19V viral load was significantly increased when viral RNA was detected in EMB samples compared to latent infection with B19V [80]. In accordance with results from publication 2, a high viral load was associated with myocardial inflammation and furthermore, a clinically relevant threshold of B19V viral load was identified by Bock *et* al [11]. Beneficial effects of IVIg treatment reported from Dennert *et* al. therefore might be attributed to the fact that the majority of patients enrolled in the trial potentially suffered from

an active B19V infection of the myocardium which is correlated with a high B19V DNA load [30]. This finding also implicated that the majority of patients enrolled in the trial from Hazebroek *et* al. might be affected by a latent B19V infection thus no beneficial effects of IVIg therapy could have been expected for this subgroup [44].

Differential expression of VP1/2 and NS1 transcripts was observed during publication 2 [80]. As it was shown *in vitro,* replication of B19V in permissive cells is a regulated process being characterized by differential expression of viral transcripts to ensure effective production of progeny virions [15, 69]. In semi-permissive cells, such as endothelial cells of the myocardium, viral precursor transcripts are polyadenylated at polyadenylation site proximal due to the absence of cellular host factors [43]. This leads to an increased expression and accumulation of NS1 intermediates [43]. In permissive cells, replication of the viral genome during the late phase of infection triggers readthrough polyadenylation site proximal and results in expression of VP1/2 RNA [43]. Since endothelial cells of the myocardium are semi-permissive for B19V infection, these do not support replication of viral genome and infection with B19V leads to an incomplete viral replication cycle [16, 69]. As a consequence, VP1/2 RNA should theoretically not be expressed in the myocardium, however, was detectable in the present study [80]. Clinical significance of either VP1/2, NS1, or expression of both viral transcripts is yet to be clarified. However, a significantly reduced LVEF at baseline was shown for B19V infection with active viral transcription, despite this finding could not be correlated to any of the subgroups characterized by differential expression of viral transcripts [80]. So far, the expression rate of B19V VP1/2 compared to NS1 RNA in the myocardium has not been investigated.

To conclude, the clinical relevance of cardiac B19V infection has been underestimated since the majority of studies did not consider to discriminate between active or persistent infection leading to seemingly ambiguous results [30, 44]. Future studies should also aim to correlate B19V viral gene expression in the myocardium to viral protein expression. Deciphering the mechanism of viral persistence and reactivation might also help to develop novel antiviral treatment options against active B19V infection of the myocardium.

3.3 SARS-CoV-2 as a viral pathogen with potential cardiotropism

Proof of SARS-CoV-2 genomes by PCR in the myocardium has been established during the present study and only recently, these findings were confirmed by others [12, 34, 66, 96, 106]. As an example, Tavazzi *et* al. investigated EMB samples of a patient suffering from cardiogenic shock during COVID-19 [96]. SARS-CoV-2 viral particles were detected by transmission electron microscopy in interstitial cytopathic macrophages and surrounding areas and these

results were confirmed by positive PCR testing [96]. Cardiac tropism of SARS-CoV-2 could not be confirmed during this study since viral particles were not localized in cardiomyocytes. In contrast, viral particles could be observed in cardiomyocytes of EMB tissue samples of a single COVID-19 patient by using transmission electron microscopy during a study from Bojkova *et* al. [12]. Whether cardiac damage could be attributed to virus-induced cytopathic effects or resulted from secondary cardiac damage as a consequence of ischemia could not be addressed by study design of publication 3 [34]. Impaired endothelial function during COVID-19, as reported in the present study, was confirmed by others [34]. Varga *et* al. established evidence of direct infection of endothelial cells by electron microscopy leading to endotheliitis in different organs including the heart of COVID-19 patients [100].

Further studies that analyzed EMBs of COVID-19 patients yielded negative PCR results for SARS-CoV-2-specific RNA in the myocardium [65, 86, 104]. Two case studies report cardiac complications during COVID-19 in the absence of clinical signs of interstitial pneumonia or when nasopharyngeal swabs tested negative for viral genomes which support findings from the present study [34, 52, 106]. Of note, when serial PCR testing of nasopharyngeal swab samples was performed in the same patient, negative results for SARS-CoV-2 RNA and dynamic changes of test positivity were reported, despite lasting clinical signs of COVID-19 [71]. Similar variation in SARS-CoV-2 PCR results were observed when virus-positivity by PCR was compared with findings from chest computed tomography [3]. These findings emphasize, that gaps in knowledge on the course of the disease still complicate molecular diagnostic of SARS-CoV-2 and nasopharyngeal swab testing should not be regarded as an exclusion criterion of COVID-19.

A wide expression across different cell types and organs, including endothelial cells, fibroblasts, and cardiomyocytes, of ACE2 acting as the primary receptor for SARS-CoV-2 might explain detection of the virus in different tissue, organs, and body fluids [68]. Despite ACE2 exhibits a higher expression in the heart in contrast to the lungs, cellular transmembrane protease, serine 2 (TMPRSS2) , which is responsible for priming viral entry, is expressed at a significantly lower rate in the heart [68]. Thus, viral entry of SARS-CoV-2 into cardiomyocytes might by restricted, however, expression of other proteases might compensate for the low expression rate of TMPRSS2 [68]. Productive infection and resulting cytopathic effects of cardiomyocytes were shown *in vitro* leading to activation of pro-apoptotic pathways which impaired beating function of cardiomyocytes [12]. Hypothetically, the process of apoptosis is not associated with immune cell infiltration which in turn represents a hallmark of myocarditis

[10]. The present study and others observed inflammation in the heart of COVID-19 patients [34, 84, 100, 104]. Other studies report no association between cardiac SARS-CoV-2 infection and inflammation of the heart muscle [66].

Certain limitations applied to publication 3 that arose from retrospective study design. Small sample size of EMB hampered the use of extensive diagnostic methods such as proteomics or RNA localization methods, e.g., *in situ* hybridization or single cell sequencing. Repeated PCR testing of samples and targeting of different viral genome regions and subsequent sequencing were not possible. Furthermore, repeated testing of patients by EMB was not feasible since EMB as an invasive procedure requires hospitalization. Specifically-preserved blood samples that could be used to monitor a possible contamination of EMB samples by systemically circulating viral particles were not available [34]. However, viral particles were absent or detected only in a low concentration in peripheral blood samples from COVID-19 patients during a study from Wang *et* al. and thus possibly would not have led to contamination of small sample sized EMB [103]. In addition to positive stranded genomic RNA of SARS-CoV-2, viral antisense RNA is transcribed from the viral genome and serves as a matrix during viral genome replication [108]. Detection of subgenomic RNA as a proof of active viral genome replication remains to be investigated in the heart muscle. A study reported an approximately 1000 times lower expression rate of subgenomic RNA in relation to SARS-CoV-2 genomic RNA in clinical samples of the respiratory tract [108]. Thus, detection of subgenomic RNA in EMB samples will hypothetically be hampered by the detection limit of PCR.

The limited number of cases studied may explain the heterologous clinical presentation of patients and variation in histopathological or molecular results of EMB analysis from COVID-19 patients across different studies. To conclude, cardiac involvement during COVID-19 is still poorly understood and large cohort studies investigating histopathological samples of the heart muscle are needed to unravel the course of the disease. These studies should focus on elucidating the cellular mechanism of cardiac damage and should also apply next generation sequencing to investigate intrahost evolution as a potential factor that might be responsible for retargeting of SARS-CoV-2 to other organs than lungs.

4 Conclusion and outlook

The results of the present project have proven its clinical usefulness to further optimize EMB-based molecular diagnostics of cardiac diseases. Improvements in molecular diagnostics of viral pathogens by detection of active B19V infection and SARS-CoV-2 of the myocardium,

could be subsequently translated into routine clinical diagnostics. Applying the IGCM-specific gene profile, it could be shown that true incidence of IGCM is underestimated. Further development of cardiovascular disease-specific gene profile should especially focus on rare cardiovascular diseases, such as eosinophilic myocarditis. Occurrence of these might be highly underestimated. The results obtained from publication 1 should be considered to develop and evaluate non-invasive diagnostic methods. In particular, clinical studies are needed to correlate results of EMB-based diagnostics with data from systemic parameters or imaging techniques, such as cardiac magnetic resonance imaging. As molecular methods continue to develop rapidly, sensitivity and cost-effectiveness of next generation sequencing and RNA-sequencing will increase and will allow for investigating large patient cohorts and routine diagnostics. Future studies should continue to explore the possibilities of molecular diagnostics of EMB by high-throughput screening for disease-specific marker genes and thus will contribute to achieving significant progress in personalized medicine.

5 Appendix

5.1 References

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5.2 Eidesstattliche Versicherung und Anteilserklärung

Ich, Heiko Pietsch, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Differentiated infection pathology and gene profile analysis of myocardial tissue for extended diagnostics of patients with unexplained heart failure (Differenzierte infektionspathologische und Genprofilanalysen im Myokardgewebe zur Erweiterung der Diagnostik von Patienten mit unklarer Herzinsuffizienz)“ 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 dem/der Erstbetreuer/in, 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

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Unterschrift, Datum und Stempel der erstbetreuenden Hochschullehrerin

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Unterschrift des Doktoranden/der Doktorandin

Anteilserklärung

Heiko Pietsch hatte folgenden Anteil an den vorgelegten Publikationen:

Publication 1

Escher F, Pietsch H, Aleshcheva G, Wenzel P, Fruhwald F, Stumpf C, Westermann D, Bauersachs J, Enseleit F, Ruschitzka F, Nägele H, Laugwitz KL, Haake H, Frey N, Brachmann J, Huber K, Braun-Dullaeus RC, Bergmann MW, Strotmann J, Grönefeld G, Krülls-Münch J, Westenfeld R, Skurk C, Landmesser U, Pieske B, Gross UM, Morawietz L, Schultheiss HP.

Evaluation of Myocardial Gene Expression Profiling for Superior Diagnosis of Idiopathic Giant-Cell Myocarditis and Clinical Feasibility in a Large Cohort of Patients with Acute Cardiac Decompensation.

J Clin Med. 2020 Aug 19;9(9):2689. Doi: 10.3390/jcm9092689. PMID: 32825201; PMCID: PMC7563288.

Beitrag zur Publikation: Basierend auf den Ergebnissen der histopathologischen Untersuchungen von EMB-Proben entwickelte Herr Pietsch das Versuchsdesign und traf eine Auswahl an potentiellen Markergenen zur molekularen Diagnostik der Riesenzellmyokarditis

(Table S.2). Er führte die qPCR Genexpressions-Messungen durch und analysierte die Genexpressionsdaten (Figure 2). Herr Pietsch wertete die Genexpressions- und histopathologischen Daten statistisch aus um das Genexpressionsprofil zu erstellen (Table 2, Table 3, Figure S.2, Table S.3, Figure S.3) und validierte die molekularen Genexpressionsdaten nach Behandlung (Table 3,4, Figure 5). Er führte die Literaturrecherche durch, schrieb die Erstfassung des Manuskripts und war an der Revision bis zur Publikation beteiligt.

Publication 2

Pietsch H, Escher F, Aleshcheva G, Lassner D, Bock CT, Schultheiss HP.

Detection of parvovirus mRNAs as markers for viral activity in endomyocardial biopsy-based diagnosis of patients with unexplained heart failure.

Sci Rep. 2020 Dec 18;10(1):22354. Doi: 10.1038/s41598-020-78597-4. PMID: 33339949; PMCID: PMC7749156.

Beitrag zur Publikation: Herr Pietsch war am Studienkonzept und Versuchsdesign maßgeblich beteiligt, wie z.B. durch die Etablierung eines qPCR assays zum Nachweis des B19V NS1 Transkripts zur Unterscheidung von transkriptionell aktiver und latenter Infektion des Myokards (Figure 1, Figure S.1), und validierte die PCR Performanz (Figure S.2). Herr Pietsch führte die methodischen Arbeiten selbstständig durch und wertete die erhaltenen Daten statistisch aus (Figure 2,3). Die Interpretation der Ergebnisse erfolgte in Kooperation mit den Betreuern. Herr Pietsch führte die Literaturrecherche durch, schrieb die Erstfassung des Manuskripts und war an den Revisionen bis zur Publikation beteiligt.

Publication 3

Escher F, Pietsch H, Aleshcheva G, Bock T, Baumeier C, Elsaesser A, Wenzel P, Hamm C, Westenfeld R, Schultheiss M, Gross U, Morawietz L, Schultheiss HP.

Detection of viral SARS-CoV-2 genomes and histopathological changes in endomyocardial biopsies.

ESC Heart Fail. 2020 Oct;7(5):2440-2447. Doi: 10.1002/ehf2.12805. Epub 2020 Jun 12. PMID: 32529795; PMCID: PMC7307078.

Beitrag zur Publikation: Herr Pietsch war am Studienkonzept und Versuchsdesign maßgeblich beteiligt. Herr Pietsch validierte die PCR-Performanz, und führte die aufwendigen molekularen Messungen an Herzmuskel-Biopsien durch (Table 3). Herr Pietsch führte die Analyse und die Interpretation der Daten durch. Er führte die Literaturrecherche durch, schrieb die Erstfassung des Manuskripts und war an den Revisionen bis zur Publikation beteiligt.

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Unterschrift, Datum und Stempel der erstbetreuenden Hochschullehrerin

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Unterschrift des Doktoranden

5.3 Printed versions of publications

Extract from Journal Summary List [Intranet der Charité] (ISI Web of KnowledgeSM)

(https://intranet.charite.de/medbib/impact_faktoren_2018_fuer_zeitschriften_nach_fachgebieten/)

Journal Data Filtered By: **Selected JCR Year: 2018** Selected Editions: SCIE,SSCI
Selected Categories: **"MEDICINE, GENERAL and INTERNAL"**
Selected Category Scheme: WoS
Gesamtanzahl: 160 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	NEW ENGLAND JOURNAL OF MEDICINE	344,581	70.670	0.686700
2	LANCET	247,292	59.102	0.427870
3	JAMA-JOURNAL OF THE AMERICAN MEDICAL ASSOCIATION	156,350	51.273	0.300810
4	Nature Reviews Disease Primers	4,339	32.274	0.019740
5	BMJ-British Medical Journal	112,901	27.604	0.152760
6	JAMA Internal Medicine	15,215	20.768	0.095580
7	ANNALS OF INTERNAL MEDICINE	57,057	19.315	0.096020
8	PLOS MEDICINE	30,689	11.048	0.071200
9	Journal of Cachexia Sarcopenia and Muscle	2,799	10.754	0.005870
10	BMC Medicine	13,630	8.285	0.045220
11	Cochrane Database of Systematic Reviews	67,607	7.755	0.158690
12	MAYO CLINIC PROCEEDINGS	14,695	7.091	0.025750
13	CANADIAN MEDICAL ASSOCIATION JOURNAL	15,351	6.938	0.016500
14	JOURNAL OF INTERNAL MEDICINE	10,547	6.051	0.015700
15	Journal of Clinical Medicine	2,315	5.688	0.007210
16	MEDICAL JOURNAL OF AUSTRALIA	11,134	5.332	0.012600
17	PALLIATIVE MEDICINE	5,682	4.956	0.009860
18	AMYLOID-JOURNAL OF PROTEIN FOLDING DISORDERS	1,335	4.919	0.003270

Journal of
Clinical Medicine

MDPI

Article

Evaluation of Myocardial Gene Expression Profiling for Superior Diagnosis of Idiopathic Giant-Cell Myocarditis and Clinical Feasibility in a Large Cohort of Patients with Acute Cardiac Decompensation

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Abstract: *Aims*: The diagnostic approach to idiopathic giant-cell myocarditis (IGCM) is based on identifying various patterns of inflammatory cell infiltration and multinucleated giant cells (GCs) in histologic sections taken from endomyocardial biopsies (EMBs). The sampling error for detecting focally located GCs by histopathology is high, however. The aim of this study was to demonstrate the feasibility of gene profiling as a new diagnostic method in clinical practice, namely in a large cohort of patients suffering from acute cardiac decompensation. *Methods and Results*: In this retrospective multicenter study, EMBs taken from $n = 427$ patients with clinically acute cardiac decompensation and suspected acute myocarditis were screened (mean age: 47.03 ± 15.69 years). In each patient, the EMBs were analyzed on the basis of histology, immunohistology, molecular virology, and gene-expression profiling. Out of the total of $n = 427$ patient samples examined, GCs could be detected in 26 cases (6.1%) by histology. An established myocardial gene profile consisting of 27 genes was revealed; this was narrowed down to a specified profile of five genes (*CPT1, CCL20, CCR5, CCR6, TLR8*) which serve to identify histologically proven IGCM with high specificity in 25 of the 26 patients (96.2%). Once this newly established profiling approach was applied to the remaining patient samples, an additional $n = 31$ patients (7.3%) could be identified as having IGCM without any histologic proof of myocardial GCs. In a subgroup analysis, patients diagnosed with IGCM using this gene profiling respond in a similar fashion to immunosuppressive therapy as patients diagnosed with IGCM by conventional histology alone. *Conclusions*: Myocardial gene-expression profiling is a promising new method in clinical practice, one which can predict IGCM even in the absence of any direct histologic proof of GCs in EMB sections. Gene profiling is of great clinical relevance in terms of (a) overcoming the sampling error associated with purely histologic examinations and (b) monitoring the effectiveness of therapy.

Keywords: idiopathic giant-cell myocarditis; gene-expression profiling; endomyocardial biopsy

1. Introduction

Cardiac inflammatory processes involving giant cells comprise a diverse group of disorders [1–4] Idiopathic giant-cell myocarditis (IGCM) is regarded as a distinct clinical and pathological entity having an exclusively cardiac manifestation. This rapidly progressive disease is associated with myocyte necrosis and poor cardiac outcome [5,6]. IGCM has been shown to involve multinucleated giant cells (GCs) which have thus far been discovered mainly in lymphocytic infiltrates and among myocytolytic tissue and eosinophils [7–9]. Since GCs tend to be focally distributed within endomyocardial biopsies (EMBs), their presence is very often missed by conventional histologic evaluation due to the sampling error involved. Thus, the actual incidence of IGCM could well be higher than its detection rate. The earlier IGCM is diagnosed and immunosuppressive treatment is initiated, the better the patient recovers, given that permanent myocardial damage can be prevented (or at least minimized), thereby improving the prognosis and possibly avoiding the need for heart transplantation. Recently published studies have shown [10,11] that myocardial gene-expression profiling defines a distinct gene expression pattern which serves to indicate the presence of IGCM even without any histologic detection of GCs. Generally

speaking, specific gene-expression profiles describe the time-specific and disease-specific synthesis of cytokines and adhesion molecules, thereby defining the activation states of pro-inflammatory and anti-inflammatory intracellular pathways [12–14]. Thus, gene-expression profiling has recently been assumed to play an increasingly important diagnostic role for rejection surveillance after cardiac transplantations [15–17]. Given the focal infiltration pattern which GCs exhibit in cardiac tissue, novel methods for diagnosing IGCM are urgently needed. This multicenter study addresses the clinical evaluation of gene profiling for purposes of identifying patients afflicted with IGCM. The aim of this study was (a) the identification of a distinct gene-profiling, (b) to demonstrate the feasibility of gene profiling in clinical practice within a large cohort of patients, and (c) to show the efficacy of subsequently applied immunosuppressive treatment regarding the prognosis of the disease.

2. Patients and Methods

2.1. Patients

This retrospective multicenter study evaluated the EMB specimens of $n = 427$ patients suffering from clinically unexplained acute decompensation; these specimens had been sent to the IKDT (Institute for Cardiac Diagnostic and Therapy Berlin, Germany). Analysis included histology, immunohistochemistry, molecular virology, and gene profiling. The suspected clinical diagnoses had been made by clinicians at the relevant medical centers. In order to develop gene-expression profile that could serve as a novel tool for the diagnosis of GCs, twenty-three age-matched and gender-matched patients without intramyocardial inflammation or viral infection were used as a peer group to create the control-group profiles. They were referred for evaluation of repeated chest discomfort but had no symptoms of heart failure. Patient characteristic and hemodynamic data are summarized in Table 1 (see also Supplemental Data Table S1 for EMB results).

Table 1. Control Group Patient Characteristics and Echocardiographical Data.

n	23
Age (years)	48.5 ± 12.9
LVEF (%)	51.5 ± 15.4
LVEDD (mm)	55.5 ± 9.3
TAPSE (mm)	23 ± 4.1
IVSD (mm)	11.8 ± 3.1
LVPW (mm)	11.2 ± 2.8

Note: LVEF = left ventricular ejection fraction; LVEDD = left ventricular end-diastolic diameter; TAPSE = tricuspid annular plane systolic excursion; IVSD = intraventricular septum diameter; LVPW = left ventricular posterior wall. The data are presented as mean ± standard deviation, and as No. (%) of subjects.

2.2. Analysis of Myocardial Morphology and Inflammation

Histologic evaluations were performed on paraffin sections of two to three EMBs using standard procedures, e.g., formaldehyde or RNA*later* fixation, paraffin embedding, staining with hematoxylin and eosin, elastic van Gieson stain (EvG) and Azan stain. The EMB diagnosis of active myocarditis was based on the histomorphologic criteria according to the Dallas Classification [18]. Immunohistochemistry was used to characterize the inflammatory infiltrates and was carried out on RNA*later*-fixed samples (two EMBs). Myocardial inflammation was diagnosed by $CD3^+$ T-lymphocytes/mm^2 (Dako, Glostrup, Denmark), $CD11a^+$/LFA-1^+ lymphocytes/mm^2 (Immuno Tools, Friesoythe, Germany), $CD11b^+$/Mac-1^+ macrophages/mm^2 (ImmunoTools), $CD45R0^+$ T memory cells (Dako, Glostrup, Denmark), perforin$^+$ cytotoxic cells/mm^2 (BD Bioscience, San Jose, CA, USA). Inflammatory cells were quantified using quantitative digital-imaging analysis, reported elsewhere [19]. Intramyocardial inflammation was categorized according to the *ESC* Statement [20]. We also analysed macrophages (threshold >40.0 $CD11b^+$/Mac-1^+ macrophages/mm^2),

CD45R0$^+$ T Memory cells (threshold > 40 cells/mm^2), and perforin-positive cytotoxic cells (threshold > 2.9 cells/mm^2).

2.3. Nucleic Acid Isolation, Reverse Transcription (RT) and nPCR for cDNA

Genomic DNA from two to three EMBs was extracted using Puregene Core Kit A (Qiagen, Hilden, Germany). Total RNAs were isolated during routine EMB diagnostics using Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA); these were treated with DNAse (PeqLab, Erlangen, Germany) to remove any traces of genomic DNA and were then reverse-transcribed to cDNA with the High Capacity Kit (Thermo Fisher Scientific) using random hexamer primers. DNA and cDNA concentrations were quantified using the PCR-based Quantifiler™ Human DNA Quantification Kit (Thermo Fisher Scientific).

2.4. Preamplification and Gene-Expression Analysis

Given the limited amounts of extracted myocardial cDNA available, a gene-specific PCR-based pre-amplification technique was applied. Gene expression was then determined via qPCR amplification of generated preAMP-DNA. The expression level was calculated in relation to the housekeeping gene *HPRT* while applying the Delta-Delta-Ct method [21]. All predesigned gene-expression qPCR assays were purchased from Thermo Fisher Scientific and applied in keeping with the manufacturer's instructions. To ensure the technical integrity of the gene profile test, samples that produced low amounts of RNA were excluded from further analysis. The cut-off values were set at Ct < 25 for *HPRT* detection and for Quantifiler^{TM} analysis, respectively. Samples of each patient were run in parallel with *HPRT* for quantification of mRNA and internal assay amplification control to ensure standardization of PCR. Previous *in vivo* and *in vitro* microarray-based studies had identified a set of 27 genes that have been shown to be deregulated by inflammatory cardiomyopathy. These genes serve as coding for cellular receptors or immune-response mediators, or are part of energy metabolism pathways [11] (Table S2). The evaluation of the myocardial gene-expression profiles revealed a specific expression pattern encompassing five specific genes: chemokine receptor 5 (*CCR5*), chemokine receptor 6 (*CCR6*); carnitine palmitoyltransferase I (*CPT1*), toll-like receptor 8 (*TLR8*), and chemokine (C-C motif) ligand 20 (*CCL20*) [22–27]. Expression of *CPT1* was found to be downregulated in IGCM.

2.5. Statistics

The quantitative results of the analysis were expressed as mean ± SD (standard deviation) values. The parametric paired *t*-test was used to analyze data within a group, whereas the parametric unpaired *t*-test was used to compare different groups. Once it had been established that none of the data were distributed normally, the non-parametric Mann-Whitney U test for group comparisons and Wilcoxon's signed rank test for comparisons between baseline and follow-up were utilized. The non-parametric Spearman correlation method was used for correlation analysis. *P*-values below 0.05 were treated as indicators of statistical significance. All statistical analyses were performed using Version 23.0 of the SPSS software, (IBM Corp. Armonk, NY, USA), as well as the GraphPad Prism 7.04 software (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

The patients included in this study were evaluated by means of extensive EMB analysis, including histologic, immunohistochemical, molecular virology analyses and gene profiling. A summary overview of the patients' characteristics, hemodynamic data and suspected clinical diagnoses can be found in Table 2.

In $n = 26$ patients, the incidence of IGCM could be determined through histopathologic analyses of EMBs. All the patients included in this study were considered for purposes of establishing the specific gene-expression profile.

Histology-proven IGCM was found to be present in $n = 26$ patients. In this patient group, $n = 23$ patients fulfilled the criteria for active myocarditis according to the Dallas Classification; in the remaining $n = 3$ patients, borderline cases of myocarditis were indicated.

Table 2. Patients' Characteristics, Hemodynamic Data and Suspected Clinical Diagnoses within the Entire Study Group.

Patient Data	Entire Cohort
n	427
Age (years)	47.03 ± 15.69
LVEF (%)	38.54 ± 17.89
LVEDD (mm)	54.51 ± 8.75
TAPSE (mm)	22.40 ± 5.93
IVSD (mm)	11.31 ± 2.80
LVPW (mm)	10.84 ± 2.40
NYHA I/II/III/VI (*n*)	0/0/250/177
Suspected clinical diagnoses (No., *n*):	
- AMC	180
- IGCM	30
- DCMi	100
- EOM	11
- cardiac sarcoidosis	20
- unexplained acute heart failure	86

Note: LVEF = left ventricular ejection fraction; LVEDD = left ventricular end-diastolic diameter; TAPSE = tricuspid annular plane systolic excursion; IVSD = intraventricular septum diameter; LVPW = left ventricular posterior wall; NYHA = New York Heart Association Classification; AMC = acute myocarditis; IGCM = idiopathic giant cell myocarditis; DCMi = dilated inflammatory cardiomyopathy; EOM = eosinophilic myocarditis; The data are presented as mean ± standard deviation, or as No. of subjects (No., *n*).

Representative images of the histologic and immunohistologic findings are shown in Figure 1.

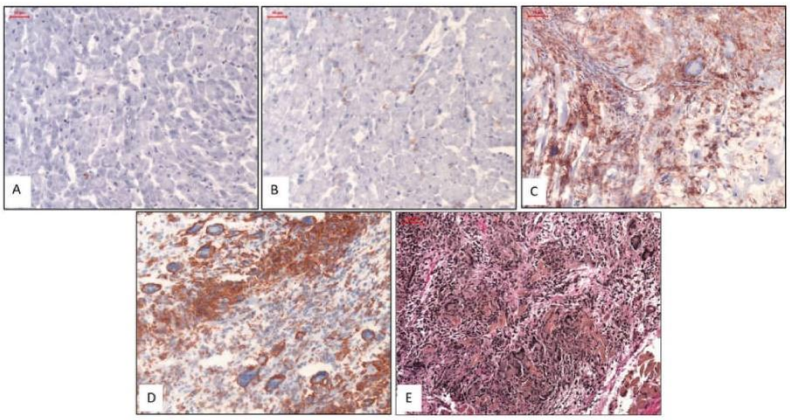

Figure 1. Representative Histologic and Immunohistochemical Images. Images (**A**) & (**B**): Patients with normal myocardium; i.e., no CD3 stain (**A**) and no Mac1 stain (**B**). Images (**C**) & (**D**): Patients with IGCM presenting GCs surrounded by diffuse infiltration of massively increased T lymphocytes (CD3) (**C**) and macrophages (Mac1) in immunohistologic staining (**D**). Image (**E**): EvG staining from a patient with severe active myocarditis and giant cells. Magnification ×200.

3.1. Distinctive Myocardial Gene-Expression Profiles Which Serve to Identify IGCM

EMBs taken from the 26 patients with histologically confirmed IGCM were analyzed. The gene-expression profile could be used to successfully identify histologically proven IGCM with a high degree of specificity in 25 of the 26 patients (96.2%) (Figure 2, Figure S1, Table S3).

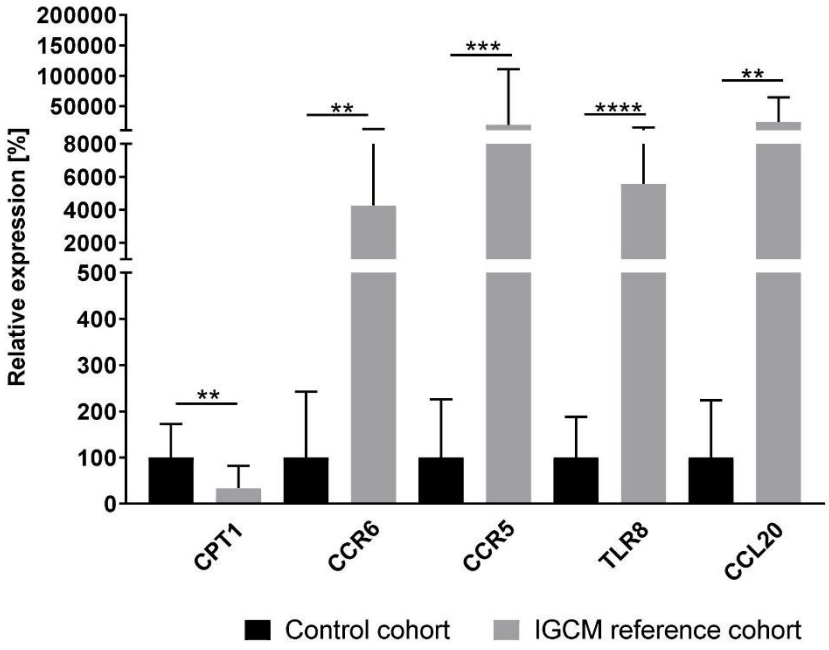

Figure 2. Gene-expression profiles for IGCM patients (n = 26) in relation to inflammation-negative patients/control cohort (n = 23). The figure shows a distinct gene-expression pattern with high statistical significance, as derived from the unpaired t-test. P-values are denoted by asterisks: ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

3.2. Application of IGCM-Specific Gene Profiling across the Entire Cohort of Patients with Acute Cardiac Decompensation

As a next step, the gene-expression data of the remaining patients (n = 401) who exhibited acute cardiac decompensation but no histologically detected GCs were evaluated based on the new numeric thresholds of the five genes identified for the IGCM-specific gene profile. When this newly derived profiling was applied, an additional n = 31 patients could be identified as having a distinctive gene-expression pattern suggestive of IGCM; these patients were therefore classified as suspected cases of IGCM despite the absence of histologic proof for the presence of myocardial GCs.

In particular, a gene profile matching the criteria for multinucleated giant cells could be observed in n = 14 of the patients with clinically suspected acute myocarditis. In n = 10 of the patients presenting a positive gene profile for GCs, an inflammatory cardiomyopathy had been clinically suspected; in n = 7 patients, unexplained acute heart failure had been diagnosed (Figure 3). Out of these 31 patients, an evaluation of EMBs determined that n = 6 patients actually had active myocarditis according to the Dallas Classification based on histology. In the remaining n = 25 patients, borderline myocarditis was identified (Figure 3).

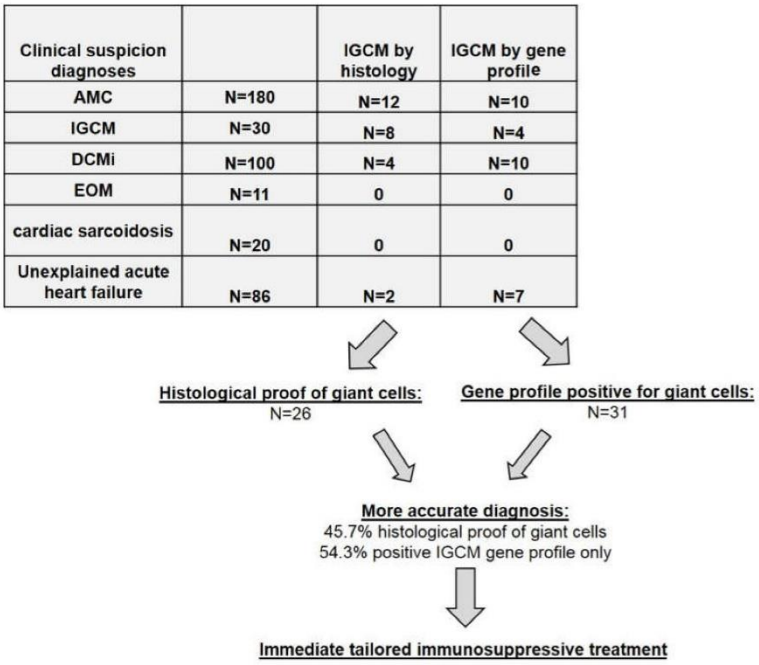

Figure 3. Clinically Suspected Diagnoses and IGCM Histologically Proven or Detected by Gene-Profiling. Note: AMC = acute myocarditis; IGCM = idiopathic giant cell myocarditis; DCMi = dilated inflammatory cardiomyopathy; EOM = eosinophilic myocarditis.

Including samples taken from the 427 patients suffering from acute cardiac decompensation, multinucleated giant cells were ultimately detected in a total of 26 (6.1%) of the patients by means of histology. However, based on the improved diagnostics offered by myocardial gene profiling, an additional $n = 31$ patients of the remaining cohort could also be identified as having giant cells even without any direct histologic proof of such cells. Thus, out of the overall cohort of 427 patients with acute cardiac decompensation, a total of 57 patients (13.3%) could be diagnosed as having IGCM. Only 26 of these 57 (45.6%) had a histologic presentation of GCs. In other words, $n = 31$ of those 57 (54.4%) would have been overlooked if conventional histology alone had been used.

3.3. Immunohistologic Analysis of Intramyocardial Infiltration in GCM Patients

When it comes to the immunohistologic staining found among the IGCM patient samples, the number of infiltrative cells involved ranges widely. See Table 3 for a breakdown of IGCM diagnoses based on conventional, histologic proof and those based on gene profiling.

3.4. Correlation of Immunohistochemical Markers with Deregulated Genes

In the total patient cohort, gene-expression data for the five deregulated genes in the IGCM-specific profile were correlated with the number of digitally measured immune cell numbers in the cardiac EMBs. Analysis revealed a weak correlation between Mac-1$^+$ macrophages and CD45R0$^+$ T memory-cells on the one hand and the computed IGCM-specific gene-profile score on the other ($p \leq 0.05$; $r = 0.158$ and $r = 0.161$). For CD3$^+$ lymphocytes, perforin$^+$ cytotoxic cells, and LFA-1$^+$ lymphocytes, no correlation was observed (n.s.; $r = 0.090$, $r = 0.048$ and $r = 0.042$) (see Supplemental Data Figure S2).

Table 3. Immunohistologic EMB Analysis of Intramyocardial Infiltration in GCM Patients Based on Conventional Histology or on Gene Profiling.

Patient Data	IGCM (By Histology)	IGCM (By Gene Profiling)
Immunohistology		
- $CD3^+$ lymphocytes infiltration/mm^2	312.4 ± 297.3	125.8 ± 196.3 *
- LFA-1^+ lymphocytes infiltration/mm^2	462.6 ± 413.8	183.4 ± 215.0 *
- $CD45R0^+$ T memory cell infiltration/mm^2	533.3 ± 349.6	114.9 ± 502.3 *
- perforin$^+$ cell infiltration/mm^2	16.23 ± 26.00	14.14 ± 32.23
- Mac-1^+ macrophages infiltration/mm^2	428.8 ± 344.0	181.1 ± 227.1 *

Note: Immunohistologic marker: CD3 = T-lymphocytes; LFA-1 = leukocyte function antigen-1; Mac-1 = macrophage-1 antigen; CD45R0 (UCHL1) = leucocyte common antigen; perforin = cytotoxic cells. The data are presented as mean ± standard deviation. Asterisk (*) indicates significant variance between the incidence of IGCM derived from histology and that derived from gene profiling.

3.5. EMB-Based Diagnosis Out of The Entire Study Group

See Supplemental Table S4 for the EMB-based diagnostic findings for the entire study group.

3.6. Clinical and Hemodynamic Outcome of IGCM Patients at Follow-Up after Immunosuppressive Treatment

In a subgroup analysis we evaluated the response of patients with gene-profiling diagnosis of IGCM to immunosuppressive therapy. Therefore, we evaluated the observed, clinical hemodynamic outcome at follow-up (mean follow-up time: 6.4 ± 4.3 months) in patients who had received immediate immunosuppressive therapy as well as heart-failure medication after receiving an EMB-based diagnosis of IGCM. In the process, the clinical outcomes were compared between those patients whose IGCM had been histologically confirmed (n = 17) and those whose ICGM had been diagnosed by gene profiling (n = 23) (Table 4).

Table 4. Clinical, Hemodynamic, and Immunohistologic EMB-analysis of Intramyocardial Infiltration in GCM patients (n = 40) Based on Conventional histology and on Gene Profiling at Baseline and After Immunosuppressive Therapy.

Patient Data	IGCM (By Histology) *At baseline*	IGCM (By Histology) *After therapy*	IGCM (By Gene Profiling) *At baseline*	IGCM (By Gene Profiling) *After therapy*
n	17	17	23	23
LVEF (%)	19.0 ± 14.22 *	47.25 ± 12.27	31.3 ± 15.0 *	49.9 ± 12.4
LVEDD (mm)	56.23 ± 5.23	55.62 ± 8.43	56.43 ± 7.28	55.93 ± 4.29
TAPSE (mm)	20.48 ± 5.13	21.23 ± 5.34	21.96 ± 7.53	21.81 ± 6.33
IVSD (mm)	10.98 ± 3.15	10.42 ± 4.21	11.12 ± 3.15	11.02 ± 4.12
LVPW (mm)	10.14 ± 2.41	10.05 ± 2.24	10.25 ± 2.16	10.58 ± 2.07
NYHA I/II/III/VI	0/0/6/11	0/11/6/0	0/0/11/12	0/18/5/0
Immunohistologic Analysis				
$CD3^+$ T lymphocytes infiltration/mm^2	397.3.4 ± 305.3 **	23.57 ± 19.23	169.1 ± 111.5 **	15.72 ± 17.94
LFA-1^+ lymphocytes infiltration/mm^2	612.6 ± 405.5 **	40.91 ± 21.00	190.8 ± 119.6 *	23.77 ± 20.69
$CD45R0^+$ T memory cells/mm^2	584.5 ± 340.5 *	63.95 ± 59.47	208.1 ± 158.3	33.99 ± 25.62
perforin$^+$ cytotoxic cells/mm^2	21.70 ± 28.42	0.97 ± 0.72	39.22 ± 48.64	2.17 ± 4.73
Mac-1^+ macrophages infiltration/mm^2	569.3 ± 311.5 **	59.44 ± 18.92	200.5 ± 111.3 **	40.10 ± 26.42

Note: LVEF = left ventricular ejection fraction; LVEDD = left ventricular end-diastolic diameter; TAPSE = tricuspid annular plane systolic excursion; IVSD = intraventricular septum diameter; LVPW = left ventricular posterior wall; NYHA = New York Heart Association Classification. Immunohistologic marker: CD3 = T-lymphocytes, LFA-1 = leukocyte function antigen-1, Mac-1 = macrophage-1 antigen, CD45R0 (UCHL1) = leucocyte common antigen, perforin = cytotoxic cells. The data are presented as mean ± standard deviation, and as No. of subjects. Significant variance between the value at baseline and after therapy are indicated (* $p \leq 0.05$; ** $p \leq 0.01$).

The entire cohort of treated patients exhibited a significant improvement of LVEF (26.6 ± 15.6% to 48.9 ± 12.1%; $p < 0.0001$) following immunosuppressive treatment. Further subgroup analysis during the follow-up to immunosuppressive treatment revealed a significant improvement of LVEF (19.0 ± 14.22% to 47.25 ± 12.27%; $p = 0.0049$) in patients ($n = 17$) whose GCs had been proved by histology, i.e., through EMBs. Similarly, a significant increase of LVEF (31.3 ± 15.0% to 49.9 ± 12.4%; $p = 0.0028$) was also observed at follow-up (see Figure 4) in those cases where the patient exhibited a positive myocardial gene profile but where there was no direct histologic proof of multinuclear giant cells ($n = 23$).

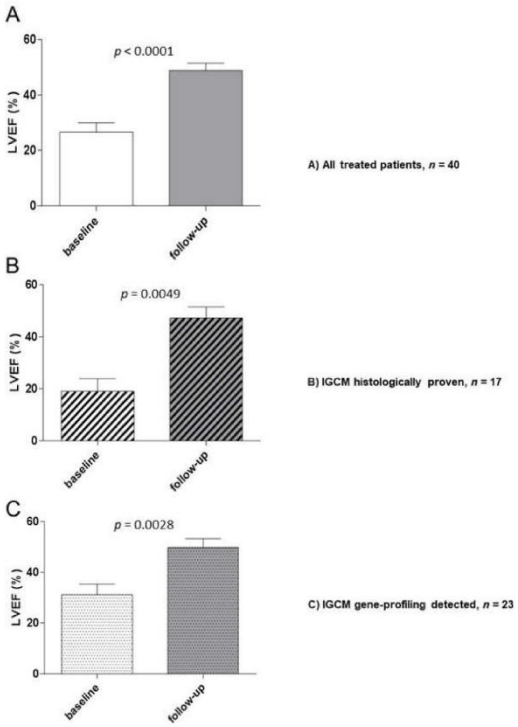

Figure 4. Baseline and Follow-up Hemodynamic Outcomes After Immunosuppressive Therapy for Patients with Histologically Proven or Gene-Profile-Detected IGCM. LVEF (%) was measured at baseline and during the follow-up period. (**A**) All treated patients. (**B**) Patients with histologically proven IGCM. (**C**) Patients with gene-profile-detected IGCM. The figures shown are mean values ± standard deviation; *P*-values compared to the baseline EMB are indicated.

The improvement in LV function was accompanied by a reduction or complete absence of intramyocardial inflammation in the follow-up EMB (Table 4). Only two patients were found to have persistent inflammation. After extension of immunosuppressive therapy (>6 months), even these two patients became immunohistologically negative. At the time of the baseline EMB, all the patients had presented a specific pattern of deregulated genes relevant for IGCM. In the follow-up phase, the genes which had been differentially expressed in the EMBs were found to have normalized, indicating that treatment had been successful for 21 of the patients (90.0%) (Figure 5). Two patients who exhibited persistent inflammation exhibited a gene profile that was still suggestive of IGCM. Immunosuppressive treatment was therefore continued and the gene-expression values eventually normalized in accordance with clinical and histologic parameters. None of the patients died during the observation.

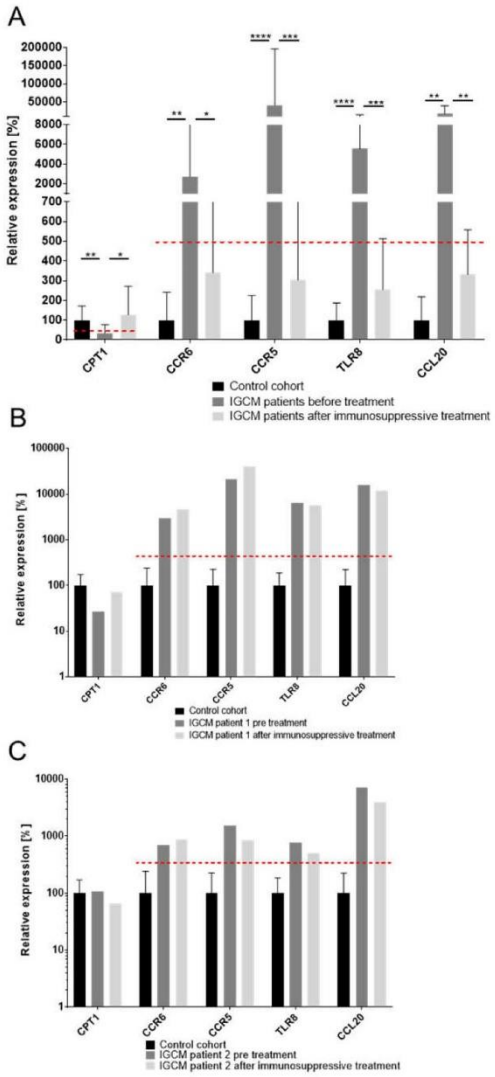

Figure 5. Analysis of Gene-Expression Dynamics Before and After Immunosuppressive Therapy of Patients with Histologically Proven and Gene Profile-Detected IGCM. (**A**) The gene expression of marker genes for IGCM were measured before and after applying immunosuppressive therapy and were compared to a healthy control cohort in patients with normalized gene-expression profiles at follow-up; the results indicated successful treatment. The mean expression for marker genes *CPT1*, *CCR6*, *CCR5*, *TLR8* and *CCL20* normalized at follow-up and fell below a clinically relevant threshold but did not reach the expression level of the control cohort. A dashed line indicates a clinically relevant threshold for the specific gene expression. (**B**) and (**C**) The gene expression of marker genes observed in two IGCM patients who exhibited persistent inflammation at follow-up along with a gene profile that suggested persistence of GCs after treatment. *P*-values are denoted by asterisks: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

4. Discussion

The main findings of this study are: (1) Of a selected panel of 27 genes previously identified to be regulated in patients with IGCM, five genes (*CPT1, CCL20, CCR5, CCR6, TLR8*) are highly specific for patients with IGCM compared with controls; (2) Our study suggests that by using this panel of five genes, 54.3% of all IGCM cases would have been missed by using conventional histologic examination alone; (3) Patients diagnosed with IGCM using this gene profile panel respond in a similar fashion to immunosuppressive therapy as patients diagnosed with IGCM by conventional histology alone, supporting the clinical utility of gene profiling in patients presenting with unexplained acute heart failure.

In this multicenter study, myocardial gene-expression profiling to diagnose IGCM was applied in clinical practice for the first time, namely within a large cohort of patients suffering from unexplained acute cardiac decompensation. This new gene-profiling approach significantly improves the diagnosis rate for clinically suspected myocarditis and unexplained acute cardiac decompensation and helps to identify GCs which would otherwise have been missed in a purely conventional, histological examination. Our study population is unique in that it is by far one of largest group of IGCM patients ever evaluated. Fraction of IGCM-positive patients as indicated by gene profile analysis was unexpectedly high as suspected clinical diagnosis of IGCM was only assumed in 12 of 57 patients (21%) by the clinician.

Thanks to this new diagnostic test, moreover, giant-cell myocarditis is sure to be diagnosed more widely going forward. This in turn will have dramatic prognostic and therapeutic relevance for patients. After all, IGCM is the most aggressive form of active myocarditis and often has a fatal outcome. Thus, there is a high medical need to detect IGCM as early as possible so as to avoid progressive myocardial tissue damage, the eventual need for transplantation, or even cardiac death. It bears noting that, according to various multicenter studies, the five-year transplant-free survival rate is no better than 10% [3,28–31].

The only way to directly confirm the presence of giant cells is to analyze histologic sections in the form of EMBs [32], but that can be problematic [30,33]. Previous publications have postulated that immunohistologic examinations of EMBs exhibit a high sensitivity because of the diffuse inflammatory infiltration of cardiac tissue in IGCM [32]. Our study suggests, however, that there is a lack of sensitivity relying solely on conventional histopathological analysis when investigating EMB for presence of IGCM. This is where gene profiling, as a novel diagnostic tool, has several major advantages over conventional EMB analysis. For one thing, the complete biopsy can be used for nucleic acid extraction. For another, the results obtained from gene-expression profiling are more conclusive and less dependent on the operator's experience than those obtained from optical analysis.

Although immunosuppressive therapy is considered the mainstay of medical treatment for IGCM, there is currently no consensus on how it should be executed; the optimal duration of treatment also remains undefined [22]. Moreover, the taking of additional EMBs is often needed in order to confirm a diagnosis, which can delay the initiation of treatment [22]. Also, the fulminant future course of the disease is often not foreseeable at the time the EMB is taken, so that an IGCM diagnosis is not expected. With gene-profiling, the repeated taking of EMBs could be avoided, immunosuppressive therapy could be started immediately, and optimal treatment duration could be effectively monitored.

Interestingly, we were able to show that 23 of the 26 patients with histologically proven IGCM exhibited active myocarditis according to the Dallas Classification. By contrast, active myocarditis could be observed in only $n = 6$ of the patients who presented positive gene profiles for GCs but, possibly due to the sampling error of histologic EMB analysis, presented no direct histologic proof of GCs. Moreover, the levels of lymphocytic and macrophage infiltration were significantly higher in patients who did exhibit histologic evidence of GCs than in patients who merely exhibited positive gene profiles. Normally, no GCs would have been expected in the latter cases. It follows, therefore, that the suspected diagnoses adopted by the clinicians must be viewed critically, given that they often failed to even suspect IGCM. These observations underscore the risk posed by a sampling error and the importance of new, supplementary diagnostic methods. One way to overcome these limitations and the general sampling error of purely conventional histologic examination is to apply specific gene

profiling to look for indicators that multinucleated giant cells may be present in the human hearts being examined [10,11,23].

Our study was furthermore able to demonstrate, using quantitative immunohistologic staining, that the number of infiltrative cells in IGCM patients ranges widely. As expected, most patients experience a massive increase in infiltrative cells. However, our data do show that there are also patients with low inflammation and IGCM, possibly due to a focal inflammatory process. This may explain the frequently faulty suspected diagnosis made by the clinician. We consider this observation extremely important for clinicians and/or pathologists because the presence of a low inflammation in the EMB should not become an exclusion criterion for IGCM.

Another aim of the present study was to show the efficacy of immunosuppressive treatment in terms of achieving improved clinical outcomes and preventing a fatal course of IGCM [34–38]. Subgroup analysis of the clinical hemodynamic outcome of $n = 40$ GC patients undergoing immediate immunosuppressive therapy revealed a significant improvement of LVEF at follow-up in those patients whose GCs were proven by EMB histology. At the same time, EMB analysis revealed a significant reduction in the quantified number of inflammatory infiltrates.

Similarly, a significant increase of LVEF after treatment was also observed in patients who exhibited positive myocardial gene profiles for GCs. EMB analysis also revealed a significant reduction in the quantified number of inflammatory infiltrates. These results underscore the clinical importance of our gene-profiling analysis, for it allows the relevant, prognostic immunosuppressive therapy to be started immediately, potentially a critical success factor. None of the patients in the overall patient cohort died during the observation period.

5. Conclusions

Based on 427 examined patient samples, multinucleated giant cells could be detected in only 26 patients (6.1%) through the use of histology alone. When this was supplemented by gene profiling, however, IGCM could be diagnosed in further 31 patients (7.2%), even in the absence of any direct histologic proof of giant cells. This means that 54.3% of all IGCM cases would have been missed by using conventional histologic examination alone. Which in turn highlights the importance of this new diagnostic approach. Our results show that: (1) the evaluation of EMBs is essential in successfully diagnosing IGCM, that (2) an analysis of gene-expression profiles in EMBs is of great clinical and prognostic importance when it comes to compensating for the sampling error which occurs when IGCM is diagnosed through a purely histologic examination of EMBs, and (3) patients diagnosed with IGCM using this gene profile panel respond in a similar fashion to immunosuppressive therapy as patients diagnosed with IGCM by conventional histology alone.

6. Limitations

This said, our study admittedly remains subject to certain caveats. For one thing, the limitations typical for retrospective cohort studies apply to our analyses. These include, among other factors, a lack of extended clinical data for all of the patients covered in this multicenter study. Furthermore, the patients in our cohorts were mainly of Caucasian ethnicity (due to the location of our study centers), which may possibly limit the applicability of our findings to other ethnic groups. On the other hand, the validity of our results tends to be corroborated by the large number of cases investigated at the core centers and by the fact that the histologic slides were independently reviewed by cardiac pathologists.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0383/9/9/2689/s1, Table S1: Detailed Endomyocardial Results in Control Group ($n = 23$); Table S2: Potential marker genes for gene profile development; Figure S1: Gene profile performance analysis for IGCM detection; Table S3: ROC of gene profile performance analysis for IGCM detection; Table S4: EMB-Based Diagnostic Findings for the Entire Study Group; Figure S2: Correlation of IGCM-specific gene-expression profile score with the number of digitally measured immune cells in EMBs in the total patient cohort.

Author Contributions: Conceptualization, F.E. (Felicitas Escher) and H.-P.S.; Data curation, G.A., P.W., F.F., C.S. (Christian Stumpf), D.W., J.B. (Johann Bauersachs), F.E. (Frank Enseleit), F.R., H.N., K.-L.L., H.H., N.F., J.B. (Johannes Brachmann), K.H., R.C.B.-D., M.W.B., J.S., G.G., J.K.-M., R.W., C.S. (Carsten Skurk), U.L. and B.P.; Funding acquisition, H.-P.S.; Investigation, F.E. (Felicitas Escher) , H.P. and D.W.; Methodology, H.P., G.A., U.M.G. and L.M.; Project administration, F.E. (Felicitas Escher) and H.-P.S.; Supervision, C.S. (Christian Stumpf), N.F., J.K.-M., R.W. and H.-P.S.; Validation, F.E. (Felicitas Escher) and L.M.; Visualization U.M.G.; Writing—original draft, F.E. (Felicitas Escher), H.P., G.A. and H.-P.S.; Writing—review & editing, P.W., J.B. (Johann Bauersachs), C.S. (Carsten Skurk) and U.M.G. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

CCL20	chemokine (C-C motif) ligand 20
CCR5	chemokine receptor 5
CCR6	chemokine receptor 6
CPT1	carnitine palmitoyltransferase I
DCM	dilated cardiomyopathy
DCMi	inflammatory DCM
EMB	endomyocardial biopsy
EOM	eosinophilic myocarditis
EvG	elastic van Gieson stain
GCs	giant cells
IGCM	idiopathic giant cell myocarditis
IVSD	intraventricular septum diameter
LVEDD	left ventricular end-diastolic diameter
LVEF	left ventricular ejection fraction
LVPW	left ventricular posterior wall
NYHA	New York Heart Association Classification
RT-qPCR	reverse transcription-quantitative polymerase chain reaction
TAPSE	tricuspid annular plane systolic excursion
TLR8	toll-like receptor 8

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2	SCIENCE	680,994	41.037	1.070190
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4	Science Advances	21,901	12.804	0.110010
5	Nature Communications	243,793	11.878	1.103290
6	Nature Human Behaviour	1,230	10.575	0.006550
7	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA	661,118	9.580	1.022190
8	Science Bulletin	3,569	6.277	0.009840
9	Scientific Data	3,240	5.929	0.015610
10	Frontiers in Bioengineering and Biotechnology	1,994	5.122	0.006540
11	Journal of Advanced Research	2,691	5.045	0.004780
12	Research Synthesis Methods	1,932	5.043	0.005420
13	GigaScience	2,674	4.688	0.012510
14	Annals of the New York Academy of Sciences	46,385	4.295	0.025840
15	Scientific Reports	302,086	4.011	1.061540
16	Journal of the Royal Society Interface	12,933	3.224	0.029190
17	NPJ Microgravity	203	3.111	0.000670
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Detection of parvovirus mRNAs as markers for viral activity in endomyocardial biopsy-based diagnosis of patients with unexplained heart failure

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Erythroparvovirus **(B19V) genomes have been detected in various organs of infected individuals including endothelial cells of the heart muscle. However, the role of B19V as a causative pathogen of myocardial damage is still unknown. The majority of reports focus on the presence of viral DNA ignoring proof of viral RNAs as important markers for viral activity. During this study, we established (RT-) qPCR to characterize expression of B19V RNAs (NS1 and VP1/2) in endomyocardial biopsies (EMBs) of 576 patients with unexplained heart failure. 403/576 (70%) EMBs were positive for B19V DNA. B19V mRNAs NS1 and/or VP1/2, indicating viral activity, could be detected in 38.5% of B19V DNA positive samples using the newly established B19V RT-PCRs. 22.1% of samples were characterized by only NS1 mRNA detection while 6.0% revealed only VP1/2 mRNA expression. Detection of both intermediates was successful in 10.4% of samples. Applying the molecular testing, our study revealed that a high proportion (38.5%) of B19V DNA positive EMBs was characterized by viral transcriptional activity. Further prospective studies will evaluate relevance of viral transcription intermediates as a diagnostic marker to differentiate between latent B19V infection and clinically relevant transcriptionally active B19V-infection of the heart muscle.**

Primate parvovirus (B19V), a non-enveloped single stranded linear DNA virus, belongs to the genus *Erythroparvovirus*. Infection with B19V is widespread in human population and leads to lifelong viral persistence[1]. As a consequence, B19V prevalence is increasing with age. Seroprevalence has been reported from 40 to 60% for young adults (< 20 years) to 78% at > 50 years of age[2]. B19V infection is usually asymptomatic or shows only mild symptoms. In contrast, B19V infection of immunosuppressed patients can cause transient aplastic crisis and persistent B19V infection developing as pure red cell aplasia and chronic anaemia[3]. During pregnancy B19V infection is associated with an increased risk for foetal loss or foetal hydrops[3]. B19Vgenomes have been detected in various organs such as heart, liver, kidney and skin, and different diseases such as rheumatoid arthritis and cutaneous T cell lymphomas[4–6].

Myocarditis as an inflammatory disease of the myocardium may be idiopathic, infectious, or autoimmune. It may heal or lead to dilated cardiomyopathy (DCM) that is characterized by dilatation and impaired ventricular contraction. Myocarditis and DCM represent acute and chronic stages, whereas DCM is the third most common cause of heart failure[7].

The classical viral pathogen associated with myocarditis or inflammatory dilated cardiomyopathy (DCMi) is Coxsackievirus B. However, B19V genomes are the most frequently detected viral genomes in endomyocardial biopsies (EMBs) of patients with suspected heart failure[8,9]. Since B19V DNA is frequently found in both, symptomatic and asymptomatic patients, the clinical relevance of B19V is still a matter of discussion[10–12].

The 5.6 kb linear single stranded DNA genome contains two major open reading frames (ORFs) coding for the NS1 (non-structural protein) and VP1 and VP2 (capsid) proteins being flanked by inverted terminal repeat regions (ITRs) that are necessary for self-priming during viral genome replication. Two minor ORFs code

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Characteristic	Value
Male, n (%)/female, n (%)	413 (71.7)/163 (28.3)
Age, years ± SD	53.6 ± 15.7
LVEF, % ± SD	34.1 ± 15.7
B19V genome detection, n/N (%)	403/576 (70)
B19 genomes viral load, median [GE/µg] (range)	944.3 (51,103)
Left/right ventricular catheterization, n (%)	303 (52.6%)/273 (47.4%)

Table 1. Baseline characteristics of the study population (N = 576).

for a 9 and 11 kDa protein of largely unknown function. Transcription activity is driven by a single promoter sequence (P6) while enhancer sequences upstream of the P6 promoter region recruit different cellular transcription factors[13]. From a single precursor (pre-) mRNA, 12 mature mRNAs are generated through alternative splicing that coordinates polyadenylation[14]. B19V infection induces a cell cycle arrest at late S phase and induces DNA damage response which will supply enzymes that facilitate B19V replication[15]. The expression of B19V transcription intermediates depends on splicing and polyadenylation efficiency and thus it depends on host cell factors. In semi-permissive cells, such as endothelial cells, transcripts are polyadenylated at polyadenylation site proximal (pA)p leading to an increased expression of NS1 intermediates. In permissive cells, however the blockade at (pA)p is overcome by replication of the viral genome in the late phase of infection and readthrough (pA)p leads to expression of VP1/2[16].

Cardiomyocytes cannot be infected by B19V[8]. Host cell tropism of B19V is restricted to erythroid progenitor cells. Since endothelial cells of the myocardium express the primary erythrovirus receptor, the P-antigen, and co-receptors such as integrin α5β1 and KU80, infection with B19V of these semi-permissive cells leads to an incomplete viral replication cycle[17]. Due to the absence of certain host cell factors, B19V is not able to complete the viral replication cycle in endothelial cells, and thus no infectious progeny virions are produced[18]. Instead, infection of endothelial cells leads to endothelial dysfunction[18]. Several mechanisms may explain B19V mediated cytotoxicity, such as direct damage through the NS1 endonuclease domain or upregulation of pro-apoptotic signalling molecules e.g. TNFα or IL-6[19,20]. Furthermore, the VP1-unique region exerts phospholipase A2 activity that promotes inflammatory signalling and impairs endothelial function[21]. In addition, NS1 transactivates viral and host gene expression and is essential for viral replication due to its DNA nickase and helicase activity.

During this study, we established a molecular approach using newly generated RT-qPCRs to show viral activity by the detection and characterization of B19V NS1 and VP1/2 mRNAs in EMBs of patients with unexplained heart failure. Detection of viral RNA intermediates demonstrates viral activity and thus can be useful as a biomarker for B19V replication that allows to differentiate between latent and active B19V infection.

Results

Study subjects. EMBs of 576 patients with diagnosed unexplained heart failure were collected from 74 German clinical centres and sent to the Institute for Cardiac Diagnostics and Therapy GmbH for molecular analysis. The cohort comprised EMBs of 413 (71.7%) male and 163 (28.3%) female patients at a mean age of 53.6 ± 15.7 years with a mean left ventricular ejection fraction (LVEF) of 34.1% ± 15.7% at the date of hospitalization (Table 1). Routine analysis of B19V genome detection revealed that 403/576 (70%) of EMBs were positive for B19V DNA with a median viral load of 944.3 GE/µg ranging from 15 to 51,118 GE/µg (Table 1). However, determination of latent versus active B19V infection is pending and requires the analysis of viral transcription intermediates expression.

Establishment of B19V qPCR for B19V NS1 DNA and RNA detection. In order to assess the presence of B19V genomes and to assess viral activity of transcription in EMBs of patients with unexplained heart failure, PCRs targeting the B19V NS1 and VP1/2 regions were applied.

Prototype genome sequences for the main B19V genotype 1 (J35; AY386330.1) and genotype 2 (LaLi; AY044266.2) were retrieved from NCBI GenBank. The sequences were globally aligned using MEGA Version X-Software (Version 10.0.5) by the implemented MUSCLE algorithm[22]. The obtained consensus sequence was then used as a reference sequence to design primers and probes for qPCR within the NS1 region (Fig. 1A). To account for differences in genotype-specific nucleotide sequences, degenerated nucleotides for primer design were used. The probes of the NS1 detection system were designed genotype-specific to allow for multiplex detection (Fig. 1A) (Table 2). Pairwise local alignment through Basic local alignment search tool (BLAST-algorithm) of each primer and probe sequence within the NCBI GenBank database confirmed the specificity of the designed sequences.

QPCR performance testing to detect B19V. The PCR was tested for residual cross contamination after DNA or RNA extraction. A representative image of agarose gel electrophoresis of a B19V DNA and RNA-positive EMB using VP1/2 specific nPCR primers was shown (Fig. 1B, Supplementary Fig. S1, Table 2). The amplicon was detectable after DNA extraction (Fig. 1B lane 6) and after RNA extraction, DNAse treatment and reverse transcription (Fig. 1B lane 7). No amplicon was detectable when DNAse treatment after DNA extraction was applied (Fig. 1B lane 5). After RNA extraction and RNAse treatment and reverse transcription PCR,

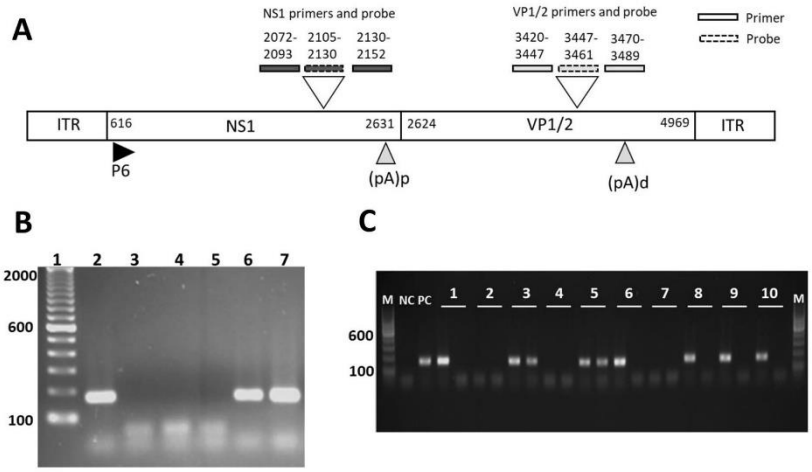

Figure 1. (**A**) Schematic description of the B19V genome organization and PCR design. Gene locus and primer and probe localization for NS1 and VP1/2 are indicated by numbers representing the nucleotide position. *ITR* inverted terminal repeat, *NS1* non structural protein 1, *VP1/2* capsid proteins, *P6* P6 promotor, *(pA)p* polyadenylation site proximal, *(pA)d* polyadenylation site distal. (**B**) Representative agarose gel electrophoresis gel blot image of a B19V-VP1/2 DNA and RNA-positive EMB using VP1/2 specific nested-PCR. Amplicon length 173 bps. 1 = DNA-Marker 100 bps; 2 = positive control; 3 = negative control; 4 = PCR after DNA extraction and DNAse treatment; 5 = PCR after RNA extraction, RNAse treatment and RT-PCR; 6 = PCR after DNA extraction; 7 = PCR after RNA extraction and DNAse treatment and RT-PCR. Complete gel blot image of figure (**B**) was shown in Supplementary Fig. S1. (**C**) Representative agarose gel electrophoresis gel blot image of 10 EMB samples following VP1/2 specific nested PCR. DNA (first lane) and cDNA (second lane) of each EMB were analysed. Amplicon length 173 bps EMBs 1, 6, 8, 9 and 10 were tested positive for viral DNA and negative for viral RNA. EMBs 3 and 5 were positive for both, viral RNA and DNA. EMBs 2, 4 and 7 were virus negative without any viral DNA nor RNA being detectable. *M* 100 bps marker, *NC* negative control, *PC* positive control.

Primer/probe name	Nucleotide sequence (5′–3′)	Nucleotide position[a]	1st, 2nd (RT)-nPCR/qPCR
NS1-FW	TCCCTGGAATWAATGCAGATGC	2072–2093	Sense B19V NS1 qPCR
NS1-RV	CACTGCTGCTGAYACTGGTGTCT	2130–2152	Antisense B19V NS1 qPCR
NS1-GT1-probe	6FAM-ACCTCCAAACCACCCCAATTGTCACA-TAMRA	2105–2130	Probe B19V NS1 qPCR
NS1-GT2-probe	VIC-ACCTCCAAACCGTCCCCATTGTCGCA-TAMRA	2105–2130	Probe B19V NS1 qPCR

Table 2. Primer sequences used for detection of B19V. [a]Nucleotide position according to reference sequence (AY386330.1).

no amplicon was detectable (Fig. 1B lane 4). The negative control (Fig. 1B lane 3) and the positive control with detectable amplicon at 173bps (Fig. 1B lane 2) were shown. Therefore, the DNAse treatment ensured that no DNA contamination is present in the sample after RNA extraction (Fig. 1B). A representative agarose gel of 10 EMBs following VP1 nPCR was shown (Fig. 1C). For each EMB, the viral DNA (first lane) and RNA (second lane) were shown (Fig. 1C). Whereas EMBs 1, 6, 8, 9, and 10 were positive for viral DNA, EMBs 3 and 5 were positive for both, viral RNA and DNA (Fig. 1C). EMBs 2, 4, and 7 were virus negative without any viral DNA nor RNA being detectable (Fig. 1C).

A serial dilution of the control plasmid (pParvovirus B19) ranging from 16,000 to 0.16 GE/µl demonstrated the performance of the NS1 qPCR (Supplementary Fig. S2). In each dilution stage, four replicates were measured and the mean value of corresponding Ct values ± SD were calculated (Supplementary Fig. S2). Successful NS1 detection was verified by VP1/2 PCR detection showing identical viral loads.

B19V detection in EMBs of patients with unexplained heart failure. The majority of EMBs (70%; n = 403) of the total cohort (N = 576) showed B19V positive genome detection (DNA) using B19V-specific PCR (Fig. 2). B19V RNA detection using RT-qPCR assays revealed that 155/403 (38.5%) of analysed EMBs with detectable B19V DNA were positive for B19V RNA (Fig. 2A). 248/403 (61.5%) of these EMBs were characterized

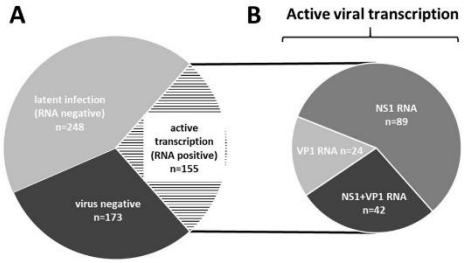

Figure 2. (**A**) B19V genome detection and detection of viral transcription activity in EMBs of patients with unexplained heart failure (N = 576). (**B**) The group composition of EMBs with detectable active viral transcription (VP1/2-RNA-, NS1-RNA and VP1/2 and NS1-RNA positive samples) was shown in detail. Numbers represent the amount of EMBs.

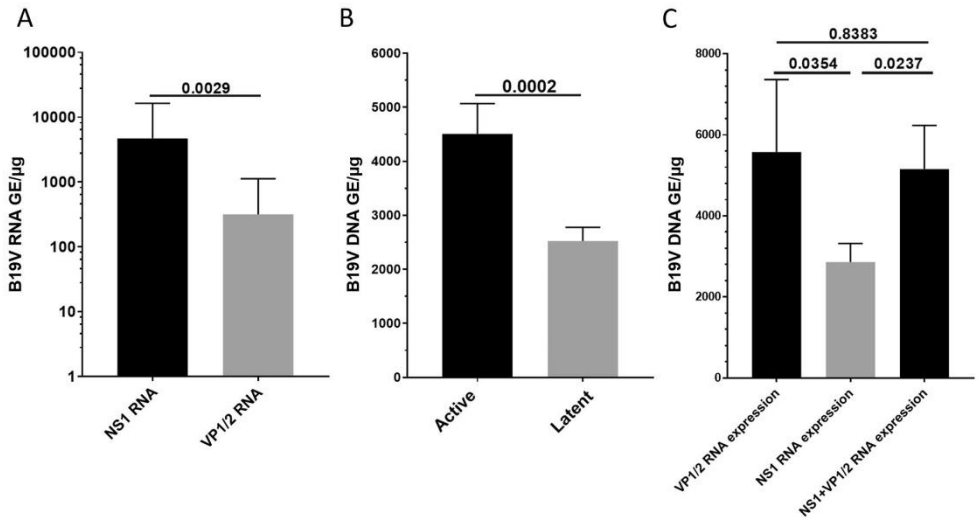

Figure 3. (**A**) The number of viral transcripts of NS1 compared to VP1/2. (**B**) Viral DNA loads in EMBs with active or latent infection. (**C**) Viral DNA load compared between EMBs with detectable VP1/2-RNA, NS1-RNA or NS1- and VP1/2-RNA expression (ANOVA p = 0.0427). Numbers above the bars represent p-values.

by a latent infection without any viral transcription intermediates being detectable (Fig. 2). Further analyses in terms of NS1 and VP1/2 RNA transcription determination in EMBs using NS1 and VP1/2-specific RT-qPCR showed that NS1 RNA could be detected in 89/403 EMBs (22.1%) positive for B19V genomes and expression of both, NS1 and VP1/2 RNA, in 42/403 samples (10.4%), respectively (Fig. 2B). However, in 24/403 samples (6%) only VP1/2-RNA intermediates were observed (Fig. 2B).

Analysis of viral transcription and viral genome copy number. Detailed analysis on the occurrence of different forms of transcription intermediates demonstrated differences in viral transcription intermediates and viral genome copy number among these subgroups. These might therefore represent different entities of the disease.

Interindividual variation in DNA copy number (6545 ± 13,077 GE/µg) and RNA transcript number (4709 ± 11,713 GE/µg) were observed. Viral DNA load of NS1 correlated significantly with VP1/2-DNA load ($p = 0.0148$; Pearson $r = 0.1228$; $r^2 = 0.01509$) and furthermore correlation of NS1- to VP1/2-RNA loads was highly significant ($p \leq 0.0001$; Pearson $r = 0.8817$; $r^2 = 0.7775$).

Expression level of NS1 transcripts (4709 ± 1023 GE/µg) differed significantly from the expression of VP1/2 transcripts (319.2 ± 100 GE/µg) ($p = 0.0029$) (Fig. 3A). EMBs of patients with active viral transcription being

detectable presented with significantly increased DNA loads (4508 ± 562.6 GE/µg) when compared to EMBs of latently B19V infected patients (2526 ± 253.6 GE/µg) (p = 0.0002) (Fig. 3B). When both transcription intermediates (5156 ± 1076 GE/µg) or only VP1/2-RNA (5577 ± 1786 GE/µg) were detectable, the load of viral genomes was significantly increased compared to EMBs of patients that expressed solely NS1 RNA (2863 ± 454.2 GE/µg) (p = 0.0237; p = 0.0354) (Fig. 3C) Overall difference of viral load between these three groups was statistically significant (ANOVA p = 0.0427). Neither frequency of B19V detection nor viral load (p = 0.0763) or load of viral transcription intermediates (NS1: p = 0.9544; VP1: p = 0.3156; NS1 and VP1: p = 0.5190) were significantly different when left or right ventricular biopsy were compared.

166/248 (66.9%) of EMBs with latent viral infection exhibited a viral load of > 500 GE/µg. Significantly more EMBs with active viral transcription (124/155 (80%)) were characterized by a viral load of > 500 GE/µg (p = 0.0045). Baseline LVEF of patients affected by viral transcriptional activity (30.7 ± 13.1%) was significantly reduced when compared to virus free (35.5 ± 15.9%; p = 0.0254) or latently infected (34.7 ± 15.7%; p = 0.0432) patients, with no significant differences between virus free and latently infected patients (p = 0.7045).

Discussion

B19V is a pathogen with broad clinical manifestations including myocarditis and chronic dilated cardiomyopathy (DCM). B19V genomes (B19V DNA) can be frequently detected in the routine molecular-pathology diagnostic in EMBs of unexplained heart failure, while with 36.7% B19V is the leading cardiotropic pathogen[12,23]. Since the first description of B19V as a cardiotropic pathogen in dogs and later on in humans, the clinical relevance of B19V infection of the heart muscle is a matter of discussion until to date[24]. However, since fulminant acute B19V infection in acute myocarditis can be causative for the heart disease is unquestionable, B19V infection in chronic myocarditis or DCM is still being discussed controversially[10–12]. Routine molecular diagnostic of cardiotropic pathogens is done using PCR techniques. However, B19V infection of the heart muscle is diagnosed only by B19V genome detection that cannot differentiate between latent and active B19V infection. B19V transcription intermediates such as B19V RNAs can therefore serve as markers of viral activity. Hence, we developed a new molecular approach to determine and quantify B19V RNAs in EMBs of patients with unexplained heart failure. Detection of B19V RNAs can differentiate between latent and active infection and therefore may serve as a marker for clinically relevant infection of the heart muscle.

In this regard, the present study demonstrated the successful detection of B19V genomes and the detection of B19V transcription intermediates (B19V RNAs) in a cohort of patients with unexplained heart failure. The newly established qPCRs targeting the NS1 and VP1/2 regions of the B19V genome demonstrated high specificity and sensitivity (Fig. 1, Supplementary Fig. S2). Among the EMBs of 576 consecutive patients suffering from unexplained heart failure who underwent first diagnostic endomyocardial biopsy, B19V genomes could be identified in 70% of all EMBs analysed. Furthermore, viral activity was detected in 26.9% of all EMBs analysed by detection of viral mRNA transcripts using the newly generated B19V-specific RT-qPCR.

The finding of 70% of B19V positivity in EMBs of the total cohort is in line with observations of a previous report[25]. A previous meta-analysis revealed that the overall detection rate of B19V genomes by PCR in different tissue types was 44.8% as summarized from 18 studies[4]. Since B19V prevalence increases with age, the rate of persistently infected individuals will be affected by the mean age of the sample population. A seroprevalence of 78% has been reported for individuals > 50 years of age that corresponds well to the data of our study population (mean age 53.6 ± 15.7 years)[2]. However, the high detection rate of active viral transcription by RT-qPCR (38.5% of all B19V positive EMBs) has not been reported before. We could also show that it was crucial to search for both replication intermediates in EMBs, the NS1 and VP1/2 viral RNAs. For that reason, our data demonstrated that for NS1 and VP1/2 RNAs in 57.4% and 15.5%, respectively, of B19V-positive EMBs an active viral transcription will remain undetected if only VP1/2 or NS1 intermediates are detected during molecular diagnostics using our newly established molecular approach. Left or right ventricular catheterization did not bias detection rate or viral load of B19V. Results were in accordance with a previous study reporting no significant difference in B19V genome detection rate when left and right ventricular EMBs were analysed in parallel[26].

Previous studies reported that persistence of B19V DNA in the heart muscle is a common finding in EMB-based analysis and could not be correlated to clinical symptoms[10,11,27,28]. In contrast, other reports revealed that B19V infection of the heart muscle may lead to diastolic dysfunction and was associated with DCMi[8,29]. Furthermore, progressive cardiac dysfunction in the course of B19V infection has been linked to viral persistence[30]. To date only few studies investigated the pathogenic effects of B19V with respect to viral activity[31]. EMBs from healthy donors were not available for ethical reasons in the present study and comparison of B19V transcriptional activity between control and cardiac patients is pending and will be accomplished in future studies. The high number of B19V genome detection both, in healthy controls and in EMBs from patients with myocarditis or dilated cardiomyopathy, may be due to the fact that up to 70% of healthy individuals suffered from a past B19V infection depending on progressing age[1,28]. However, viral genomes in all tissues including the heart muscle will not be cleared following infection but remain generally as a latent infection[1]. The expression of viral transcription intermediates represents a distinct entity of the disease with clinical consequences for the patient. From a cohort of 416 B19V DNA positive cardiac patients, in only a small fraction (15.9%) active viral transcription was detectable[31]. Bock et al. reported that viral transcripts were only detectable in patients with myocardial inflammation and were absent in persistently infected patients without viral transcription[12]. Viral transcription might be associated with local increase in inflammatory signalling while triggering of signalling cascades may lead to increased expression of IL-6 or TNF-α[19,20,32]. Since detection of B19V genomes is a common finding in various diseases, the viral activity as measured by transcription of viral mRNAs, will be of potential diagnostic relevance. We hypothesize that reduced LVEF at baseline of patients presenting with viral transcription activity compared to latently infected or virus free patients might be associated with pathogenic effects of B19V transcription activity.

However, this finding must be confirmed by further prospective studies also assessing long-term effects of B19V in the myocardium. Variation in viral loads and viral RNA expression among samples were observed which may be attributed to different phases of the B19V replication cycle. As we could demonstrate here, the highest amount of viral DNA was found in those samples when both viral RNAs, NS1 and VP1/2, or only VP1/2-RNA were detectable (Fig. 3C). This finding is in line with few studies that used both, capsid (VP1/2) and non-structural protein (NS1) specific sequences to detect B19V genomes by PCR, while discrepancies in the detection rate were reported[4]. Furthermore, our analysis revealed that EMBs of patients with active transcription demonstrated a significantly increased viral load (Fig. 3B). This result is in agreement with a previous study by Kuehl et al. showing that the expression of viral VP1/2 intermediates lead to significant increase in viral genomes while a correlation between the number of viral RNA transcripts and the viral genome copy number was observed[31]. Our data are furthermore supported by in vitro studies showing that replication of B19V proceeds in different phases and viral DNA and RNA concentrations may vary during these phases. The early phase of replication is characterized by alternative splicing and internal polyadenylation of pre-mRNA and leads to expression of NS1[33]. Whereas the replication of the viral genome and the formation of double-stranded viral DNA initiates the shift of gene expression towards an increase in VP1/2 during the late phase of infection[33].

Notably, in vitro studies revealed that in semi-permissive cells such as endothelial cells the replication cycle of B19V is not able to complete due to absence of cellular host factors that drive the viral genome replication. Parvoviral replication is limited to erythroid progenitor cells in vivo and to certain cell types in vitro. Therefore, infection of endothelial cells does not lead to a productive infection. As a consequence, VP1/2 expression is low and NS1 is expressed at a higher rate[34]. Accumulation of NS1 may trigger the hosts' immune response leading to auto-immunity that could be responsible for cardiac damage and clinical symptoms. Endothelial dysfunction then leads to secondary necrosis of myocardial cells[29]. Furthermore, NS1 exhibits cytotoxicity through mitochondria-mediated reactive oxygen species accumulation and apoptosis. For porcine parvovirus, it has been reported that NS1 expression leads to a downregulation of antiapoptotic molecules Bcl-2 and Mcl-1 and enhanced expression of proapoptotic molecules Bax, P21, and P53[35]. In addition, a direct damage of B19V in vivo might be possible through its NS1 endonuclease function[36].

Only recently, novel antiviral treatment options against B19V have been discussed. In this regard, telbivudine, a nucleoside analogue used for Hepatitis B-virus therapy, seemed to be promising[37]. In order to monitor B19V replication activity under antiviral treatment, e.g. with telbivudine, viral RNA detection will be a pivotal diagnostic method.

Study limitation

Limitations typical for retrospective cohort studies apply to our analyses. These include, among other factors, a lack of extended clinical data for all of the patients covered in this study. Prospective studies must investigate long-term effects of B19V activity in the myocardium. EMBs from healthy donors were not available for ethical reasons in the present study and comparison of B19V transcriptional activity of control and cardiac patients is pending and must be addressed in future studies. Limitation in sample material available of each patient did not allow for intensive investigation, such as virus-host interactions.

Conclusion

In this study, we developed new molecular approach for B19V genome detection in order to discriminate a latent B19V infection from a B19V infection that is characterized by viral transcriptional activity in EMBs of patients suffering from unexplained heart failure. Retrospective analysis of 576 EMB samples demonstrated the feasibility of the method in a clinical setting of molecular diagnostics. The detection of B19V RNA replication intermediates can serve as a novel biomarker to differentiate between clinically relevant and non-relevant B19V infection.

Methods

Acquisition of endomyocardial biopsy samples. EMBs of 576 consecutive patients with clinical evidence of symptomatic heart failure of unknown cause after invasive exclusion of coronary artery disease by left heart catheterization (e.g., acute cardiac decompensation and suspected acute myocarditis) were collected from 74 German clinical centres and were sent to the Institute for Cardiac Diagnostics and Therapy GmbH for molecular diagnostics. All patients enrolled were catheterized for EMB during the period of 03/16/2011 to 08/25/2008 (median: 11/16/09) in the clinical centres. Since consecutive patients were investigated in the present study, the cohort represents a cross-section of cardiac patients catheterized during acute or stable phase. Patients suffering from a severe course of disease, such as fulminant acute forms of heart muscle disease, were excluded. The cohort comprised EMBs from 413 (71.7%) male and 163 (28.3%) female patients at a mean age of 53.6 ± 15.7 years with a mean left ventricular ejection fraction of 34.1% ± 15.7% at the date of hospitalization. All samples were retrospectively and anonymously tested for B19V. The extended diagnostic approach of the present study was approved by and performed within European Research Area Network on Cardiovascular Diseases (ERA-CVD; JTC2016-40-158).

Sample preparation. Immediately after taking EMBs at the hospitals, samples were transferred to RNAlater solution (Thermo Fisher Scientific, Waltham, MA, USA) stabilizing the nucleic acids of the EMBs[38]. DNA from two to three EMBs was extracted by Puregene Core Kit A (Qiagen, Hilden, Germany) according to manufacturer's instructions[31,38,39]. Total RNA was isolated using TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA), treated with DNAse (PeqLab, Erlangen, Germany) to remove any traces of DNA, and reverse-transcribed to cDNA with High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) using random hexamer primers according to the manufacturers protocol (Thermo Fisher Scientific,

Waltham, MA, USA)[31,38–40]. Following cDNA synthesis or DNA extraction, samples were stored at − 80 °C until further evaluation[40]. Nucleic acid concentration was measured by PCR-based Quantifiler Human DNA Quantification Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer´s instructions[38,40].

Molecular diagnostics. *VP1/2-detection.* Nested polymerase chain reaction (nPCR) and quantitative real time PCR (qPCR) targeting the VP1/2 region of B19V were applied to detect B19V genomes (DNA) and VP1/2 mRNA as described previously[12,17].

NS1-DNA detection. For the newly developed NS1-qPCR, 4 µl of extracted sample DNA and primers NS1-FW and NS1-RV and probes NS1-GT1-probe and NS1-GT2-probe were used. The PCR reaction was carried out in a 96-well microtiter plate format (Applied Biosystems, USA) according to the manufacturer's instructions using TaqMan Universal PCR Master Mix (Applied Biosystems, USA) on a QuantStudio 12 K Flex Real-Time PCR System (Applied Biosystems, USA) (Table 2). Serial dilutions of a plasmid (pParvovirus B19) containing the NS1 sequence (3.5 to 3.5×10^4 GE/µl) (GenExpress, Berlin, Germany) were simultaneously amplified for quantification and standardization as described previously[41] (Table 2). Viral load was calculated by ratio of viral genome copy number to amount of total DNA extracted and was given as viral genome equivalents/µg genomic DNA (GE/µg). For qPCR of NS1 the following reaction conditions were used: initial denaturation for 10′ at 95 °C, followed by 40 cycles of denaturation for 15″ at 95 °C and a combined annealing and extension step for 60″ at 60 °C.

NS1-RNA detection. The newly developed NS1-qPCR was carried out in a 96-well microtiter plate format (Applied Biosystems, USA) according to the manufacturer's instructions using TaqMan Universal PCR Master Mix (Applied Biosystems, USA) using 4 µl of extracted sample cDNA and primers NS1-FW and NS1-RV and probes NS1-GT1-probe and NS1-GT2-probe on a QuantStudio 12 K Flex Real-Time PCR System (Applied Biosystems, USA) (Table 2). Serial dilutions of a plasmid (pParvovirus B19) containing the NS1 sequence (3.5 to 3.5×10^4 GE/µl) (GenExpress, Berlin, Germany) were simultaneously amplified for quantification and standardization as described previously[41] (Table 2). Copy numbers of viral RNA were normalized by quantification of isolated total mRNA measured as expression of the house-keeping gene HPRT. Expression of HPRT was measured using predesigned primers and probe (TaqMan gene expression assay; Hs99999909_m1) (Applied Biosystems, Germany) and therefore also served as an internal quality control for extraction efficiency or possible sample degradation. Serial dilutions (25–2 ng/µl) of reverse transcribed Total RNA Control (Human) (Applied Biosystems, Germany) were used to quantify HPRT expression. The PCR reaction was conducted according to the manufacturer's instructions using TaqMan Universal PCR Master Mix (Applied Biosystems, USA). For qPCR of NS1 the following reaction conditions were used: initial denaturation for 10′ at 95 °C, followed by 40 cycles of denaturation for 15″ at 95 °C and a combined annealing and extension step for 60″ at 60 °C.

Statistical analysis. Results for quantitative analysis are given as mean value ± SD (standard deviation). Fisher's exact test and Chi-square test were used to compare frequency distribution of dichotomic variables among two or more groups. To compare continuous variables between two groups, parametric unpaired Student's *t*-test or non-parametric Mann–Whitney U test was used in the case of not normally distributed data, respectively. To test for statistical significance between more than two groups, ANOVA or Kruskal–Wallis test was used. For correlation analysis, Pearson correlation coefficient (r^2) was assessed. P-values below 0.05 were considered to indicate statistical significance. All statistical analyses were performed using GraphPad Prism 7.04 software (GraphPad Software Inc., La Jolla, CA, USA). All graphics were created using GraphPad Prism 7.04 software (GraphPad Software Inc., La Jolla, CA, USA).

Ethical approval. Extended routine diagnostic has been applied for and was approved within the European Research Area Network on Cardiovascular Diseases (ERA-CVD; JTC2016-40-158). The study conformed to the principles outlined in the Declaration of Helsinki. Patients' data were anonymized for analyses. All experimental methods applied during the study were approved by the ethics committee of Charité-Universitätsmedizin Berlin, Germany, (Ethikkommission, Ethikausschuss 4 am Campus Benjamin Franklin, Charitéplatz 1, 10117 Berlin) (Ethikvotum Berlin 225-07) within the SFB Transregio 19 (Deutsche Forschungsgemeinschaft (DFG), project number 5486135).

Informed consent. Informed written consent was obtained from each study patient.

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Author contributions

C.T.B. and H.P. conceived and designed the experiments; H.P. carried out the experiments; H.P.S. provided samples and materials; H.P. and C.T.B. analysed the data; G.A., F.E., D.L. and H.P.S. provided comments and valuable feedback; H.P. and C.T.B. wrote the manuscript; G.A., F.E., D.L. and H.P.S. revised and proof-read the manuscript. All data generated or analysed during this study are included in this article.

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Competing interests

The authors declare no competing interests.

Additional information

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Gesamtanzahl: 136 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	EUROPEAN HEART JOURNAL	57,358	23.239	0.125920
2	CIRCULATION	166,484	23.054	0.211290
3	JOURNAL OF THE AMERICAN COLLEGE OF CARDIOLOGY	100,986	18.639	0.193290
4	Nature Reviews Cardiology	6,301	17.420	0.018820
5	CIRCULATION RESEARCH	52,988	15.862	0.072290
6	EUROPEAN JOURNAL OF HEART FAILURE	13,107	13.965	0.027620
7	JAMA Cardiology	3,280	11.866	0.019320
8	JACC-Cardiovascular Imaging	8,801	10.975	0.026160
9	JACC-Cardiovascular Interventions	11,555	9.544	0.033640
10	JACC-Heart Failure	3,537	8.910	0.016830
11	JOURNAL OF HEART AND LUNG TRANSPLANTATION	12,436	8.578	0.027310
12	CARDIOVASCULAR RESEARCH	21,828	7.014	0.021500
13	European Heart Journal-Cardiovascular Pharmacotherapy	442	6.723	0.001430
14	Circulation-Heart Failure	6,900	6.526	0.022830
15	BASIC RESEARCH IN CARDIOLOGY	4,137	6.470	0.005590
16	PROGRESS IN CARDIOVASCULAR DISEASES	4,055	6.162	0.008860
17	JOURNAL OF THE AMERICAN SOCIETY OF ECHOCARDIOGRAPHY	10,478	6.111	0.016060
18	EUROPACE	10,908	6.100	0.025320
19	Circulation-Cardiovascular Interventions	5,289	6.060	0.016640

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
20	Cardiovascular Diabetology	5,392	5.948	0.011550
21	Circulation-Cardiovascular Imaging	5,456	5.813	0.018480
22	European Journal of Preventive Cardiology	4,782	5.640	0.013370
23	CANADIAN JOURNAL OF CARDIOLOGY	6,710	5.592	0.018500
24	JOURNAL OF THORACIC AND CARDIOVASCULAR SURGERY	29,599	5.261	0.036950
25	European Heart Journal-Cardiovascular Imaging	5,498	5.260	0.021650
26	HEART RHYTHM	12,344	5.225	0.029030
27	REVISTA ESPANOLA DE CARDIOLOGIA	3,566	5.126	0.004640
28	HEART	18,063	5.082	0.030620
29	JOURNAL OF CARDIOVASCULAR MAGNETIC RESONANCE	5,113	5.070	0.014020
30	JOURNAL OF MOLECULAR AND CELLULAR CARDIOLOGY	14,143	5.055	0.020450
31	Circulation-Arrhythmia and Electrophysiology	6,432	4.968	0.017840
32	Clinical Research in Cardiology	3,022	4.907	0.006760
33	Circulation-Cardiovascular Genetics	3,441	4.864	0.010500
34	Journal of the American Heart Association	13,230	4.660	0.060340
35	TRENDS IN CARDIOVASCULAR MEDICINE	2,667	4.462	0.003930
36	Circulation-Cardiovascular Quality and Outcomes	4,531	4.378	0.014350
37	ATHEROSCLEROSIS	23,442	4.255	0.033500
38	CARDIOVASCULAR DRUGS AND THERAPY	2,109	4.181	0.003140
39	JOURNAL OF NUCLEAR CARDIOLOGY	3,711	4.112	0.004480

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Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
40	AMERICAN JOURNAL OF PHYSIOLOGY-HEART AND CIRCULATORY PHYSIOLOGY	27,828	4.048	0.022820
41	AMERICAN HEART JOURNAL	20,811	4.023	0.026780
42	EuroIntervention	6,097	4.018	0.016840
43	HEART FAILURE REVIEWS	2,598	4.015	0.005300
44	ANNALS OF THORACIC SURGERY	36,145	3.919	0.040630
45	JOURNAL OF CARDIAC FAILURE	5,339	3.857	0.009350
46	EUROPEAN JOURNAL OF CARDIO-THORACIC SURGERY	17,156	3.847	0.026410
47	European Heart Journal-Acute Cardiovascular Care	1,466	3.734	0.005330
48	INTERNATIONAL JOURNAL OF CARDIOLOGY	30,479	3.471	0.080570
49	ESC Heart Failure	680	3.407	0.002020
50	NUTRITION METABOLISM AND CARDIOVASCULAR DISEASES	5,821	3.340	0.010180
51	CURRENT PROBLEMS IN CARDIOLOGY	574	3.333	0.000700
52	Journal of Cardiovascular Computed Tomography	1,711	3.316	0.004430
53	Global Heart	881	3.238	0.003800
54	RESPIRATORY MEDICINE	11,846	3.237	0.015840
55	CIRCULATION JOURNAL	9,904	3.025	0.016510
56	JOURNAL OF THROMBOSIS AND THROMBOLYSIS	2,789	2.941	0.005860
57	JOURNAL OF CARDIOVASCULAR ELECTROPHYSIOLOGY	7,508	2.910	0.010700
58	Annals of Cardiothoracic Surgery	1,528	2.895	0.004950
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ORIGINAL RESEARCH ARTICLE

Detection of viral SARS-CoV-2 genomes and histopathological changes in endomyocardial biopsies

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Abstract

Aims Since December 2019, the novel coronavirus SARS-CoV-2 has spread rapidly throughout China and keeps the world in suspense. Cardiovascular complications with myocarditis and embolism due to COVID-19 have been reported. SARS-CoV-2 genome detection in the heart muscle has not been demonstrated so far, and the underlying pathophysiological mechanisms remain to be investigated.

Methods and results Endomyocardial biopsies (EMBs) of 104 patients (mean age: 57.90 ± 16.37 years; left ventricular ejection fraction: 33.7 ± 14.6%, sex: *n* = 79 male/25 female) with suspected myocarditis or unexplained heart failure were analysed. EMB analysis included histology, immunohistochemistry, and detection of SARS-CoV-2 genomes by real-time reverse transcription polymerase chain reaction in the IKDT Berlin, Germany. Among 104 EMBs investigated, five were confirmed with SARS-CoV-2 infected by reverse real-time transcriptase polymerase chain reaction. We describe patients of different history of symptoms and time duration. Additionally, we investigated histopathological changes in myocardial tissue showing that the inflammatory process in EMBs seemed to permeate vascular wall leading to small arterial obliteration and damage.

Conclusions This is the first report that established the evidence of SARS-CoV-2 genomes detection in EMBs. In these patients, myocardial injury ischaemia may play a role, which could explain the ubiquitous troponin increases. EMB-based identification of the cause of myocardial injury may contribute to explain the different evolution of complicated SARS-CoV-2-infection and to design future specific and personalized treatment strategies.

Keywords SARS-CoV-2-infection; COVID-19; Endomyocardial biopsy; Myocarditis; Heart failure

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Introduction

In December 2019, a novel coronavirus with potential zoonotic origin, named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was identified as the causative agent of a cluster of suspicious pneumonia cases in Wuhan, Hubei, China. The incredible fast worldwide spread of the coronavirus disease 2019 (COVID-19) prompt the World Health Organization (WHO) to declare COVID-19 as a pandemic on 11 March 2020.[1] More than 1 776 867 confirmed cases of COVID-19 and more than 111 828 fatalities in 185 countries have been attributed to SARS-CoV-2 as of 14 April 2020 (https://who.sprinklr.com/).

Molecular tests (real-time reverse transcriptase polymerase chain reaction, RT-qPCR) were generally used to confirm the clinical diagnosis of COVID-19. A recent report showed

that SARS-CoV-2 could be detected in different types of clinical specimens such as Broncho alveolar lavage, sputum, nasal swabs, feces, blood, and urine.[2] The ubiquitous distribution of the main viral entry receptor angiotensin converting enzyme 2 (ACE2) for SARS-CoV-2 entry into the target cells led to the hypothesis of the involvement of other potential target organs for SARS-CoV-2 besides the respiratory tract, for example, the heart, the liver, the brain, the pancreas, or the kidneys.[3]

Infection with the SARS-CoV-2 is associated with systemic illness by hyper-inflammation.[4] Cardiovascular complications with embolism due to COVID-19 have been reported recently.[5–8] Acute myocardial injury associated with COVID-19 manifested as an increase of high-sensitivity cardiac troponin levels.[1] However, direct SARS-CoV-2 RNA in the heart muscle has not been demonstrated so far. The damage caused by SARS-CoV-2 to the cardiovascular system and the underlying mechanisms remain to be investigated. Accordingly, we prospectively analysed endomyocardial biopsies (EMBs) from a cohort of 104 samples of patients with suspected myocarditis or unexplained heart disease for the presence of SARS-CoV-2 RNA by RT-qPCR and hints for histopathological injury.

Methods

Up to 8 EMBs each of 104 patients [mean age: 57.90 ± 16.37 years; left ventricular ejection fraction (LVEF): 33.7 ± 14.6%, sex: *n* = 79 male/25 female] with suspected myocarditis or unexplained heart failure were analysed between 3 February and 26 March 2020 in German clinical centres in accordance with SARS-CoV2 spread in Germany. EMBs were routinely taken from left ventricle. In 60.4% a hypertrophy was seen according possible due to a cardiac oedema. Coronary artery disease was excluded angiographically in all patients prior to EMB. The suspected diagnosis had been made by clinicians. EMBs were send for further diagnosis to the laboratory IKDT (Institute for Cardiac Diagnostic and Therapy Berlin, Germany). Analysis included histology, immunohistochemistry, and molecular virology. Following EMB extraction, samples were transferred to formalin for histological analyses and to RNAlater™ solution (Thermo Fisher Scientific, Waltham, MA, USA) for immunohistological and molecular analyses. DNA was extracted by Puregene Core Kit A (Qiagen, Hilden, Germany) according to manufacturer's instructions. Total RNA from one EMB was isolated using TRIzol Reagent™ (Thermo Fisher Scientific, Waltham, MA, USA), solubilized in DEPC-H_2O, and treated with DNAse (PeqLab, Erlangen, Germany) to remove any traces of DNA followed by reverse transcription with High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Random hexamer primers (5 μM) were used in addition to specific primers targeting the E-gene of SARS-CoV2 (0.2 μM each). DNA and cDNA concentrations were measured by PCR-based Quantifiler™ Human DNA Quantification Kit (Thermo Fisher Scientific, Waltham, MA, USA) or by expression of housekeeping gene HPRT, respectively. Detection of other cardiotropic viruses (Enteroviruses, Adenoviruses, Human Herpesvirus 6, Epstein-Barr virus, parvovirus B19) using (RT-) qPCR or nested PCR was applied as described elsewhere.[9,10] Commercially available RT-qPCR kits targeting the E-gene and RdRp-gene (TIB MOLBIOL, Roche Diagnostics, Germany) and the assay N2 (N-gene) published by the CDC were chosen to initially screen samples for presence of SARS-CoV2 genomes. As aforementioned assays were proven to be the most robust, RdRp-gene assay was used for confirmation of results.[11]

All RT-qPCR assays were performed using TaqMan Universal PCR MasterMix (Thermo Fisher Scientific, Waltham, MA, USA) in a 20 μL reaction mix consisting of 2× PCR buffer including enzyme mix, primers, and probes concentrations as recommended by manufacturers and 21.5 μL and 5 μL of cDNA. Thermal cycling was carried out as recommended by manufacturers on either ABI QuantStudio 12k flex or BIORAD CFX96 thermal cyclers. In brief, real-time RT-PCR was performed with 45 cycles using 1.5 μL of cDNA. However, for validation of our PCR results additional PCR runs were performed with 5 μL of cDNA not altering the results obtained by the 1.5 μL approach. Synthetic *in vitro* RNA of E-gene and RdRP-gene assays were diluted 1:10 prior to cDNA synthesis and plasmid positive control of N-gene assay was diluted 1:104 prior to RT-qPCR to account for an expected low yield in total RNA extracted from EMB samples.

Histology was developed from formalin-fixed tissue by haematoxylin & eosin (HE); Azan, and Periodic acid–Schiff (PAS) staining in light microscopy. For immunohistological evaluation, specimens were RNAlater fixed, embedded in Tissue Tec (SLEE, Mainz, Germany) and immediately snap-frozen in methyl butane which had been cooled in liquid nitrogen and then stored at −80°C until processing. Embedded specimens were cut into cryosections placed on 10% poly-L-lysine-precoated slides. Myocardial inflammation was diagnosed by $CD3^+$ t-lymphocytes/mm^2 (Dako, Glostrup, Denmark), $CD11a^+$/LFA-1^+ lymphocytes/mm^2 (Immuno Tools, Friesoythe, Germany), $CD11b^+$/Mac-1^+ macrophages/mm^2 (Immuno-Tools, Friesoythe, Germany), $CD45R0^+$ t memory cells (Dako, Glostrup, Denmark), perforin$^+$ cytotoxic cells/mm^2 (BD Bioscience, San Jose, California). In addition, we stained intercellular adhesion molecules and MHC class II cell surface receptor (CD54/ICAM-1 and HLADR, Immunotools, Friesoythe, Germany). Staining were quantified by digital image analysis.[12]

Ethical approval

Approval was not required.

Results

Endomyocardial biopsy results of total patient cohort are summarized in *Table 1*. Out of the 104 EMB samples, five patients were positive for SARS-CoV-2 E-gene specific sequences indicating the first description of SARS-CoV-2 presents in a case series.

Besides latent infection with parvovirus B19, no other viral pathogens were detectable in SARS-CoV-2 positive samples.

Based on the clinical history, the clinicians expressed a suspicion of a previous COVID-19 infection, but they were not tested with throat swab sample during admission to the hospital. The clinical courses of the five patients were different and showed highly acute to mild forms.

Table 1 Clinical characteristics and biopsy findings

Group	All patients
Number of patients, *n* (%)	104 (100)
Men, *n* (%)	79 (76)
Age at diagnosis, mean ± SD (years)	57.9 ± 16.4
LVEF at diagnosis, mean ± SD (%)	33.7 ± 14.6
Diagnosis, *n* (%)	
• Active myocarditis	14 (13.4)
• Inflammatory cardiomyopathy	34 (32.6)
• Borderline myocarditis	3 (2.9)
• Dilated cardiomyopathy	43 (41.3)
• Amyloidosis	10 (9.6)
EMB results	
CD3+ count in EMB Mean ± SD (cells/mm^2)	24.1 ± 54.0
CD45R0+ count in EMB Mean ± SD (cells/mm^2)	87.9 ± 96.4
LFA-1+ count in EMB Mean ± SD (cells/mm^2)	29.9 ± 48.3
Mac-1+ count in EMB Mean ± SD (cells/mm^2)	70.3 ± 106.7
Perforin+ count in EMB Mean ± SD (cells/mm^2)	1.3 ± 3.8
CD54+ count in EMB Mean ± SD (%Area fraction)	2.7 ± 1.5
HLADR+ count in EMB Mean ± SD ((%Area fraction)	4.6 ± 2.0
SARS-CoV-2, *n* (%)	5 (4.8)
B19V, *n* (%)	70 (67.3)
HHV6, *n* (%)	8 (7.7)
ADV, *n* (%)	0 (0.0)
EBV, *n* (%)	4 (3.8)
COX, *n* (%)	1 (1.0)

EMB, endomyocardial biopsy; LVEF, left ventricular ejection fraction. Immunohistological marker: CD3, T-lymphocytes; LFA-1, leukocyte function antigen-1; Mac-1, macrophage-1 antigen; CD45R0 (UCHL1), leucocyte common antigen; perforin, cytotoxic cells; CD54/ICAM-1, intercellular adhesion molecule-1; HLADR, MHC class II cell surface receptor; B19V, Parvovirus B19; HHV6, Human Herpesvirus 6; ADV, Adenovirus; EBV, Epstein–Barr-Virus; COX, Coxsackivirus. The data are presented as mean ± standard deviation.

Patient 1

Patient 1 was a 48-year-old male with newly diagnosed heart failure and significantly reduced systolic function (EF 22%). Suspected diagnosis was acute myocarditis. He described sudden onset of high-grade fever and dyspnoea within a few days. In addition, he suffered from thrombi and embolia. He reported a prior vacation in Tyrol, Austria. This patient showed a highly acute status was admitted to the intensive care unit (ICU) and due to severe infection. The diagnosis of a small-vessel vasculitis was established, and cyclophosphamide and additional steroids were initiated. The patient recovered adequately. After receiving EMB results, immunosuppressive treatment was stopped immediately.

Patient 2

Patient 2 was a 62-year-old male with mildly reduced EF (40%) and moderate LV-hypertrophy, and without respiratory infect. This patient had a new cardiac impairment of LV function since January 2020. The cause was unknown, so a possible myocarditis was assumed. With the exception of cardiac symptoms, this patient had a mild course and did not need to be monitored by ICU.

Patient 3

Patient 3 was a 60-year-old female with heart failure symptoms but preserved EF (60%) with pronounced LV-hypertrophy. Initially, she was admitted to the ICU with severe acute respiratory syndrome. Blood tests revealed elevated levels of markers of myocyte injury (see *Table 2*), which remained positive during the first days of her hospitalization. After respiratory improvement the EMB was carried out 4 weeks after onset of syndromes. In this interesting case, the cardiac symptoms occurred with a pronounced relapse after the initial event.

Patient 4

Patient 4 was a 36-year-old male with a significantly reduced systolic function (EF 25%) with a history of mild respiratory infect 3 weeks ago. The clinical course developed without complications and ICU surveillance. During hospitalization, the levels of troponin decreased laboratory values on day 15 were in reference range, and he recovered during this time.

Table 2 Characteristics of patients

Patient	1	2	3	4	5
Age at diagnosis (years)	48	62	60	36	39
Clinical suspected diagnosis	Acute myocarditis	Unexplained heart failure	Unexplained heart failure	Inflammatory cardiomyopathy	Acute myocarditis
Diagnosis	Active myocarditis	Inflammatory cardiomyopathy	Inflammatory cardiomyopathy	Inflammatory cardiomyopathy	Borderline-myocarditis
Sex	M	M	F	M	M
LVEF at diagnosis (%)	22	40	60	25	55
Laboratory parameters:					
High sensitive Troponin (pg/mL)	3264	-	83	56	379
BNP (pg/mL)	12 232	-	113	258	109
EMB analysis:					
Myocyte diameter (μm)	18	18	32	22	19
CD3+ count in EMB (cells/mm^2)	106.98	7.0	20.54	4.97	18.74
CD45R0+ count in EMB (cells/mm^2)	156.23	14.0	96.15	61.47	162.38
LFA-1+ count in EMB (cells/mm^2)	83.15	-	24.36	16.95	102.6
Mac-1+ count in EMB (cells/mm^2)	155.34	39,5	91.56	49.09	154.35
Perforin+ count in EMB (cells/mm^2)	1.79	-	1.74	0.00	4.01
CD54+ count in EMB (%Area Fraction)	6.42	-	1.91	1.90	4.05
HLADR+ count in biopsy (%Area Fraction)	7.25	0,5	3.78		7.14

EMB, endomyocardial biopsy; LVEF, left ventricular ejection fraction. Immunohistological marker: CD3, T-lymphocytes; LFA-1, leukocyte function antigen-1; Mac-1, macrophage-1 antigen; CD45R0 (UCHL1), leucocyte common antigen; perforin, cytotoxic cells; CD54/ICAM-1, intercellular adhesion molecule-1; HLADR, MHC class II cell surface receptor.

Patient 5

Patient 5 was a 39-year-old male with heart failure symptoms but preserved EF with suspected diagnosis of acute myocarditis. The patient had a history of upper airway infection with headache and fever up to 4 weeks before admission. He suffered from shortness of breath, T-wave inversions in the anterolateral leads in ECG, elevated cardiac troponin I, and cardiac magnetic resonance imaging compatible with myocarditis. The course of this patient was acute and required ICU treatment.

In patients 2–5, treatment strategies were not modified after receiving the result of SARS-CoV-2 RT-qPCR in EMB. They were treated symptomatically, in part with initiation of guideline-directed medication for heart failure.

Patient characteristics and EMB results are summarized in *Table 2*.

Analysis of endomyocardial biopsies

SARS-CoV-2 loads determined in the EMBs were low (Ct values: 36.66 ± 1.99) corresponding to less than 100 to 500 viral copies/reaction. Viral loads were determined from the internal SARS-CoV-2 positive control with a Ct value of 32.73 ± 1.12 corresponding to approximately 10E + 4 copies/reaction while the Ct values of SARS-CoV-2 negative samples were below 40 cycles and thus below detection limit. Results from RT-qPCR are shown in *Table 3*.

Histological assessment of EMBs revealed an active myocarditis according to the Dallas criteria in patient 1[13,14](*Figure 1A*). Histological analysis could also show necrosis of myocytes and interstitial tissue and granulation tissue in the periphery of necrosis of the type observed after an infarction (*Figure 1A*).

Immunohistochemical EMB analysis confirmed pronounced intramyocardial inflammation. Analysis of immune cell infiltrates of SARS-CoV-2 genome positive EMBs showed elevated number of T-cells, macrophages, lymphocytes, and T-memory cells (CD45R0) in four of the five patients (*Figure 1A–C, E, F*). Moreover, all SARS-CoV-2 patients exhibited an elevated number of cell adhesion molecules (CD54/ICAM-1). Patient 2 showed inflammatory response on limit values.

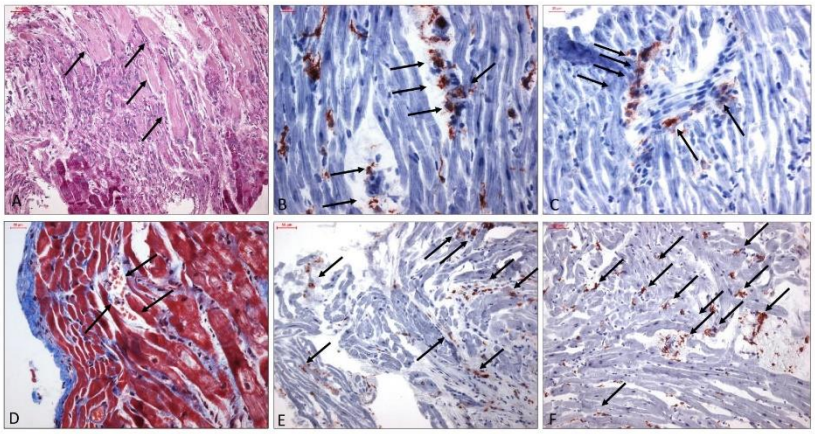
We could show that the inflammatory process in cardiac tissue seemed to permeate vascular wall. The inflammatory process was leading to arterial obliteration and damage (*Figure* 1B–D). The final mechanism of tissue damage in

Table 3 RT-qPCR results of SARS-CoV-2 detection in EMBs

Patient	Ct (E-Gen)
1	36.79 ± 2.48
2	38.65 ± 8,9
3	33.36 ± 1.9
4	37.6
5	36.91 ± 3.7
positive control	32.73 ± 1.12

Ct, threshold cycle; RT-qPCR, quantitative polymerase chain reaction.

Figure 1 (A) Myocardium with recent necrosis (arrows) in the upper part, below granulation tissue with moderate infiltrates of inflammatory cells. At the bottom persisting myocytes. PAS. Bar 50 μm. (B) Immunohistochemical staining of CD11b + macrophages. Vessels are involved in the inflammation process. Bar 20 μm. (C) Perforin positive cells in the neighbourhood of an oblique cut blood vessel (arrow) within the myocardium. Staining by antibody against perforin, hemalum. Bar 20 μm. (D) Five to six myocyte layers below the endocardium ruptured capillary and bleeding (arrow). Azan staining. Bar 20 μm. (E) Increase of T-lymphocytes stained by CD3 antibody. Bar 50 μm. (F) Increase of CD45RO positive T memory cells. Bar 50 μm.



consequence of vascular obliteration appears to be similar to systemic forms of vasculitis leading to ischaemia. The neighbouring myocardium displayed vacuoles in myocytes as a sign of restricted metabolism. Perivascular fibrosis with variation of fibre densities could be seen in cases 2 to 5 (not shown in figures). This phenomenon indicated relicts of previous damage.

Discussion

In this study, we established for the first time the evidence of SARS-CoV-2 genome detection in 5 of 104 EMBs of patients with suspected myocarditis or unexplained heart failure.

After the first cases describing pneumonia of unknown origin in Wuhan, China, SARS-CoV-2 rapidly spread worldwide with critical challenges for the public health and medical communities. Cardiovascular involvement in COVID-19 seems to be a notable complication. First single case reports could show viral particles in interstitial cytopathic macrophages and their surroundings in EMB of a severe COVID-19 shock patient by electron microscopic analyses. Whether direct myocardial injury due to viral involvement or the effect of systemic inflammation appear to be the most common mechanisms responsible for cardiogenic shock situation needs to be further investigated.[19]

In the analytic stage, real-time RT-PCR assays remain the molecular test of choice for the aetiologic diagnosis of SARS-CoV-2 infection. Specificity of E-gene and RdRP-gene assays tested with clinical respiratory samples and SARS-CoV and MERS-CoV did not result in cross-reactivity and false positive results. High sensitivity of both assays as indicated by low PCR limit of detection for purified RNA could also be confirmed for RNA spiked into and extracted from swab samples.[15] However, direct SARS-CoV-2 RNA detection in the myocardium has not been demonstrated so far. Herein, we demonstrated by RT-qPCR that SARS-CoV-2 genomes is present in different cases.

In this study, we described series of different histories of cardiovascular patients admitted to the hospital. One main clinical finding is that cardiac involvement with positive SARS-CoV-2 genomes in EMBs can either occur acutely or with latency after onset of symptoms of infection.

Based on the results of currently published research, it seems important to discuss the manifestations and characteristics of myocardial damage induced by COVID-19.[16] Herewith, we validated the direct cardiac involvement associated with intramyocardial inflammation in patients with SARS-CoV-2 genome positivity in EMBs. In patient 1, we could show an active myocarditis and in patient 5 a borderline-myocarditis according the Dallas criteria. In the remaining patients, an inflammatory cardiomyopathy was determined.

Recent literature data have shown that cardiac Troponin I concentration is increased in all patients with SARS-CoV-2 infection, and values exceeding the 99th percentile in the upper reference limit can be observed in 8–12% of positive cases.[17] Moreover, patients with COVID-19 are known to be

at higher risk of acute pulmonary embolism, and elevated d-dimer levels on admission are predictive of adverse outcomes for patients with COVID-19.[5] The first vascular sign has been referred to as 'vascular thickening' or 'vascular congestion' in the lung. Bai *et al.*[18] reported vascular thickening to be significantly associated with COVID-19 compared with non–COVID-19 pneumonia (59% vs. 22%, $P < 0.001$). The physiopathologic mechanisms behind these changes remain unclear, but their role in diagnosis and possible future treatment strategies is substantial.

In this regard, a very recent report showed that pericytes demonstrating high ACE-2 expression might act as target cells for SARS-CoV-2, while pericyte injury can result in endothelial cell dysfunction.[22] Recent reports showed that besides pericyctes ACE-2 is expressed to different levels also in cardiomyocytes, endothelial cells, fibroblasts, and leucocytes.[23] However, ACE-2 expression does not argue for permissive infection of a respective target cell by SARS-CoV-2. On the other hand, recent reports have demonstrated that SARS-CoV-2 genomes could be detected besides airway epithelium cells also in the intestinal enterocytes, spleen, liver, kidney, and heart.[2,24] In addition, recent histologically post-mortem analyses in COVID-10 positive patients revealed lymphocytic endotheliitis in different organs with evidence of direct viral infection, indicating endothelial dysfunction as a possible principle determination of microvascular dysfunction by shifting the vascular equilibrium towards more vasoconstriction with subsequent organ ischaemia and inflammation.[21] Although nearly all organs seemed to be affected by COVID-19, we currently do not know in-depth details about the organ-specific infection by SARS-CoV-2. In this regard, Tavazzi and coworkers have shown recently in their case description using electron microscopy on EMBs of a patient with COVID-19 in cardiogenic shock that SARS-CoV-2 particles could be localized to interstitial macrophages and their surroundings but not in cardiomyocytes.[19] As to whether this observation is due to a transient viraemia or infected macrophage migration from the lung has to be evaluated. Our finding of SARS-CoV-2 genome detection in EMBs of patients suffering from myocarditis/inflammatory cardiomyopathy cannot rule out or confirm the infection of cardiac cells but revealed incremental insights into organ-specific infection of SARS-CoV-2 using possibly macrophage migration as a shuttle from the lung to the heart.

In this study, we investigated histopathological changes in myocardial tissue in the series of SARS-CoV-2 positive EMBs. In line with the recently published study, we could show that the inflammatory process in EMBs seemed to permeate vascular wall leading to small arterial obliteration. The final mechanism of tissue damage in consequence of vascular obliteration appears to be similar to systemic forms of vasculitis. We therefore hypothesize that in these patients, myocardial injury ischaemia may play a role, which could explain the ubiquitous troponin increases. As a result, this ischaemia could trigger possible cardiac arrhythmias.

Limitation of study

A limitation of this study is that we did not had enough material for SARS-CoV-2 genome in depth analysis to certainly exclude cross reaction with other corona virus strains due to the limited material available by the EMBs. However, the high sensitivity and specificity of the used PCR systems to detect solely SARS-CoV-2 genomes have been demonstrated recently.[15]

Another limitation is that we cannot completely exclude that the detection of SARS-CoV-2 genomes in the heart might result from contamination of circulating blood. Unfortunately, we have no blood samples to the corresponding EMBs on hand to analyse this aspect. However, SARS-CoV-2 load in blood seemed to be low in comparison with other clinical types of specimens.[2]

Nevertheless, SARS CoV-2 can potentially bind to its cellular ACE2 receptor in heart tissue cells and can therefore be detected in the heart muscle. In this regard, a recent report showed that pericytes in the heart demonstrating high ACE-2 expression might act as target cells for SARS-CoV-2 while pericyte injury can result in endothelial cell dysfunction.[20,22] If SARS CoV-2 can replicate in these target cells of the heart, this has to be investigated in subsequent analysis. The low detection rate and low viral loads of SARS-CoV-2 genomes may be due to the limited number, size, and quantity of EMBs. Heart tissue cells (e.g. pericytes) are not the main target cells of SARS-CoV-2 while specimens of the main target the lung of infected patients are easier and in larger quantity to obtain than EMBs and may contribute to the low detection rate in EMBs. However, we showed that SARS-CoV-2 is detectable in the heart muscle but can only speculate about the clinical relevance of SARS-CoV-2 infection of the heart. As to whether SARS-CoV-2 infection may induce myocarditis is questionable, however, may trigger an ongoing progress to myocarditis of other reason.

In conclusion, in this study, we could show for the first-time evidence of SARS-CoV-2 genome detection in 5 of 104 patients with suspected myocarditis or unexplained heart failure with different history of symptoms and time duration. In addition to inflammation and consequential damage, one possible histopathological mechanism may be vascular involvement with arterial obliteration which can lead to ischaemia. A possible SARS-CoV-2 infection should therefore be considered in patients with acute unexplained heart failure or new cardiac arrhythmias. We believe that recognition by the scientific community of myocarditis as a possible complication associated with COVID-19 may be helpful for strict monitoring of affected patients. EMB-based identification of

the cause of myocardial injury may contribute to explain the different evolution of complicated SARS-CoV-2-infection and to design future specific treatment strategies. An antiviral therapy is not yet available. Based on our histopathological results, possible anticoagulant/antiaggregation therapy should be investigated.

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Conflict of interest

None declared.

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5.4 Curriculum vitae

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

5.5 Complete list of publications

Table 2. Complete list of publications (2018 - 2021)

Escher F, Pietsch H, Aleshcheva G, Wenzel P, Fruhwald F, Stumpf C, Westermann D, Bauersachs J, Enseleit F, Ruschitzka F, Nägele H, Laugwitz KL, Haake H, Frey N, Brachmann J, Huber K, Braun-Dullaeus RC, Bergmann MW, Strotmann J, Grönefeld G, Krülls-Münch J, Westenfeld R, Skurk C, Landmesser U, Pieske B, Gross UM, Morawietz L, Schultheiss HP. *Evaluation of Myocardial Gene Expression Profiling for Superior Diagnosis of Idiopathic Giant-Cell Myocarditis and Clinical Feasibility in a Large Cohort of Patients with Acute Cardiac Decompensation.* J Clin Med. 2020 Aug 19;9(9):2689. doi: 10.3390/jcm9092689. PMID: 32825201; PMCID: PMC7563288.

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Baumeier C, Escher F, Aleshcheva G, Pietsch H, Schultheiss HP. *Plasminogen activator inhibitor-1 reduces cardiac fibrosis and promotes M2 macrophage polarization in inflammatory cardiomyopathy*. Basic Res Cardiol. 2021 Jan 11;116(1):1. doi: 10.1007/s00395-020-00840-w. PMID: 33432417.

Schultheiss HP, Bock T, Pietsch H, Aleshcheva G, Baumeier C, Fruhwald F, Escher F. *Nucleoside Analogue Reverse Transcriptase Inhibitors Improve Clinical Outcome in Transcriptional Active Human Parvovirus B19-Positive Patients.* J Clin Med. 2021 Apr 29;10(9):1928. doi: 10.3390/jcm10091928. PMID: 33946917.

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