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Direttore: Chiar.mo Prof. Federico Rea

Tesi di laurea

GENETIC CHARACTERIZATION OF BIOPSY-PROVEN MYOCARDITIS:

A PROSPECTIVE STUDY

Relatrice: *Chia.ma Prof.ssa Alida Linda Patrizia Caforio*

Correlatrice: *Chia.ma Prof.ssa Kalliopi Pilichou*

Laureando

Tommaso Latella

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A Sara,

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LIST OF ABBREVIATIONS

ACE: Angiotensin Converting Enzyme

ACM: Acute Cardiomyopathy

AHA: Anti-Heart Antibodies

AIS: Autoinflammatory Syndrome

AML: Acute Myeloid Leukemia

ANA: Anti-Nucleus Antigens

ANCA: Anti Neutrophil Cytoplasm Antibodies

AOSD: Audlt-Onset Still Disease

ARB: Angiotensi Receptor Blockers

ARVC: Arrhythmogenic Right Ventricular Cardiomyopathy

BNP: Brain Natriuretic Peptide

CAR: Coxasckievirus Adenovirus Receptor

CCC: Cronic Chagas' Cardiomyopathy

CMR: Cardiac Magnetic Resonance

CMV: Citomegalovirus

CRP: C Reactive Protein

CRT: Cardiac Resynchronization Therapy

CVID: Common Variable Immunodeficency

DALY: Disability-Adjusted Life Year

DCM: Dilated Cardiomyopathy

DES= Desmin

DSP= Desmoplakin

EBV: Ebstein Barr Virus

ECG: Elettrocardiogram

ECV: Extracellular Volume

EDD: End Diastolic Diameter

EDV: End Diastolic Volume

EF: Ejection Fraction

EGE: Early Gadlinium Enhancement

EGPA: Eosinophilic Granulomatosis with Poliangiitis

ELISA: Enzyme-Linked Immunosorbent Assay

EMB: Endomyocardial Biopsy

ESC: European Society of Cardiology

FAC: Fractional Area Change

FLNC=Filamin C

GCM: Giant Cells Myocarditis

GWAS: Genomic Wide Assciation Study

HES: Hyper Eosinophilic Syndrome

HF: Heart Faliure

HHV6: Human Herpesvirus 6

HIV: Human Immunodeficiency Virus

HLA: Human Leucocyte Antigen

ICD: Implantable Cardioverter Defibrillator

IQR: Interquartile Range

IT: Immunotherapy

IVIG: Intravenus Immunglobulins

LGE: Late Gadolinium Enhancement

LV: Left Ventricle

MG: Myasthenia Gravis

MINOCA: Myocardial Infarction with Non-Obsructive Coronary Arteries

MRA: Mineralcorticoid Receptor Antagonist

MYBPC3= Myosin Binding Protein C3

MYH7= MutY Homolog7

NYHA: New York Heart Association

PCR: Polymerase Chain Reaction

PET: Positron Emission Tomography

PID: Primary Immunodefincy

P/LP: Pathologic/Likely Pathologic

PVB19: Parvovirus B19

RA: Reumathoid Arthritis

SID: Sistemic Immune-mediated Disease

SLE: Sistemic Lupus Erythematosus

SNP: Single Nucleotide Polymorphism

SS: Sistemic Sclerosis

TAPSE: Tricuspid Annular Plane Systolic Excursion

TMPT: Thiopurine S-methyltransferase

TNFα: Tumor Necrosis Factor alpha

TNNT= Troponin T2

TTE: Transtoracic ecography

TTN= Titin

VAD: Ventricular Assistence Device

WHO: World Health Organization

XLA: X-linked Agammaglobulinemia

ABSTRACT (italiano)

Presupposti dello studio: La miocardite è una patologia infiammatoria del miocardio, definita da criteri istologici, immunologici ed immunoistochimici, ed è caratterizzata da eterogeneità eziologica (principalmente virale o autoimmune) e clinica. Il gold standard diagnostico è la biopsia endomiocardica (BEM), ma le linee guida internazionali ammettono anche una diagnosi di esclusione di miocardite clinicamente sospetta. Una predisposizione poligenica alla miocardite è già stata dimostrata. Sebbene recenti studi abbiano investigato il ruolo diagnostico e prognostico di singole mutazioni in coorti composte prevalentemente da pazienti con miocardite clinicamente sospetta, tale aspetto non è ancora stato specificamente indagato nei pazienti con miocardite biopticamente provata.

Scopi dello studio: 1) Determinare la prevalenza di varianti patogene/probabilmente patogene (P/LP) di un subset di 174 geni correlati alle cardiomiopatie in una coorte prospettica di pazienti con miocardite biopticamente provata; 2) indagare un possibile ruolo diagnostico e prognostico dell'analisi genetica nella miocardite biopticamente provata.

Materiali e metodi: A partire dal luglio 2020, sono stati sottoposti ad analisi genetica i pazienti con miocardite biopticamente provata in follow-up presso l'ambulatorio di Cardioimmunologia del nostro Centro, di cui sono state registrate le principali caratteristiche anamnestiche, cliniche, bioumorali e strumentali alla diagnosi e ad ogni follow-up ambulatoriale. Il test genetico prevedeva il sequenziamento mediante Next Generation Sequencing delle regioni codificanti di 174 geni associati alla Cardiomiopatia Dilatativa, Aritmogena ed Ipertrofica; sono state considerate esclusivamente le varianti classificate come P/LP. I dati clinici sono stati confrontati tra pazienti positivi (gen+) e negativi (gen-) al test genetico. Le variabili continue sono state confrontate mediante i test di Wilcoxon o Kruskal–Wallis, e le variabili categoriche mediante i test di χ^2 e Fisher. Visto il basso numero di eventi cardiovascolari maggiori durante il follow-up, sono stati individuati due outcome compositi: classe funzionale definita secondo la New York Heart Association (NYHA)>I e/o frazione di eiezione ventricolare sinistra (FEVS) <50% durante tutto il follow-up (outcome primario), o solamente all'ultimo follow-

up (outcome secondario). La sopravvivenza libera dagli outcome compositi è stata valutata mediante il metodo di Kaplan–Meier e mediante modelli di Cox.

Risultati: Sono stati prospetticamente inclusi nello studio 84 pazienti (età mediana 46 anni, 50 maschi) affetti da miocardite biopticamente provata. Sono state identificate varianti P/LP in 16 pazienti (19%): 8 del gene Titina, 3 del gene Desmoplachina e una variante ciascuno dei geni Troponina T2, Filamina-C, Proteina Legante la Miosina C3, Desmina e Catena Pesante della Miosina 7. La presenza di varianti geniche mutate non ha influenzato né il tipo di presentazione clinica (la presentazione tipo scompenso cardiaco è stata la prevalente in entrambi i gruppi, 44% sia in gen+ sia gen-, p=0.9) né la classe funzionale alla diagnosi (NYHA I nel 50% dei casi in gen+ vs 44% gen-, p=0.2). La FEVS alla diagnosi era ridotta in entrambi i gruppi (44% gen+ vs 33% gen-, p=0.5). Il tipo istologico prevalente era miocardite linfocitaria in entrambi i gruppi (78% gen -vs 56% gen+, p=0.14), soprattutto virus-negativa (87% gen- vs 82% gen+, p>0.9). Una proporzione simile di pazienti di entrambi i gruppi è stata sottoposta a terapia immunosoppressiva (49% gen- vs 75% gen+, p=0.056). All'ultimo follow-up, la FEVS era normalizzata in entrambi i gruppi (55% gen-vs 54% gen+, p=0.4). Solo un paziente gen+ è stato sottoposto a trapianto cardiaco durante il follow-up, e nessuno è deceduto; non sono state rilevate differenze nella sopravvivenza libera dagli outcome compositi tra i due gruppi (p=0.2 per l'outcome primario e p=0.62 per l'outcome secondario).

Conclusioni: Nel nostro studio, sebbene il 19% dei pazienti affetti da miocardite biopticamente provata abbia presentato una positività per varianti P/LP dei geni associati alle cardiomiopatie, le mutazioni non sembrano aver esercitato alcuna influenza sul fenotipo della malattia. Infatti, la presenza di mutazioni genetiche non ha determinato alcuna differenza nelle caratteristiche cliniche, strumentali o istologiche alla diagnosi o al follow-up rispetto ai casi geneticamente negativi.

ABSTRACT

Background: Myocarditis is an inflammatory disease of the myocardium, defined by histological, immunological and immunohistochemical criteria, and characterized by etiological heterogeneity (mainly viral or autoimmune) and clinical variability. The gold standard for diagnosis is represented by endomyocardial biopsy (EMB), and, when it is not performed, international guidelines allow for a diagnosis of exclusion as clinically suspected myocarditis. A polygenic predisposition to myocarditis has already been demonstrated. Although recent studies have already investigated the diagnostic and prognostic role of single-gene mutations in cohorts of patients predominantly with clinically suspected myocarditis, this issue has not been specifically investigated in patients with biopsy-proven myocarditis.

Study aims: 1) To determine the prevalence of pathogenic/likely pathogenic (P/LP) variants in a subset of 174 genes associated with cardiomyopathies in a prospective cohort of biopsy-proven myocarditis patients. 2) To investigate the potential diagnostic and prognostic role of genetic analysis in biopsy-proven myocarditis.

Materials and Methods: Starting from July 2020, patients with biopsy-proven myocarditis who were followed up at the Cardioimmunology outpatient clinic of our Centre underwent genetic analysis. The main anamnestic, clinical, biomarker, and instrumental characteristics at diagnosis and each outpatient follow-up were recorded. The genetic test consisted of Next Generation Sequencing of the coding regions of 174 genes associated with Dilated, Arrhythmogenic, and Hypertrophic Cardiomyopathy. Only variants classified as P/LP were considered. Clinical data were compared between patients that tested positive (gen+) or negative (gen-) at the genetic test. Continuous variables were compared using the Wilcoxon or Kruskal-Wallis tests, and categorical variables were compared using the Chi-square and Fisher's tests. Due to the low incidence of major cardiovascular events during the follow-up period, two composite outcomes were identified: New York Heart Association functional class (NYHA)>I and/or left ventricular ejection

fraction (LVEF) <50% throughout the entire follow-up (primary outcome), or only at the last follow-up (secondary outcome). The survival free from the composite outcomes was evaluated through the Kaplan-Meier method and Cox models.

Results: Eighty-four patients (median age 46 years, 50 males) with biopsy-proven myocarditis were prospectively included in the study. P/LP variants were identified in 16 patients (19%): 8 in the Titin gene, 3 in the Desmoplakin gene, and one variant each in the Troponin T2, Filamin-C, Myosin Binding Protein C3, Desmin, and Myosin Heavy Chain 7 genes. The presence of gene variants did not influence either the type of clinical presentation (heart failure was the predominant presentation in both groups, 44% in both gen+ and gen-, p=0.9) or the functional class at diagnosis (NYHA class I in 50% of cases in gen+ vs. 44% in gen-, p=0.2). Left ventricular ejection fraction (LVEF) was reduced in both groups (44% in gen+ vs. 33% in gen-, p=0.5). The predominant histological type was lymphocytic myocarditis in both groups (78% in gen- vs. 56% in gen+, p=0.14), mainly virus-negative (87% in gen- vs. 82% in gen+, p>0.9). A similar proportion of patients in both groups received immunosuppressive therapy (49% in gen- vs. 75% in gen+, p=0.056). At the last follow-up, LVEF was normalized in both groups (55% in gen- vs. 54% in gen+, p=0.4). Only one gen+ patient underwent cardiac transplantation during the follow-up period, and no one died; no difference was observed in the survival free from the two composite outcomes between the two groups (p=0.2 for the primary outcome and p=0.62 for the secondary outcome).

Conclusions: In our study, although 19% of patients with biopsy-proven myocarditis presented a P/LP variant in cardiomyopathy-associated genes, these mutations did not appear to exert any influence on the disease phenotype. In fact, the presence of genetic mutations did not result in any difference in the clinical, instrumental, or histological features at diagnosis or during follow-up compared to gene negative cases.

1.INTRODUCTION

1.1 Myocarditis

Myocarditis is an inflammatory disease of the myocardium that can be caused by a wide range of infectious and non-infectious agents. The inflammatory process mainly affects the heart muscle cells, but it can extend to all the structures of the heart including the pericardium and the endocardium. Myocyte damage may lead to impaired contractile heart function and, in some cases, acute or chronic heart failure. Clinical presentation of myocarditis can range from pauci-symptomatic to severe symptoms, such as dyspnea, chest pain and/or arrhythmias. Myocarditis diagnosis is complex, since symptoms may be similar to other heart diseases (1). The diagnostic gold standard is endomyocardial biopsy (EMB), and, when this is not performed, the 2013 ESC criteria allow a diagnosis of clinically suspected myocarditis, mainly based on exclusion of coronary artery disease by coronary angiography and typical cardiac magnetic resonance (CMR) findings (2). Myocarditis management depends on the severity of the symptoms. Treatment may include immunosuppressive/immunomodulating drugs, inotropic and antiarrhythmic agents, as well as the general management of acute and chronic heart failure. In some severe cases, heart transplantation (3) and the implantation of ventricular assist devices (VAD) may be necessary (4,5).

Historically, myocarditis has been considered a rare and poorly understood entity for a long time, with a difficult clinical, diagnostic and therapeutic approach. Conversely, current knowledge shows that myocarditis is not a rare disease, but its diagnosis is still complex, due to its heterogeneous clinical presentation, lacking pathognomonic clinical and instrumental findings. In fact, its symptoms can simulate the onset of numerous non-inflammatory heart syndromes, such as ischemic heart disease when presenting with chest pain, or dilated cardiomyopathy, when presenting as acute or chronic heart failure. In the absence of EMB, myocarditis is a diagnosis of exclusion and not of certainty. However, EMB is an invasive procedure that is recommended with higher priority for cases judged to be at high risk (2,6,7). The prognosis is equally heterogeneous, ranging from spontaneous “restitutio ad integrum” to progressive heart failure, death or heart

transplantation. Although the peak incidence is in young males, myocarditis is a hardly predictable condition that can affect any age. Despite the heterogeneity and complexity of the topic, in recent decades myocarditis is becoming increasingly central in cardiology. The interest and the amount of scientific research in myocarditis have considerably increased, leading to a better definition of the diagnostic and pathogenetic aspects of the disease, along with new therapeutic approaches, mainly in the field of cardioimmunology (8,9).

1.1.1 Historical perspective on the definition of myocarditis

The concept of myocarditis has evolved over time reflecting advances in the progressive understanding of the pathogenesis, clinical aspects and therapeutic approaches of this disease. The following is a brief review of the key points in the history of myocarditis research:

- 1837: The term "myocarditis" is coined by Jean Cruveilhier, a French pathologist who described inflammation and necrosis in the heart muscle of patients dying of rheumatic fever (10).
- 1899: Paul Ehrlich, a German pathologist, demonstrates that the injection of diphtheria toxin in animal models causes myocarditis and proposes a similar mechanism for the human disease (11,12).
- 1900s and 1920s: Numerous studies report the presence of lymphocytic infiltrates in the myocardium of patients with heart failure, rheumatic fever and influenza, suggesting that an inflammatory component might be related to myocardial damage (11,13).
- 1930s and 1940s: Histopathology and electron microscopy techniques are developed which will be later used to obtain a more detailed characterization of the cellular and subcellular changes in myocarditis (13–16).
- 1950s and 1960s: Improved immunological and virological techniques allow to identify viral causative agents in myocarditis, including Coxsackie B virus, adenovirus, and cytomegalovirus. Thanks to these technologies, the production of autoantibodies directed against myocardial antigens is

demonstrated, favoring the hypothesis of the presence of an autoimmune component in the pathogenesis of the disease (16,17).

- 1970s and 1980s: Introduction of non-invasive imaging diagnostic techniques, such as ultrasound and scintigraphy, allows the study of myocardial dysfunction and of the abnormalities in myocardial perfusion in the course of myocarditis. At the same time, there is improvement in the EMB technique using King's bioptome (18). Woodroof JF et al. in 1982 presents a publication with a modern and comprehensive approach to myocarditis and the proposal of an etiopathogenetic classification (19).
- 1990s and 2000s: The use of EMB in the clinical field is widespread, and EMB becomes the gold standard for myocarditis diagnosis due to the ability to detect viral genomes and immune cells directly within the heart tissue. At the same time the use of CMR develops as a non-invasive means for clinically suspected myocarditis diagnosis and follow-up. Neu et al. report a mouse model of autoimmune myocarditis induced by immunization with cardiac myosin (20). Cooper et al. perform a retrospective multicentric study demonstrating the effectiveness of immunosuppressive therapy in giant cell myocarditis (21).
- 2010s and beyond: Research continues in investigating pathogenic mechanisms and therapeutic targets in new areas such as the role of innate immunity, self-inflammation and modulation of cardiac metabolism.
- The concept of myocarditis has evolved over time from a mere descriptive term for inflammatory changes in the heart muscle to a complex pathological reality with a multiform presentation, etiology and pathophysiology. This process of refinement and understanding has been made possible by the research and contribution of numerous clinicians and scientists by means of histopathological, immunological, virological, radiological and clinical studies. Multidisciplinary collaboration is now favored by the creation of global data registers and international collaboration groups, e.g. the Myocardial and Pericardial Working Group of the ESC (European Society of Cardiology), founded in 2007 to allow data

sharing, standardization of protocols, drafting common guidelines and consensus documents and finally dissemination of knowledge.

1.1.2 Definitions and classifications

1.1.2.1 Dallas criteria

The first definition of myocarditis was provided by the Dallas criteria in 1987 by a group of American pathologists to standardize the pathological diagnosis of myocarditis and to establish a clear definition of the disease for research and clinical purposes. These criteria were elaborated based on literary review, discussion and consensus among experts in the field of cardiovascular pathology. According to these criteria, myocarditis is defined as an inflammatory disease of the heart muscle characterized by the presence of inflammatory infiltrates in the myocardium in association with myocytic lesions and/or necrosis not typical of myocardial infarction (22). These criteria are based on the evaluation of a first biopsy compared to a subsequent one, in order to allow comparative analysis of the evolution in time of histological aspects. Based on the histopathological finding at the first biopsy, myocarditis can be diagnosed as:

- Active myocarditis with/without fibrosis, i.e presence of inflammatory infiltrate and necrosis/degeneration of cardiomyocytes not typical of myocardial infarction.
- Borderline myocarditis: presence of inflammatory infiltrate in the absence of necrosis/degeneration of cardiomyocytes.
- Absence of myocarditis: both inflammatory infiltrate and necrosis/degeneration of cardiomyocytes are missing.

Histopathological evaluation at the control biopsy defines pathology as:

- Persistent myocarditis with/without fibrosis.
- Healing myocarditis with/without fibrosis.
- Healed myocarditis with/without fibrosis.

1.1.2.2 WHO/ISFC Report 1995

Later on, the 1995 report by the WHO/ISFC (World Health Organization/International Society and Federation Cardiology) refines the definition of myocarditis offered by the Dallas classification by adding immunological and immunohistochemical parameters in addition to histology alone.

Myocarditis is defined as an inflammatory disease of the myocardium diagnosed by histological, immunological and immunohistochemical criteria, on endomyocardial biopsy. Inflammatory cardiomyopathy is diagnosed when there is association of myocarditis and heart dysfunction.

For the first time, myocarditis is included among the specific cardiomyopathies. This 1995 document also states that the diagnosis of myocarditis must be based on the combination of clinical, laboratory, imaging and histological findings when possible (23).

1.1.2.3 AHA 2006 and ESC 2008 Definitions

The importance of the clinical context and non-invasive investigations is highlighted in the subsequent definitions proposed in parallel by the AHA (American Heart Association) in 2006 and by the ESC in 2013 (2,24). These definitions present a more comprehensive and multidisciplinary approach to the diagnosis of myocarditis. The 2013 ESC definition defines myocarditis as:

"An inflammatory myocardial disease diagnosed by established histological, immunological and immunocytochemical criteria"

In both the ESC and AHA documents, myocarditis is included in the classification of cardiomyopathies. The AHA classification system categorizes myocarditis according to both clinical and histological criteria into acute, fulminant, lymphocytic, giant cell, eosinophilic and mixed.

1.1.2.4 ESC Position Statement 2013

The 1995 WHO/ISFC definition was later expanded by the 2013 Position Statement of the ESC Working group on Myocardial and Pericardial Diseases, with the addition of molecular analysis for the viral genome on EMB among the diagnostic

tools. In fact, the detection of molecular nucleic acids of a viral pathogen allows to achieve an etiological diagnosis of infectious myocarditis (25). The ESC 2013 document reinforces the WHO/ISFC definition of biopsy-proven myocarditis and the concept that EMB provides both a diagnosis of certainty as well as of myocarditis etiology; in addition, it provides a new rigorous definition of clinically suspected myocarditis to refine the probability of myocarditis in cases that do not undergo EMB.

1.1.2.5 ESC guidelines 2021

Finally, in 2021 the European Society of Cardiology published the most recent guidelines for the diagnosis and treatment of heart failure; a special section is dedicated to the management of heart failure patients with certain or suspected myocarditis (26).

Compared to the 2013 Position Statement by the ESC Working group on Myocardial and Pericardial Diseases, the 2021 ESC Guidelines provide more comprehensive and detailed recommendations, in particular with regard to the use of non-invasive imaging techniques such as CMR and the role of EMB in the diagnosis of myocarditis. In fact, the role of BEM has been confirmed as fundamental for myocarditis diagnosis, especially in some peculiar clinical scenarios: literary review, discussion and consensus among experts in the field of cardiovascular pathology. According to these criteria, myocarditis is defined as an inflammatory disease of the heart muscle characterized by the presence of inflammatory infiltrates in the myocardium. Such criteria require the presence of inflammatory infiltrates in the myocardium in association with myocytic lesions and/or necrosis (22). These criteria are based on the evaluation of a first biopsy compared to a subsequent one, in order to allow comparative analysis of the evolution in time of the histological aspects. Based on the histopathological finding at the first biopsy, myocarditis can be diagnosed as:

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1.1.2.2 WHO/ISFC Report 1995

Later on, the 1995 report by the WHO/ISFC (World Health Organization/International Society and Federation Cardiology) enhanced the definition of myocarditis offered by the Dallas by adding the immunological and immunohistochemical parameters in addition to the histology alone.

Myocarditis has therefore been defined as an inflammatory disease of the myocardium diagnosed through histological, immunological and immunohistochemical criteria, found on EMB. Inflammatory cardiomyopathy is diagnosed when there is association of myocarditis and heart dysfunction.

For the first time, myocarditis was also included among specific cardiomyopathies. This 1995 document also states that the diagnosis of myocarditis must be based on the combination of clinical, laboratory, imaging and histological findings when possible (23).

1.1.2.3 AHA 2006 and ESC 2008 Definitions

The importance of the clinical context and non-invasive investigations were highlighted in the subsequent definitions proposed in parallel by the AHA (American Heart Association) in 2006 and by the ESC in 2008 (17). These definitions present a more comprehensive and multidisciplinary approach to the diagnosis of myocarditis. The 2008 ESC definition defines myocarditis as:

"An inflammatory myocardial disease diagnosed by established histological, immunological and immunocytochemical criteria, or by clinical and imaging criteria in the absence of histology"

In both the ESC and AHA documents, myocarditis is included in the classification of cardiomyopathies. The ESC document provided a probabilistic diagnosis divided in three categories: certain, probable and possible myocarditis diagnosis. In contrast, the AHA classification system categorized myocarditis according to both clinical and histological criteria into acute, fulminant, lymphocytic, giant cell, eosinophilic and mixed.

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Table I. Recommendations for endomyocardial biopsy (EMB) in patients with suspected myocarditis according to 2021 ESC HF guidelines (26).

Recommendations for endomyocardial biopsy	**Class of recommendation**
In case of acute/ fulminant myocarditis with progression or persistent cardiac dysfunction and/or malignant ventricular arrhythmias and/or atrioventricular block without expected response to standard treatment during first <1-2 weeks.	I
In patients with exacerbation of HF despite optimal treatment when there is a suspicion of specific diagnosis which can be confirmed in myocardial samples.	IIa
EMB is especially recommended in patients with acute and/or chronic HF and suspected giant cell-, eosinophilic-, ICI-related and/or lymphocytic myocarditis, vasculitis, sarcoidosis, SLE, and other auto-immune conditions.	I

Legend: HF-heart failure, ICI-immune checkpoint inhibitor, SLE-systemic lupus erythematosus.

1.2 Histopathologic classification

EMB is crucial for the proper diagnosis, management and prognostic stratification in patients with clinically suspected myocarditis. It provides a direct visualization of myocardial tissue and allows the detection of inflammatory cells, fibrosis and other pathological aspects. These data are essential to determine the etiology and clinical severity and to establish and etiology-directed therapy. In addition, EMB can help differentiate myocarditis from other heart conditions with similar clinical presentations, such as dilated cardiomyopathy, Takotsubo cardiomyopathy, etc. To date it is ascertained that EMB carries a relatively low risk of complications, especially when performed by experienced operators in high flow centers (7).

1.2.1 Dallas criteria

Historically, histological characterization of myocarditis has been based on the aforementioned Dallas criteria (22). These criteria presented several issues and have been set aside by the most recent classifications (27). Critical aspects of these criteria included: the absence of immunohistochemistry assessment of inflammatory cells and a quantitative assessment of myocardial fibrosis; the type and extent of myocardial damage were not been further specified; inflammatory infiltrates other than lymphocytes were not mentioned (i.e., eosinophilic, polymorphic, giant cell and granulomatous myocarditis); a diagnosis of healed myocarditis was not feasible at the first biopsy, but only when unequivocal myocarditis had been previously diagnosed; the term "borderline myocarditis", which also includes chronic forms, remained ambigous; any reference to etiological agents was missing; finally, a relevant inter-operator variability in pathological interpretation was allowed.

1.2.2 Immunoistopathologic classification

Histological analysis can be corroborated with additional immunohistochemical and molecular analyses, in order to overcome the critical aspects of the Dallas criteria. Routine immunohistochemistry analysis is mandatory in conjunction with histological analysis (2) and should involve the use of a recommended antibody panel, in order to characterize the cellularity of the inflammatory infiltrate. The molecular investigation, through PCR, allows the detection of viral pathogens in the sample. The combination of immune-histochemistry and PCR with histological analysis has greatly increased the sensitivity and specificity of EMB diagnosis, as well as greater reproducibility and a reduced inter-operator variability. Based on histological evidence (22), combined with immune-histopathological analysis, myocarditis can be defined as:

- Active (acute) myocarditis: detection of ≥ 14 leukocytes/mm2, of which up to 4 monocytes per mm2, with a number of CD3-positive T cells ≥ 7 cells/mm2, and presence of necrosis and/or degeneration of cardiomyocytes, while fibrosis may be present or absent (25,28–31).
- Chronic myocarditis: ≥ 14 leukocytes/mm2, of which up to 4 monocytes per mm2, with a number of CD3-positive T cells ≥ 7 cells/mm2, in the

absence of necrosis or degeneration of cardiomyocytes; fibrosis is often present.

- Inflammatory cardiomyopathy: ≥ 14 leukocytes/mm2, of which up to 4 monocytes per mm2, with a number of CD3-positive T cells ≥ 7 cells/mm2, with modification of tissue architecture in a cardiomyopathic sense, with or without concomitant fibrosis.

Taking into account all these parameters characterizing the inflammatory infiltrate of myocarditis, subtypes of this pathology can be identified with precise prognostic and therapeutic implications (1). Below is an overview of the most relevant myocarditis types according to histological, immunohistochemical and microbiological parameters.

1.2.3 Lymphocytic myocarditis

Lymphocytic myocarditis is the most common form of myocarditis. It is an acute inflammatory cardiac disease with variable etiology. It is frequently of viral, autoimmune or toxic origin. The inflammatory infiltrate is mostly made up by CD3+ T lymphocytes and, to a lesser extent, by CD68+ macrophages; few neutrophils, plasma cells and eosinophils may also be present. The disease begins as acute myocarditis according to immune-histopathological criteria, with presence of cardiomyocyte damage and necrosis, regardless of the degree of fibrosis, that may be absent or present. Subsequently, the clinical evolution may be towards a chronic form, characterized by the presence of scar fibrosis, with the permanence of the leukocyte infiltrate but in most cases in absence of necrosis.

1.2.4 Giant cells Myocarditis

GCM is a rare and aggressive form of myocarditis that is characterized by the presence of giant multinucleate cells in the myocardium, which can lead to severe and rapidly progressive heart failure. The exact cause of GCM is unknown, but it is believed to be an autoimmune disorder due to the prevalence of autoimmune and autoinflammatory extracardiac comorbidities and the frequency of recurrence after heart transplan (32). It is a rare disease that affects both men and women of all ages, but is most commonly diagnosed in middle-aged adults. The incidence of

GCM is estimated to be less than 1 case per million population per year. The clinical presentation of GCM is highly variable, ranging from acute to severe and fulminant heart failure. Patients with GCM may have symptoms of heart failure such as dyspnea, fatigue, and peripheral edema. Other symptoms may include chest pain, syncope, and unremitting arrhythmias. It often occurs in the form of fulminant myocarditis, with rapid progression towards cardiogenic shock and need of hemodynamic support (33). These patients are characterized by poor response to supportive cardiological therapy and a particularly poor prognosis. The diagnosis of GCM is based on EMB histological examination, which shows the presence of giant multinucleate cells in the myocardium. Widespread myocardial involvement accounts for the high diagnostic sensitivity of EMB. The characteristic histological findings include an extensive leukocyte infiltrate with predominance of myeloid line cells, often accompanied by the presence of eosinophil granulocytes and T-lymphocyte infiltration in the absence of defined granulomas (34). Other diagnostic tests, such as echocardiography, electrocardiography, and CMR, can be used only to support diagnosis. The prognosis of GCM is generally poor, with high mortality both in the short and long term. Patients with GCM often have a rapid decline in heart function and many require an urgent heart transplant. The overall 5-year survival rate for GCM patients is estimated to be less than 20%. Mortality is often linked to frequent complications that include heart failure, arrhythmias, sudden heart death, and the need for a heart transplant. The most relevant is the evolution to dilated cardiomyopathy with heart failure, attested in up to 80% of cases (35). Treatment of GCM requires prompt combination immunosuppressive therapy, such as corticosteroid, mycophenolate mofetil and cyclosporine, to suppress the immune system and reduce inflammation in the myocardium (36). In selected cases, intravenous immunoglobulin therapy or plasmapheresis have also been be used (37). Patients with severe heart failure may require mechanical circulatory support, such as a ventricular assist device, or a heart transplant. In the absence of immunosuppressive therapy, patients show a median survival of three months, while in patients undergoing proper immunosuppression the time between onset of symptoms and cardiac transplantation lengthens up to a year.

1.2.5 Sarcoidosis

Sarcoidosis is a multisystemic granulomatous disease of unknown etiology characterized by the formation of non-caseating granulomas. The disease mainly affects the lungs, but other organs may be involved, including the heart, liver, spleen, eyes and skin. Sarcoidosis diagnosis is based on a combination of clinical, radiological and histological findings (38,39). Cardiac involvement is a rare complication of sarcoidosis, occurring in up to 25% of affected patients (40), and in some cases it may exist in the form of isolated cardiac sarcoidosis (41)Etiology appears to be immune-mediated and seems to involve an interaction between exposure to environmental agents, genetic predisposition, and dysfunction of the immune system, in particular a dysfunction of antigen-presenting cells (APCs). Cardiac sarcoidosis can present with a wide range of symptoms, including atrioventricular block, ventricular arrhythmias, heart failure, and sudden death. Timely identification and treatment of cardiac involvement in sarcoidosis is critical to reduce morbidity and mortality. According to current guidelines, the diagnosis of suspected sarcoidosis can also be made on the basis of a combination of clinical, laboratory and instrumental criteria; cardiac imaging plays a fundamental role, and in this sense the use of RMC and PET total-body has both a diagnostic and prognostic role and for monitoring response to medical treatment(42).

Differential diagnosis for cardiac sarcoidosis includes other heart diseases that may present with similar symptoms, such as other forms of granulomatous myocarditis, dilated cardiomyopathy, and right ventricular arrhythmogenic cardiomyopathy. In other forms of granulomatous myocarditis differential histological diagnosis should include possible infectious forms (i.e. mycobacterial infections), and should consider the nature of granulomas, excluding caseous necrosis, and a comprehensive immunohistochemistry analysis. It is important to perform a thorough assessment to rule out other possible causes of heart dysfunction before making a diagnosis of cardiac sarcoidosis. In addition, it is important to distinguish between cardiac sarcoidosis and pulmonary hypertension, which can also be associated with sarcoidosis. A careful assessment of pulmonary pressures and right heart function is necessary. In conclusion,

sarcoidosis is a granulomatous disease that can involve many organs, including the heart. Cardiac sarcoidosis may be associated with significant morbidity and mortality and is characterized by the presence of non-caseous granulomas in various parts of the heart. Histological examination at EMB or post-mortem is essential for the diagnosis of cardiac sarcoidosis; in case of negative histological examinations, or isolated heart involvement, a presumptive diagnosis can be made based on a combination of clinical findings, laboratory and instrumental. Timely identification and treatment of the disease is crucial to prevent adverse outcomes (43).

1.2.6 Eosinophilic myocarditis

Eosinophilic myocarditis is a rare form of myocarditis that may be idiopathic or more often associated with forms of peripheral hypereosinophilia, such as idiopathic hypereosinophilic syndrome, eosinophilic granulomatosis with polyangiitis (EGPA, formerly known as Churg-Strauss syndrome), allergic reactions, parasitic infections, some neoplastic and autoimmune diseases. It is a potentially life-threatening condition that can lead to heart failure, arrhythmias, and sudden death if not recognized and promptly treated. The clinical presentation of eosinophilic myocarditis varies widely, from asymptomatic to fulminant heart failure. Patients may experience chest pain, dyspnea, fatigue, palpitations, and signs of heart failure, such as edema and jugular distension. The clinical presentation of eosinophilic myocarditis is highly variable, ranging from paucisymptomatic forms in which dyspnea, chest pain and fever occur, to fulminant forms characterized by acute heart failure and ventricular arrhythmias. Patients with mild disease and prompt adequate treatment have a good prognosis, while most may face complications and poor prognosis. Among the most important complications are progressive heart dysfunction, cardiogenic shock, thromboembolic events, arrhythmias and sudden death. A rare and severe form of eosinophilic myocarditis has been reported, namely acute necrotizing, characterized by marked eosinophilic infiltration, myonecrosis and interstitial fibrosis. It is considered a medical emergency, as it can rapidly evolve into heart failure and death if left untreated.

Diagnosis is usually suspected on the basis of clinical presentation and confirmed by serum biomarkers, electrocardiography, imaging findings and biopsy. Laboratory tests frequently reveal elevated levels of eosinophils in the blood and other signs of systemic inflammation, such as elevation of C-reactive protein erythrosedimentation rate. The electrocardiogram may show non-specific changes in the ST-T wave or evidence of arrhythmias. Imaging studies, such as echocardiography, CMR, or PET can reveal abnormalities in the structure or function of the heart. Histological examination of EMB samples is the reference standard for the diagnosis of eosinophilic myocarditis, as for all types of myocarditis. Typical findings include patchy myocardial infiltration of perivascular interstitial eosinophilic granulocytes. In addition to eosinophils, other white blood cells such as lymphocytes, plasma cells, and macrophages may be present. Instead of a precise entity, eosinophilic myocarditis is better described as a continuous spectrum of disease that ranges from hypersensitivity myocarditis to systemic hypereosinophilic syndrome (44). The latter is characterized by peripheral hypereosinophilia (>1500 cells/µl) lasting at least six months with concomitant multi-organ damage mediated by eosinophils. The physiopathological mechanism through which eosinophilic inflammation causes tissue damage is the release of systemic and local mediators such as pro-inflammatory cytokines, cationic proteins, and free radicals. Hypereosinophilic syndrome is characterized by three phases: an acute phase with inflammation and necrosis and thrombosis of the damaged myocardium; a subsequent subacute phase with the formation of thrombosis within the damaged myocardium; finally, a fibrotic phase accompanied by severe atrioventricular valve regurgitation and restrictive cardiomyopathy. The terminal stage of the disease is in fact called Loeffler's endocarditis.

The presence of eosinophilic infiltrates in other organs, such as the lungs, skin, or gastrointestinal tract, may support the diagnosis of hypereosinophilic syndrome. Treatment of eosinophilic myocarditis usually involves immunosuppressive therapy, such as corticosteroids, to suppress the immune response and reduce inflammation. In severe cases, additional treatments may be needed, such as intravenous immunoglobulin, plasmapheresis, or cytotoxic agents; recently, some

reports cite the possible "off label" use of biological drugs such as Mepolizumab, a monoclonal antibody directed against IL-5 (45). Depending on the clinical characteristics of the patient and the state of the disease, various regimens of therapy against heart failure are associated with specific immunosuppressive therapy (46).

1.3 Epidemiology

Myocarditis is a significant cause of morbidity and mortality worldwide but its actual incidence and prevalence are difficult to define. This is due to the heterogeneity of clinical presentations of the disease, and the poor frequency of performance of EMB to get a diagnosis of certainty. In the United States, a population study conducted in Olmsted County, Minnesota, found that the annual incidence of clinically myocarditis was 1.5 cases per 100,000 individuals, with increased incidence among males and younger age groups (47). In Denmark, a more recent national population study yielded comparable results, with a peak incidence of 20 to 29 years (48). In Japan, another population study found an incidence rate of 1.06 cases per 100,000 people-year, with a higher incidence among males and younger age groups (49). Myocarditis can affect individuals of all ages, with some subgroups having a higher incidence. In pediatric populations, DCM (Dilated Cardiomyopathy) is a major cause of heart failure and a significant proportion of cases are due to myocarditis (50). The multicenter Myocarditis Treatment Trial, conducted in the 1990s, enrolled adult patients with myocarditis confirmed by the biopsy and found that the average age of patients was 39, with a male predominance and a high proportion of patients showing symptoms of heart failure (51). Gender differences in myocarditis have also been reported by other sources, with some studies suggesting a higher incidence among males (52). A systematic analysis of the global burden of disease found that clinically suspected myocarditis was responsible for about 1.5 million disability-adjusted life years (DALY) in 2013, with the highest DALY rates observed in high-income countries (53). In this study the overall annual incidence of myocarditis was estimated to be about 22 in 100000 cases, but this may be an overestimation as no distinction has been made between biopsy-proven acute myocarditis or inflammatory cardiomyopathy and other cardiomyopathies. Myocarditis is

described in the literature with a variable incidence depending on the population under examination, with estimates between 0.2% and 12% in cases of post-mortem diagnosis. In particular, autopsy studies conducted on young adults who died suddenly show a prevalence of myocarditis ranging between 2% and 42%. In 2010, a review by Blauwet and Cooper reported an overall prevalence of 0.5% - 5% of myocarditis in consecutive autopsies (54).

1.4 Etiology

Myocarditis is a disease with heterogeneous etiology. One of the most common causes of myocarditis is viral infection, including coxsackievirus, herpes simplex virus, adenovirus and parvovirus b19. Other infectious agents, such as bacteria, fungi, and parasites, may also cause myocarditis, but they are uncommon. Apart from infections, myocarditis can be caused by toxic substances, such as alcohol, cocaine, and some chemotherapy drugs. In addition, myocarditis can arise as a result of an autoimmune reaction, due to a pathologic immune response directed against self cardiac antigens. This form of myocarditis may exist as isolated organ-specific disease, or be present in the context of systemic immune mediated diseases, such as systemic lupus erythematosus (SLE), systemic scleroderma, EGPA, etc. Understanding the etiology of myocarditis is important in order to treat it effectively. In the next section, the causes of myocardial inflammation will be investigated, focusing on infectious and viral causes, but also on toxic and autoimmune causes.

1.4.1 Infectious myocarditis

Several infectious agents can cause acute myocarditis and inflammatory cardiomyopathy, such as viruses, bacteria, parasites and fungi. In general, viral etiology is presumed to be the most frequent, while other infectious causes have a much lower incidence. However, a geographic variability exists: viruses are detected as the most frequent causative agent in European and North American countries, while in Central and South America a higher incidence of Chagas disease, caused by the protozoan Trypanosoma Cruzi, is reported.

1.4.1.1 Viral Myocarditis

Historically, enteroviruses and adenoviruses were the most common causes of viral myocarditis. However, research of viral genetic material on EMB samples is reporting an increased positivity to Parvovirus B19 (PVB19) and Human Herpesvirus 6 (HHV-6); this increase in the prevalence of such viruses may be due to the increased incidence of PVB19 and Herpesvirus infections in children and their subsequent persistence in the body (55). Other herpesviruses, including Epstein-Barr virus (EBV) and cytomegalovirus (CMV), have also been implicated in the development of myocarditis. HIV-associated myocarditis is usually associated with severe immunodeficiency in advanced stages of the disease. Infections by hepatitis C, influenza A, and B viruses have also been occasionally associated with myocarditis. Finally, it is estimated that about 30% of patients have a mixed infection, i.e. more than one virus is concomitantly found in the myocardium (56).

Based on their tropism, viruses are classified into: primary cardiotropic viruses, which directly infect cardiomyocytes and which can be eliminated by the immune response (for example, adenoviruses and enteroviruses); vasculotropic viruses, which are able to infect endothelial cells (PVB19); and lymphotropic viruses, which may persist in the body throughout the life of the individual, belonging to the family of Herpesviridae (HHV6, CMV, EBV). Some viruses can cause myocarditis indirectly, activating the immune response, including HIV, HCV, Influenza virus A and B (7). It is important to make a distinction between myocarditis induced by viral infection and myocarditis associated with infection. In the first case, for example with Coxsackievirus, viral replication itself causes cellular damage. In the second case, myocardial damage may be caused by molecular mimicry between viral antigens and myocardial proteins, which directs the immune response to self expressed antigens in the heart (57).

1.4.1.1.1 PVB19 myocarditis

The etiological role of PVB19 in the development of myocarditis is not yet clear, since it seems that myocardial damage is caused both by a direct viral action, and by the immune response directed against self antigens. Also the detection of viral DNA in healthy subjects (58) suggests that in such hearts the virus simply has an innocent bystander role (59). The cutoff to consider viral etiology from PVB19 has

been set at 500 copies per microgram cardiac DNA detected on biopsy samples (60).

1.4.1.1.2 HIV myocarditis

HIV is related to the development of myocarditis and dilated cardiomyopathy, although the virus does not have a direct cytotoxic action against cardiomyocytes. The origin of the clinical picture seems to be linked to co-infections, anti-retroviral therapy and the inhibition of cardiac contractility caused by type 1 HIV gp120 glycoprotein (61). In particular, Toxoplasma Gondii is the opportunistic pathogen that most frequently causes myocarditis in HIV-positive subjects, but also Coxsackievirus B3, EBV, CMV and HIV-1 may be involved. Another pathogenetic mechanism in the onset of cardiomyopathy may be the reduction of contractile function induced by the immune system: HIV infection leads to increased production of pro-inflammatory cytokines, such as TNF-α, which stimulates cardiomyocyte apoptosis and reduces inotropism. It is believed that an autoimmune phenomenon can also contribute to cardiomyopathy. In fact, HIV-positive patients with left ventricular dysfunction have higher levels of autoantibodies directed against cardiac antigens such as myosin (62).

HAART (Highly Active Antiretroviral Therapy) has been developed to treat HIV infection, but has also been shown to be effective in preventing HIV-associated viral myocarditis. This can reduce the prevalence of cardiomyopathy following HIV infection by 30% if accessible and properly administered (63).

1.4.1.1.3 HCV myocarditis

HCV myocarditis is one of the most common extrahepatic complications of HCV infection and can evolve into DCM. Some studies have also suggested that the presence of HCV in the myocardial tissue may result in chronic inflammation and influence the gene expression of myocytes, leading to long-term structural and functional changes. In addition, the genetic predisposition, in particular specific Human Leukocyte Antigen (HLA) might play a role in individual susceptibility to HCV myocarditis and in its evolution into DCM (64).

1.4.1.1.4 Influenza Virus Myocarditis

Influenza virus serotypes A and B can rarely cause myocarditis with an indirect physiopathological mechanism with immune-mediated damage. However, this mechanism is still poorly understood (65).

1.4.1.2 Bacterial myocarditis

There are many bacterial species that can lead to myocarditis development, although this etiology is uncommon. Cell damage can be caused by the direct action of bacteria such as streptococci, staphylococci, meningococci and listerie, or through the cytotoxic effect mediated by toxins, as in the case of infection with Corynebacterium diphtheriae and clostridia. Infection originates from sepsis progression or less frequently from bacterial endocarditis (54). The bacterial pathogens most frequently involved in the etiology of myocarditis are Staphylococcus (S.) aureus and Streptococcus pyogenes (66). In particular, S. aureus has been associated with severe infections and high mortality in patients with bacterial myocarditis (67).

Borrelia burgdorferi, the bacterium responsible for Lyme disease, may be implicated in the etiology of myocarditis. Lyme carditis, characterized by severe inflammation of the myocardium and endocardium, represents a rare but potentially lethal complication of Lyme disease. In these patients, myocarditis may manifest as complete heart block or as heart failure (68). In general, the bacterial etiology of myocarditis has been poorly studied compared to viral infections. While viruses can cause myocarditis by direct and indirect mechanisms, the pathogenesis of bacterial myocarditis remains largely unknown. However, the early identification of the pathogen causing the infection is of paramount importance for the choice of appropriate antibiotic treatment.

1.4.1.3 Parasitic myocarditis

Myocarditis can also be caused by parasitic infections of which the most frequent is Chagas disease, caused by the protozoan parasite Trypanosoma cruzi. Chagas disease is endemic in Central and South America, infecting about 6-7 million people and becoming the leading cause of heart failure and heart transplantation in Brazil (69,70). Infection with Trypanosoma Cruzi develops in three stages.

During the acute phase, EMB shows a high incidence of myocarditis, while in the latent phase, even if asymptomatic, cardiac involvement can be detected by electrocardiographic and echocardiographic evaluation. In the chronic phase, Chagas' cardiomyopathy develops, characterized by heart failure, aneurysmatic dilatation of the cardiac apex, ventricular arrhythmias and sudden death (71,72). The pathogenesis of the disease appears to involve various mechanisms, including autonomic nervous system dysfunction, microcirculation alterations and direct myocardial damage caused by the parasite, but the most important seems to be the damage mediated by the immune response due to molecular mimicry (73). The latter leads to the production of cross-reactive autoantibodies between parasite proteins and heart proteins, such as the myosin heavy chain and the β1 adrenergic receptors, which can be detected in increased concentrations in the sera of patients suffering from chronic Chagas disease (73).

1.4.2 Toxic myocarditis

Myocarditis can also be caused by toxic substances, including drugs and alcohol, heavy metals, radiation, insect bites and stings, and snake, spider and scorpion venom. The direct toxicity of these substances can cause myocarditis by inducing inflammation in the heart muscle, leading to death of myocardial cells and fibrosis. In addition, hypersensitivity disorders can also cause myocarditis, where the body's immune system mistakenly attacks heart tissue in response to exposure to toxic substances.

1.4.2.1 Drug hypersensitivity myocarditis

One of the most common causes of toxic myocarditis is drug-induced myocarditis. Various medications, such as antibiotics, antipsychotics, chemotherapy agents, and non-steroidal anti-inflammatory drugs, have been associated with the development of myocarditis. Hypersensitivity myocarditis is caused by a delayed allergic reaction to certain drugs; it results in a picture of eosinophilic myocarditis that often responds to the sole discontinuation of the drug, although in some cases it is necessary to make use of corticosteroids (54). In addition, another recently recognized rare form of drug-associated immune-mediated myocarditis is related to immune checkpoints inhibitors such as Pembrolizumab, which may

occur either in isolated forms or systemic multiorgan involvement (myocarditis, pancreatitis, etc.)(74,75).

1.4.2.2 Alcoholic myocarditis

The risk of developing alcoholic cardiomyopathy is related to both the average alcohol daily intake and the duration of the drinking habit, but a relevant individual susceptibility to the toxic effect of alcohol exists. Most patients with alcoholic cardiomyopathy have been drinking more than 80 g/day for more than 5 years. The clinical diagnosis of alcoholic cardiomyopathy reflects the coexistence of overall myocardial dysfunction in a large drinker where no other cause for myocardial disease has been found. In studies on alcoholic cardiomyopathy, myocarditis (lymphocytic infiltrate in association with myocyte degeneration or focal necrosis) has been found in about 30% of cases. Patients with alcohol abuse and myocarditis have a poor prognosis. Prevention of further alcohol intake, specific treatment of myocarditis and heart failure are necessary to reverse myocardial damage and increased susceptibility to infections (76).

1.4.2 Heavy metals myocarditis

In addition to medications and alcohol, various environmental toxins and poisons have also been linked to myocarditis. For example, heavy metals, such as lead and arsenic, have been associated with myocarditis due to their direct toxicity to the heart muscle. A case report described the development of myocarditis in a patient exposed to high levels of arsenic from agricultural sources (77).

1.4.3 Autoimmune myocarditis

Autoimmune myocarditis may manifest as an isolated autoimmune disease or in the context of an autoimmune syndrome in which extra-cardiac involvement is clinically relevant. According to molecular studies, autoimmune etiology is recognized if no infectious genetic material is detected in the biopsy sample and all other known causes are excluded. In general, an autoimmune disease develops as a result of the interaction between genetic and environmental factors, when the immune system fails to recognize endogenous antigens as self, leading to a loss of tolerance. The definition of organ-specific autoimmunity refers to the

Rose-Witebsky criteria, divided into major and minor criteria. To be defined as such, an autoimmune disease must meet at least two major criteria (78).

1.4.3.1 Rose-Witebsky Criteria

In human myocarditis, more than one of the major Rose-Witebsky criteria (Table II) are met, in particular:

- presence of a mononuclear inflammatory infiltrate, abnormal expression of HLA class II and/or endothelial adhesion molecules in the myocardium in the absence of infectious agents or other known causes (29,79,80);
- circulating autoantibodies to organ-specific autoantigens in the patient and his/her healthy relatives;
- autoreactive autoantibodies and/or lymphocytes bound to myocardial tissue;
- identification and isolation of organ-specific autoantigens involved;
- disease induced in animal models through immunization with autoantigens and/or passive transfer of serum, purified autoantibodies and/or lymphocytes;
- efficacy of immunosuppressive therapy in myocarditis with proven autoimmune pathogenesis.

In addition to these, most minor criteria are met.

Table II. Rose-Witebsky criteria met in autoimmune myocarditis/CMD (adapted from Caforio ALP (1)*).*

Major criteria
Mononuclear infiltrate and abnormal expression of HLA in the myocardium in the absence of infectious agents or other known causes
Circulating autoantibodies detectable in patients and asymptomatic relatives
Autoreactive autoantibodies and/or lymphocytes in the context of the myocardium

Identification and isolation of the organ-specific autoantigens involved	
Disease reproducible in animal models through immunisation with autoantigens, transfer of serum, purified autoantibodies and/or lymphocytes	
Efficacy of immunosuppressive therapy in biopsy-proven myocarditis	
Minor criteria	
Common to all autoimmune diseases	Family aggregation
	Association with certain HLAs
	Clinical course with acute phases and remissions
	Associated autoimmune diseases in he patient or his relatives
Peculiar to organ-specific autoimmune diseases	Autoantibodies directed against organ-specific autoantigens
	Immunopathogenesis mediated by type II, IV, V, VI reactions
	Induction to the production of autoantibodies induces organ-specific disease
	Transfer of autoantibodies leads to transfer of disease/phenotype

Legend: HLA= Human Leucocyte Antigen.

1.4.3.2 Autoantibodies

Numerous types of autoantibodies have been detected and studied in patients with myocarditis and DCM. Functional effects of some autoantibodies isolated from patients have been demonstrated in vitro, particularly those directed against

the β1-adrenergic receptor, isolated in patients with idiopathic DCM (81,82); in addition, post-myocardial DCM was reproduced in animal models immunized with cardiac antigens such as myosin, troponin I, β1-adrenergic and M2-muscarinic receptors (31,33–40). Transfer of the immune components responsible for the development of myocarditis and DCM from one animal model to another has been shown to induce pathological cardiac changes (81,83–87). Finally, an improvement in cardiac morphology and function was achieved by removing, by immunoadsorption, autoantibodies directed against the β1-adrenergic receptor (88,89).

1.4.3.2.1 Anti-heart antibodies

Autoimmunity has been implicated in the development of myocarditis, and autoantibodies, in particular AHA, play a crucial role in pathogenesis, diagnosis and prognosis of the disease (90). These autoantibodies can be detected in the serum of myocarditis patients and are associated with a more severe clinical course and worse prognosis (91). Autoantibodies can be classified as organ-specific or not organ-specific, depending on antigenic specificity. Organ-specific autoantibodies recognize antigens expressed only in a specific organ or tissue, while non-specific antigens of organ autoantibodies are expressed in various tissues.

AHA are a type of autoantibodies that recognize cardiac antigens, including myosin, troponin, and other proteins expressed in heart tissue. AHA have been used as diagnostic and prognostic markers in myocarditis. In one study, the presence of AHA was detected in 56% of patients with biopsy-proven myocarditis and 26% of patients with clinically suspected myocarditis, and much less in healthy controls (92). In addition, the presence of AHA has been associated with deterioration of the systolic and diastolic function of the left ventricle in patients with chronic myocarditis (93). In asymptomatic relatives of patients with dilated cardiomyopathy, the presence of AHA was found to be a predictor of the development of the disease (94,95). The pathogenetic role of AHA in myocarditis is not fully understood. Studies suggest that AHA may directly compromise myocardial contractility by binding to cardiac antigens and inducing complement

activation and cell-mediated cytotoxicity (91,96). Other studies suggest that AHA may indirectly contribute to myocardial damage by inducing an inflammatory response and promoting autoimmunity (97,98).

1.4.3.2.1.1 Specificity AHA patterns

To test the specificity of AHAs, standard indirect immunofluorescence (s-IFI) tests were performed on muscle tissue samples from the atrium, ventricle and intercostal muscle of blood group 0 subjects. The cardiospecificity of the diffuse pattern has been confirmed through absorption studies on homogenates of human atrium, skeletal muscle and mouse liver. Three different patterns were detected at the IFI (97):

- Organ-specific AHA: they show a spread cytoplasmic pattern over both atrial and ventricular myocardial tissue, more intense in the former, and a negative pattern on skeletal muscle tissue. In all sera tested, these were antibodies of IgG class.
- Cross-reactive AHA types 1 or partially organ-specific: they show a striated fluorescence on heart tissue, less extensive in the ventricle than in the atrium, and a negative or weakly positive pattern on the striated muscle. Again, they were antibodies of IgG class.
- Cross-reactive AHA type 2: they are completely cross-reactive with skeletal muscle tissue and show a striated pattern on both samples.

The molecular targets of organ-specific AHA are the isoforms α and β of the heavy myosin chain and are detected by immunoblotting and ELISA (92,93,96,98). They are considered organ-specific since the isoform α is expressed only at the atrial level. Loss of myosin tolerance can be caused by molecular mimicry mechanisms, cross-reaction between myosin and the β1-adrenergic receptor, or cell necrosis caused by infection or other noxae. Some *in vitro* studies have shown a direct effect of AHA on the heart, in particular a negative inotropic effect (91) and a deterioration in heart function (96).

For asymptomatic relatives of myocarditis/DCM patients with positive autoantibodies, AHA research can identify subjects at risk of developing DCM. One

study showed that AHA positivity is an independent predictor of DCM development in asymptomatic relatives at five years of follow-up (94). The AHA-ultrasound combination has been shown to have a higher positive predictive value in recognizing progression to DCM or a preclinical form than the two examinations conducted in isolation (94). In early preclinical DCM, autoantibodies are positive in the absence of echocardiographic abnormalities, while in advanced preclinical DCM, AHA and at least one of the other autoantibodies described in DCM are positive in the absence of echocardiographic alterations. Finally, in the late preclinical phase, in addition to positivity at least one antibody is present as well as left ventricular enlargement (LVE) or depressed fractional shortening (dfs) at standard transthoracic echocardiography (TTE). As for asymptomatic AHA-negative relatives and without echocardiographic alterations, 98% of subjects do not develop any form of progression at five years, but, since organ-specific autoimmune diseases have a slow progression, studies over longer periods of time are needed to confirm this result (95). According to the ESC Working Group on Myocardial and Pericardial Disease, the dosage of AHA at "time zero" and during follow-up in asymptomatic relatives is therefore recommended, while non-invasive cardiac screening by ECG and echocardiography should be more frequent in subjects with positive AHA (25,99).

1.4.3.2.2 Other antibodies

Many other types of antibodies are involved in the complex physiopathological mechanism of myocarditis:

- anti-intercalated disk (AIDA): They are considered organ-specific autoantibodies, since they are directed against intercalated discs, structures exclusively present in the heart. Immunofluorescence (IFI) reveals a characteristic linear pattern. The antigens to which they are directed have not yet been precisely defined. They have been found to be more frequent in patients with myocarditis/DCM and patients with recurrent idiopathic acute pericarditis (100).

- Anti endothelial cells antibodies (AECA): on IFI they are detected by staining the capillary walls in the context of the heart muscle. Their presence was found in EMB-proven myocarditis and DCM (95).
- Muscarinic M2 anti-receptor antibodies: were detected in 39% of subjects with CMD and only in 7% of healthy controls (101).
- β-adrenergic anti-receptor antibodies: some studies have detected IgG directed against such receptors that involved an important inhibitory activity; these antibodies were found in 30-75% of patients with DCM, 37% of patients with other non-inflammatory cardiological diseases and 18% of healthy subjects (102,103). Such antibodies were detected, by using synthetic antigens similar to the β1-adrenergic receptor, in 31% of subjects with DCM, in 12% of healthy subjects and in none of the subjects with other diseases (104). These antibodies had a positive chronotropic effect in vitro and their effect was inhibited by β-blocker drugs. Stimulatory β1-adrenergic receptor antibodies were found in 96% of patients with myocarditis, 26-95% of patients with CMD, 8-10% of controls with ischemic heart disease and 0-19% of healthy controls (82,87,95,105,106,106–114).
- Anti-troponin antibodies: direct antibodies against troponins I (TnI) and T (TnT) have been detected; TnI is thought to be an organ-specific antigen, but studies that can confirm the specificity of such antibodies for myocarditis/DCM compared to ischemic heart disease are still lacking (88,115).
- Anti-HSP antibodies: heat shock proteins are proteins produced by the cell under stress conditions; in patients with CMD autoantibodies directed against the HSP-60 and HSP-70 isoforms were found (116,117).
- Anti-Na-K-ATPase antibodies: using Na-K-ATPase derived from porcine cerebral cortex, antibodies directed against this protein have been shown to be present in 26% of CMD subjects and 2% of healthy controls and independently associated with sudden death. The same authors hypothesized that such antibodies led to electrical instability of the cell membrane due to the reduced activity of Na-K-ATPase and the altered current of calcium (118).

- Antibodies anti mitochondrial antigens: Antibodies directed against several mitochondrial antigens have been described, including the protein M7, the protein ANT (adenine nucleotide translocator) and the protein BCKD-E2. In particular, anti-M7, belonging to the IgG class, were detected in 31% of patients with DCM, in 13% of patients with myocarditis and in 33% of controls with hypertrophic cardiomyopathy, but not in controls with other cardiological diseases, immune systems and healthy controls. Moreover, thanks to immunoadsorption studies, it has been demonstrated that the M7 autoantigen is organ-specific (119).

In conclusion, AHA play a crucial role in the pathogenesis, diagnosis and prognosis of myocarditis. These autoantibodies are associated with the severity and outcome of the disease and can be used as diagnostic and prognostic markers in clinical practice. Further studies are needed to clarify the pathogenic mechanisms of AHA in myocarditis and to develop therapies aimed at modulating the immune response in these patients.

1.4.4 Myocarditis within systemic immune-mediated diseases

Systemic immune-mediated diseases (SID) are a heterogeneous group of clinical conditions that share an immunological pathogenesis and include autoimmune, granulomatous, vasculitic and autoinflammatory diseases (120,121). Cardiac involvement in SID, although often underestimated or unrecognized, is quite common and can produce severe clinical pictures with adverse prognosis (122–124). Myocarditis is a characteristic of SID, although coronary vessels, heart valves and pericardium may be involved (120).

1.4.4.1 Systemic lupus erythematosus

SLE is an autoimmune syndrome caused by the deposition of immune complexes and complement proteins that stimulate an inflammatory reaction, and can theoretically affect any organ, even if the joints, serous membranes, skin and kidneys are the most frequently affected. At the heart level it is often seen in the form of pericarditis, endocarditis, coronary artery disease and myocarditis. The latter was found in about 8% of autopsies, with a incidence rate greatly reduced

compared to the past probably in relation to improvements in immunosuppressive therapy (125); in a study of 29 SLE patients, myocarditis preceded SLE diagnosis in 58.6% of cases (126). Recognition of myocarditis is difficult, especially if an SLE diagnosis has not yet been established. In addition to the clinical signs and symptoms listed above, the diagnostic process may also use CMR to visualize myocarditis-related abnormalities in the very early stages of SLE. Assessment of the coronary artery circulation, which is designed to rule out severe coronary artery disease, and EMB, the gold standard for diagnosis, are also important for differential diagnosis. High doses of corticosteroids may be used in combination with other immunosuppressive drugs, known as steroid-sparing drugs.

1.4.4.2 Systemic sclerosis

*Systemic sclerosis (*SS) is a rare immune-mediated chronic disease with a progressive and irreversible evolution, which is characterized by generalized thickening of the skin and other soft tissues due to increased collagen deposition (127). Myocardial involvement is primarily related to microcirculation dysfunction and the presence of pulmonary hypertension (128), but myocarditis may also develop in the framework of SS-related systemic myositis (129). The incidence of myocarditis in active SS is estimated to be less than 5%, but the risk may be greater in patients with diffuse skin disease or severe interstitial lung disease (120). Patients with SS-associated myocarditis typically present with symptoms such as chest pain, dyspnea, and palpitations. Diagnosis can be difficult, as symptoms may be non-specific and ECG may not show abnormalities (130). Treatment typically involves aggressive immunosuppression (25,131–134).

1.4.4.3 Rheumatoid arthritis

In rheumatoid arthritis (RA), myocarditis is a rare but potentially life-threatening complication. RA is an autoimmune inflammatory disease that affects the joints but can also have an extra-articular involvement. The incidence of myocarditis in RA is estimated to be less than 1%, but the risk may be greater in patients with severe disease or extra-articular manifestations. The main effect at the cardiovascular level is an acceleration of the atherosclerotic process. At the myocardial level, an inflammatory process is possible in 3-30% of patients,

although studies based on CMR suggest that the prevalence of myocarditis associated with RA is higher (135). Patients with RA-associated myocarditis may experience symptoms such as chest pain, dyspnea, and palpitations. Diagnosis can be difficult, as symptoms may be nonspecific and the ECG may show abnormalities. Early diagnosis and treatment are essential to improve clinical outcomes in these patients (136–138).

1.4.4.4 Inflammatory myopathies

This is a heterogeneous group of diseases that first affect the skeletal muscle in which myocardial involvement is possible. Myocarditis was found in 30% of autopsies (139), while at EMB it is possible to diagnose both lymphocytic and giant cell myocarditis (140–142). Cardiac involvement in a picture of inflammatory myopathy is often related to worse prognosis and is an indication to enhance standard immunosuppressive therapy.

1.4.4.5 Sarcoidosis

As described above, sarcoidosis is a disease with unknown etiology characterized by the formation of granulomas in different organs. Cardiac involvement varies from case to case and may reach up to 25% of patients (40,143), Moreover, the incidence varies according to ethnicity; it has been more frequently observed in the Japanese population (50-80% of autopsies of patients with sarcoidosis had heart involvement) than in Caucasians (13-20%)(122). Cardiac sarcoidosis can occur at any stage of the disease, regardless of the involvement of other organs, and should be suspected in the presence of unexpected new-onset symptoms, such as arrhythmias, heart failure, reduced cardiac function at cardiac imaging, although sudden cardiac death may be the first clinical manifestation. In the active phase of systemic sarcoidosis an increased concentration of blood angiotensin converting enzyme (ACE) and of blood and urinary levels of calcium and phosphates can be found. For diagnosis, imaging techniques such as CMR and PET are used; EMB differentiates sarcoidosis from other diseases such as GCM and other granulomatous diseases, but because of myocardial focal involvement, EMB has low sensitivity. Therapy is based on corticosteroids possibly associated with other immunosuppressive drugs, antiarrhythmic and HF drugs. In the case of

major arrhythmias, it is often necessary to implant a pacemaker or defibrillator. In cardiac sarcoidosis prognosis is poor, with a 5-year mortality rate of about 25%. Treatment with immunosuppressive agents can improve clinical outcome (144).

1.4.4.6 Eosinophilic granulomatosis with polyangiitis

EGPA is a necrotizing vasculitis that affects small and medium-sized vessels, mainly at the level of the respiratory system, with granuloma formation, often associated with positivity to anti-neutrophil cytoplasmic antibodies (ANCA). A higher incidence of myocardial involvement is described in ANCA-negative EGPA. Myocardial damage results from infiltration and subsequent degranulation by eosinophils (46). The most frequent picture is of endomyocardial fibrosis; about 20% of patients develop an inflammatory cardiomyopathy (145), which is associated with a worse prognosis.

1.4.4.7 Granulomatosis with polyangiitis

Formerly known as Wegener's granulomatosis, granulomatosis with polyangiitis (GPA) is an ANCA-positive vasculopathy mainly affecting small and medium-sized vessels of the respiratory tract, but can also give lesions in the kidney and brain. Cardiac involvement is varied, affecting different structures, and rather uncommon, being found in about 3.3% of patients. However, echocardiographic studies conducted with speckle-tracking showed a reduction in systolic function in 73% of patients, while only 32% showed wall motion abnormalities at TTE (146).

1.4.4.8 Myasthenia gravis

Myasthenia gravis (MG) is an autoimmune disease caused by the presence of autoantibodies directed against acetylcholine receptors at the level of neuromuscular plaque, leading to a reduction in signal transmission, resulting in muscle weakness. An association between MG and myocarditis has been seen, especially in patients with thymoma; this association appears to be due to the presence, in thymoma patients, of autoantibodies directed against voltage-dependent potassium channels (anti-Kv1.4)(147).

1.4.4.9 Mixed connective tissue disease

This uncommon group of SIDs patients is characterized by overlapping clinical features of SLE, SS, and inflammatory myopathies. In the absence of lung involvement, they usually have a relatively benign prognosis. However, as in other SID, recent evidence suggests that involvement in mixed connective tissue disease (MCTD) is associated with poor prognosis (148). In addition to ischemic heart disease, the main causes of cardiac-based mortality in MCTD include myocarditis, which is probably an expression of the inflammatory background of the myositic mold frequently associated with MCTD (149). As for the LES type component, ongoing myocarditis of MCTD requires aggressive immunotherapy with prednisone usually in combination with a steroid-sparing immunosuppressive agent (150).

1.4.4.10 Autoinflammatory syndromes

Autoinflammatory syndromes (AIS) are a group of diseases characterized by recurrent systemic inflammation in the absence of antigen-specific autoantibodies or autoreactive T cells. They include a broad spectrum of disorders, including familial Mediterranean fever (FMF), periodic TNF receptor associated syndrome (TRAPS), periodic cryopyrin associated syndromes (CAPS), and others. Myocarditis has been described in association with AIS, particularly FMF and TRAPS. Myocarditis was diagnosed in 9% of FMF patients with RMC and 4% of TRAPS patients had cardiac involvement, mainly in the form of pericarditis (151). The pathogenesis of myocarditis in AIS is not fully understood, but is thought to be related to dysregulated inflammation and cytokine release. Treatment of myocarditis in AIS is complex and a multidisciplinary approach is needed. The use of biological agents targeted at pro-inflammatory cytokines has been proposed as a potential therapeutic strategy (152).

Inflammation and myocardial damage in AIS, if not readily recognized and properly treated, eventually evolve into DCM or non-dilated hypokinetic cardiomyopathy (23,25,99,153).

1.5 Pathophysiology

The pathogenesis of human myocarditis is composed, in parallel with the etiology, of infectious and autoimmune mechanisms.

1.5.1 Infectious pathophysiology

The pathophysiologic process is divided into three phases: in the acute phase, which can last 1-7 days, the virus enters the cell leading to the activation of the innate immune response; after 1-4 weeks, a subacute phase in which the adaptive immune response is activated; finally, the inflammatory process can undergo spontaneous resolution, which occurs in most cases, or, if the elimination of the virus is ineffective, it has a chronic course evolveing towards inflammatory cardiomyopathy/DCM. Viral myocarditis is a condition in which the infection causes inflammation of the myocardium, which leads to damage to the heart muscle. The exact mechanisms by which viruses cause myocarditis are not fully understood, but different hypotheses have been proposed.

1.5.1.1 Exogenous damage

Some cardiotropic viruses directly infect and damage cardiomyocytes, leading to inflammation and cell death. Viruses enter cardiomyocytes through specific receptors on the cell surface, such as the coxsackievirus-adenovirus receptor (CAR) and the decay acceleration factor (DAF)(154). Once inside the cells, some cardiotropic viruses, in particular coxsackievirus and adenovirus replicate and release viral proteins that can directly damage the cell membrane, weaken the cytoskeleton and induce cell necrosis as well as propagation of the infection to adjacent cardiomyocytes (155,156).

1.5.1.2 Endogenous damage

In addition, the immune response to viral infection may be responsible for damage to the heart muscle. The immune response involves activation of innate and adaptive immune cells, including macrophages, neutrophils, T cells, and B cells. Immune cells release cytokines, chemokines, and reactive oxygen species that can cause oxidative stress, mitochondrial dysfunction and tissue damage. In addition,

immune cells can recognize and attack heart muscle cells as if they were infected by the virus, causing autoimmune damage (157–159).

1.5.1.2 Genetic predisposition

As will be discussed in depth in the dedicated chapter, several studies have analyzed the genetic and immunological factors that can contribute to the development and severity of viral myocarditis. For example, patients with idiopathic dilated cardiomyopathy have a higher prevalence of Class II Human Leukocyte Antigen (*HLA*) alleles than healthy controls, suggesting that genetic factors may predispose individuals to viral myocarditis and related complications (160). A case of severe myocarditis, associated with the polymorphism CD45 C77G, affecting T and B cell function (157), has also been reported.

1.5.1.3 Animal models

Animal models of viral myocarditis have provided answers about the mechanisms of viral pathogenesis and the role of immune cells in the disease. For example, enteroviral infection of mice with severe combined immunodeficiency (SCID) has been shown to lead to myocardial injury and heart dysfunction, indicating that the virus may directly cause tissue damage (161). Expression of enteroviral genes, responsible for viral replication in the heart muscle, induced electromechanical dissociation and DCM in transgenic mice, providing evidence for a direct pathogenic effect of viral proteins on heart muscle cells (157). Shi et al. used cardiac specific gene deletion to demonstrate that CAR is essential for coxsackievirus B3 infection and myocarditis in vivo (162).

1.5.1.3 Molecular pathogenesis

Some studies have focused on the mechanisms of damage induced by viral proteins in the heart muscle cells. Enteroviral protease 2A cleaves dystrophin, causing cytoskeletal dysfunction in acquired DCM (155). Another key role in myocarditis pathogenesis could be played by coxsackievirus B3 proteases 2A and 3C, which induce apoptotic cell death through mitochondrial injury and eIF4GI 123 cleavage (156). The innate immune system may also contribute to inflammation and myocardial damage in acute viral myocarditis, through toll-like receptor

signaling pathway and cytokine production, i.e. interleukin-1β and tumor necrosis factor α (TNF α) (163). In addition, persistent activation of the nitric oxide pathway and synthesis contributes to myocarditis immunopathology through dysregulation in interleukin-10 production (164). Myeloid differentiation factor 88 (Myd88) has been shown to be crucial in the pathogenesis of coxsackievirus B3-induced myocarditis, affecting the production of type I interferon (165). Finally, the pathologically activated interleukin-23 signaling pathway is necessary to cause autoimmune myocarditis in the xxxxxx (GM-CSF) driven mouse model (158).

In conclusion, the pathogenesis of myocarditis is complex and multifactorial, and involves both infectious and non-infectious causes. A better understanding of the pathogenesis of myocarditis is necessary for the development of more effective therapies for this condition.

1.6 Clinical presentation

Myocarditis is a complex and heterogeneous disease that can present a variety of symptoms ranging from mild to severe and even fulminant. The diagnosis of myocarditis is often delayed due to the lack of specificity of its clinical presentation, which can mimic other non-inflammatory myocardial diseases. In some cases, the presentation may be paucisymtomatic, with slow and insidious course, which may delay the diagnosis. In other cases, the onset of cardiac signs and symptoms may be rapid and unexplained, leading to life-threatening arrhythmias and sudden death, cardiogenic shock and severely impaired left ventricular function (25). The symptoms of myocarditis are nonspecific and depend on the degree of myocardial inflammation and ventricular dysfunction. Clinical presentation may be insidious and the disease may not be recognised (29,30,50,166–168). In some cases, myocarditis may mimic other non-inflammatory heart diseases. For example, in hyperacute myocarditis, the echocardiographic appearance of increased wall thickness may resemble hypertrophic cardiomyopathy. In infarct-like myocarditis, segmental kinetic abnormalities may be similar to those observed in an acute coronary syndrome (25,60,95,169–172). Myocarditis is a diagnosis of exclusion, but clinical suspicion of the disease should be high if cardiac signs and symptoms are unexplained, occur

predominantly in young or middle-aged male patients, although it can occcur even in old age, and with little or no risk factor of coronary heart disease. Myocarditis can resolve spontaneously, recur, or progress to DCM, sudden death, heart failure, or heart transplant in about 25% of biopsy-proven cases (25). According to the 2013 ESC Position Statement on myocarditis, the clinical presentation of myocarditis can be divided into four categories below, in addition to pauci/asymptomatic presentation and mixed forms (25).

1.6.1 1.6.1 Infarct-like

Infarct-like presentation presentation of myocarditis is the most common and affects usually previously asymptomatic subjects with few risk factors for coronary heart disease, who, days or weeks after a presumably viral respiratory or gastrointestinal prodromal phase, with or without increased systemic inflammatory markers and fever, develop one or more of the following symptoms: (1) dyspnea/orthopnea, (2) palpitations, (3) intolerance to exercise/general malaise, (4) heart failure, (5) thoracic pain (which may be pleural if concomitant pericarditis is also present) and (6) cardiac troponin release with temporal kinetics similar to that observed in acute myocardial infarction or prolonged for days/weeks, occasionally months, and without coronary occlusion (7,29,48,49,112–120). This scenario, dominated by a pseudo-infarct presentation with normal coronary arteries, may become relapsing with a variable level of troponin release. The similarity of these manifestations with coronary artery disease leads to difficulties in differential diagnosis; however, patients with myocarditis are often young and do not have significant cardiovascular risk factors. Definitive diagnosis is based on the exclusion of clinically significant coronary lesions by examinations such as selective coronary angiography or coronary computed tomography, chosen based on the pre-test probability of coronary heart disease (26).

1.6.2 Acute heart failure

Myocarditis may be the underlying cause of non-ischemic acute heart failure, especially in the presence of a DCM phenotype. Acute heart failure is considered among the differential diagnosis if the symptoms develop over days or a period not exceeding 3 months. The symptoms and signs presented are those typical of

heart failure, such as dyspnea, peripheral edema, asthenia and atypical chest pain. Echocardiography or CMR may confirm a reduction of the left and/or right ventricle function, but the presence of thickening of the heart walls and dilation of the cavities may vary. Electrocardiograms may show non-specific signs such as branch blocks, atrioventricular blocks, and supraventricular/ventricular arrhythmias. It is important to exclude non-inflammatory causes of heart failure, such as coronary artery disease, valvular diseases and hypertensive cardiomyopathy. Differential diagnosis should also include Tako-Tsubo cardiomyopathy, peripartum cardiomyopathy, and other forms of cardiomyopathy.

1.6.3 Chronic heart failure

Unlike acute presentation of myocarditis, biopsy-proven myocarditis in patients with chronic symptoms may have an indolent course with history of asthenia, palpitations, dyspnea, atypical chest pain, and arrhythmias. LV and/or RV function in echocardiography or CMR may be slightly, moderately or severely impaired, with or without ventricular dilation (99,173). Tissue characterization by CMR may reveal normal findings, active myocardial inflammation, or fibrotic changes with a non-ischemic late gadolinium enhancement (LGE) pattern (30,167,168,174,175). ECG results are often abnormal and non-specific, with the presence of branch blocks, ventricular arrhythmias or AV block. A recent monocentric study investigated univariate predictors of death or heart transplantation prior to the introduction of immunosuppressive therapy in a cohort of patients with biopsy-proven myocarditis: chronic heart failure presentation, together with young age, female gender, worse NYHA class, GCM, high titre AHA, reduction of the left ventricular ejection fraction, are a negative prognostic factors both in the short and long term (100).

1.6.2 "Life-threatening" myocarditis

Myocarditis is classified as life-threatening in the presence of severe tachyarrhythmias or bradyarrhythmias with possible aborted sudden death, cardiogenic shock and severe left and/or right ventricular dysfunction. The presence of any of these conditions must be considered a medical emergency. When a fulminant form of myocarditis is suspected, particularly if

hemodynamically unstable or not responding to standard cardiological therapy, patients should immediately be sent to centers specialized in EMB and advanced mechanical support in the form of a "bridge to recovery" or heart transplantation. Performing EMB in such a setting is a Class I recommendation, as it may reveal forms of myocarditis that require specific therapy such as immunosuppression for GCM or eosinophilic myocarditis (25,28,100,172). Early diagnosis and timely treatment based on a certain etiology are crucial to prevent the risk of death in patients with life-threatening myocarditis.

1.6.4 Asymptomatic forms

Myocarditis can be diagnosed in asymptomatic patients in whom arrhythmias are occasionally detected on 12-lead electrocardiographic Holter monitoring, or with reduction of left and/or right ventricular function on echocardiography, or with suggestive myocarditic/DCM LGE patterns on CMR, performed for other reasons. Before considering a diagnosis of myocarditis all other main causes of cardiac involvement should be excluded.

1.6.5 Myocarditis in pediatric age

Clinical presentation of myocarditis in children varies depending on the patient's age. In infants, non-specific symptoms such as agitation, malaise, poor appetite, fever, tachycardia, tachypnea, cyanosis predominate; in this age group, in addition, the severity of symptoms is greater. In children over the age of two, symptoms such as chest pain, abdominal pain, myalgia, cough, peripheral edema and asthenia appear. In general, in children and especially in infants, fulminant forms of myocarditis have a higher incidence rate than adults and require in the initial stages of disease advanced support for circulation and oxigenation (54).

1.7 Diagnosis

The 2013 ESC Position Statement reconfirms EMB as the gold standard diagnostic for myocarditis. However, given the invasive nature of this procedure, clinical and laboratory criteria have also been proposed for the presumptive diagnosis of "clinically suspected myocarditis". This definition is applicable when at least one of the clinical presentation criteria is present, with or without ancillary criteria,

and at least one of the diagnostic criteria is met, in the absence of coronary artery disease, other pre-existing cardiac and/or extracardiac diseases that may justify the symptoms (eg. valvular heart disease, congenital heart disease, hyperthyroidism). If the patient is asymptomatic, at least two diagnostic criteria are required. The more criteria are met, the suspicion of myocarditis is more likely. The clinical presentation criteria may include:

- Acute, pericardial or pseudo-ischemic chest pain.
- Recent onset (within three months) or worsening of HF, with symptoms such as dyspnea at rest or from exertion, with or without signs of left and/or right heart failure.
- Subacute/chronic onset or worsening (more than three months) of symptoms such as resting or exertional dyspnea, with or without signs of left and/or right heart failure.
- Palpitations and/or arrhythmic symptoms of unknown cause and/or syncope and/or sudden cardiac death.
- Cardiogenic shock of undefined cause.

The ancillary presentations include:

- Fever of at least 38 C° at presentation or within the previous thirty days, with or without evidence of signs and symptoms of respiratory or gastrointestinal infection.
- Peripartum period.
- Previous diagnosis of EMB-proven or clinically suspected myocarditis.
- positive personal and/or family history of allergies, allergic asthma, extra-cardiac autoimmune diseases, use of toxic substances linked to the development of myocarditis.
- Family history of myocarditis or CMD.

Criteria based on instrumental diagnostic tests include:

- ECG/ECG according to Holter/Stress test: detection of new onset abnormalities such as atrioventricular blocks, branch blocks, ST and T-wave

changes, sinus arrest, tachycardia or ventricular fibrillation, asystole, atrial fibrillation, R-wave amplitude reduction, intraventricular conduction delays, pathological Q-waves, low voltages, supraventricular tachycardia, frequent premature beats.

- Myonecrosis biomarkers: increased troponin I or T.
- Cardiac imaging such as ultrasound, MRI, angiography: detection of new onset structural and functional abnormalities of the left and/or right ventricle of otherwise unexplained causes, such as changes in regional wall motion abnormalities, overall systolic or diastolic dysfunction, with or without ventricular dilation, wall thickening, pericardial effusion, endocavitary thrombosis.
- Tissue characterization on CMR: presence of myocardial pattern of edema and/or LGE.

1.7.1 Electrocardiogram

ECG is a widely available and non-invasive tool used in the diagnosis of myocarditis. ECG findings may suggest the presence of myocardial inflammation and provide important information on the extent and severity of myocardial damage (25). The results of the ECG in myocarditis can be nonspecific and variable. At the beginning of the course of the disease, the ECG may be normal. However, with the progress of myocardial inflammation, changes in the ECG may become more noticeable. Common ECG abnormalities in myocarditis include T-wave inversion, ST segment elevation, and ST-T non specific changes. Other less common ECG abnormalities that may be observed in myocarditis include atrioventricular conduction abnormalities (AV), branch blocks and ventricular arrhythmias (176).

1.7.1.1 T-wave inversion

T-wave inversion is the most commonly reported ECG abnormality in patients with myocarditis. It is observed in about 50% of patients and is most commonly found in V1 to V4 leads. T-wave inversion can be transient or persistent and can be observed in the early stages of the disease before other ECG changes become apparent (177).

1.7.1.2 ST segment elevation

ST segment elevation is another ECG abnormality that can be observed in patients with myocarditis. It is detectable in about 25% of patients and is most commonly present in V1 to V4 leads. The ST segment elevation profile is typically convex in myocarditis, unlike the concave appearance that is usually found in acute coronary syndromes (178).

1.7.1.3 ST-T changes

Nonspecific ST-T changes are common in patients with myocarditis and can be observed in up to 70% of patients. These changes may include depression of the ST segment, flattening of the T-wave, or inversion and extension of the QT segment. These changes are typically present in the early stages of the disease and may resolve as the disease progresses (179,180).

1.7.1.4 Q waves

Q waves are an important diagnostic element in the ECG of patients with acute myocarditis. However, unlike myocardial infarction, where the presence of these waves indicates irreversible tissue necrosis, Q waves tend to disappear with resolution of the inflammatory process in acute myocarditis. This disappearance is often accompanied by improved heart function and reduced thickening of the heart walls. In contrast, patients with Q-waves on ECG are more likely to have reduced left ventricle function and an increased risk of major complications such as hemodynamic instability and changes in electrical conduction. Q wave analysis can provide important information for myocarditis diagnosis and monitoring (1).

1.7.2 Echocardiography

Among the imaging techniques useful for the diagnosis of myocarditis, echocardiography is the most used, as it is non-invasive, widely available and relatively inexpensive. In particular, TTE plays a crucial role in the diagnosis and follow-up of myocarditis. TTE is indicated in all cases of suspected myocarditis and should be repeated in case of hemodynamic worsening and during follow-up to monitor the course of biventricular function (25). TTE is a useful tool for differential diagnosis, as it can rule out non-inflammatory diseases such as valvular

heart diseases. TTE also allows monitoring changes in cardiac diameters, wall thickness, ventricular function, and the presence and modification of pericardial effusion. In addition, myocarditis may present with a number of echocardiographic patterns, including myocardial edema, regional or global dysfunction, and pericardial effusion. The normal ventricles have an elliptical shape, while in the case of active myocarditis there may be a dilation of the left ventricle with reduction of the ejection fraction and spherical remodeling, with "restitutio ad integrum" within a few months after the resolution of the inflammatory process (181). Conversely, in severe cases the ventricle evolves towards a DCM-like morphology. Mitral regurgitation, often secondary to ventricular dilation, is an important identifiable TTE factor which can affect ventricular remodeling, the onset of symptoms and prognosis. In fact, functional mitral regurgitation has been identified as an independent predictor of mortality in patients with ischemic and nonischemic DCM (182).

The evaluation of left ventricular diastolic function by TTE is an important aspect in the management of myocarditis patients. The American Society of Echocardiography and the European Association of Cardiovascular Imaging have provided recommendations for the assessment of diastolic function of the left ventricle, which include the use of multiple parameters such as the E/A ratio, the E/e ratio and the E wave deceleration time (183). Diastolic dysfunction is often related to left ventricle systolic dysfunction and pulmonary hypertension, indicating disease severity and worse prognosis. Ventricular remodeling is defined as an increase in the left telediastolic ventricular diameter or a decrease in the ejection fraction (181). This dysfunction is evaluated through the pulsed wave colorDoppler (PW) of the mitral valve and tissue Doppler (TDI). The presence of a thickened and hyperechogenic myocardium may be indicative of myocardial edema, which can cause systolic dysfunction and arrhythmias. In addition, edema reduces ventricular compliance and increases wall stiffness (174). The presence of pericardial effusion is due to the extension of the inflammatory process to the pericardium, and must be assessed in terms of localization and maximum thickness, and classified as mild (<10 mm), moderate (10-20 mm) or severe (>20 mm). The presence of signs of cardiac tamponade should also be assessed (184).

TTE is useful in confirming suspected intracardiac thrombus, which can be a complication of myocarditis, especially in cases of DCM with severe left ventricular dysfunction or in eosinophilic myocarditis. In addition, TTE may be useful in patients with suspected endocarditis or pericardial effusion of undefined diagnosis (185). In conclusion, echocardiography, and in particular TTE, plays a crucial role in the diagnosis and follow-up of myocarditis. Echocardiography can provide valuable diagnostic and prognostic information and can also help in the differential diagnosis of other heart diseases.

1.7.3 Cardiac magnetic resonance

CMR is indicated in any form of clinical presentation of myocarditis. In particular,, with respect to clinically suspected myocarditis in peculiar scenarios, CMR has class Ib indication in patients with myocardial infarction in the absence of coronary obstruction (Myocardial infarction with non-obstructive coronary arteries, MINOCA)(26), in which myocarditis is an important differential diagnosis, and Ic class indication in acute and chronic heart failure (186), if there is a suspicion of myocarditis. Here we will focus on the evolution of diagnostic criteria and discuss the role of CMR, in particular the early and late gadolinium enhancement (EGE and LGE), edema, T1 and T2 mapping, in the diagnosis of myocarditis.

1.7.3.1 Lake Louise criteria

In 2009, a consensus was published by the Journal of the American College of Cardiology, proposing the Lake Louise criteria for the diagnosis of myocarditis using CMR. The Lake Louise criteria included three elements: 1) global or regional myocardial systolic dysfunction, 2) myocardial edema and 3) myocardial hyperemia or increased vascular permeability (as evidenced by the early (EGE) or late gadolinium enhancement (LGE) on CMR. A diagnosis of myocarditis required the presence of at least two of these criteria, with at least one being either myocardial oedema or myocardial LGE (174). The 2009 Lake Louise criteria were widely adopted as the standard for the diagnosis of clinically suspected myocarditis on CMR. However, these criteria had some limitations. First, the diagnostic accuracy of the criteria of myocardial edema and EGE is affected by several factors, such as movement artifacts, the moment image acquisition after

gadolinium administration and the presence of confounding factors such as chronic myocardial infarction. Secondly, these criteria did not consider the potential technical contribution of T1 and T2 mapping in the diagnosis of myocarditis.

1.7.3.2 Updated Lake Louise criteria

A new consensus document was published in 2018 to update the original Lake Louise criteria. The revised criteria added T1 and T2 mapping techniques to the diagnostic algorithm for myocarditis and provided a more specific definition of LGE characterization . The updated criteria consisted of: 1) myocardial edema on T2-weighted sequences, 2) alterations on T1-mapping or 3) myocardial LGE with non-ischemic pattern. The presence of at least two of these criteria was necessary for the diagnosis of myocarditis (187).

1.7.3.3 Edema

Edema is the characteristic sign of inflammation in the soft tissues; on CMR it causes a lengthening of the relaxation time on T1 and especially of T2 sequences , secondary to the accumulation of water in the tissue. On T2-weighed sequences STIR (short tau inversion recovery), myocardial edema appears as a hyperintense signal (188). T2 weighed sequences are highly sensitive but lack specificity, and therefore the presence of myocardial edema alone is not sufficient for the diagnosis of myocarditis. However, the presence of myocardial edema, together with other clinical and imaging findings, can increase the diagnostic accuracy of CMR for myocarditis diagnosis (189,190).

1.7.3.4 Early Gadolinium Enhancement

EGE is defined as the presence of increased gadolinium signal within the myocardium during the first minutes after contrast injection. EGE can be used as a marker of hyperemia, which occurs during the initial inflammatory phase of myocarditis. EGE sequences are highly sensitive, but lack specificity and can be seen in other cardiac conditions such as ischemia, infarction, and myocardial fibrosis. Therefore, the presence of EGE alone is not enough for the diagnosis of

myocarditis, and EGE was not included among the updated Lake Louise criteria in 2018.

1.7.3.4 Late Gadolinium Enhancement

LGE is defined as the presence of increased gadolinium within the myocardium several minutes after the contrast injection. LGE is thought to be a marker of myocardial fibrosis and is found in the chronic stage of myocarditis. Acute LGE may however be a marker of edema and disappear on a follow up CMR, which also has a prognostic value (191). LGE is highly specific, but lacks sensitivity and may be absent in the very early stages of myocarditis. Therefore, the absence of LGE does not exclude a diagnosis of myocarditis.

1.7.3.4.1 LGE Patterns

There are specific patterns for non-ischemic inflammatory myocardial damage, which facilitate differential diagnosis with other types of myocardial diseases. The lesions that are appreciated in a myocarditis can be subepicardial, intramyocardial or a combination of the two. LGE may appear as a stria or with focal, patchy distribution and mainly affects the basal and mid-inferolateral wall. An exception may occur when the inflammatory damage is very severe and the hyperintensity extends to the subendocardium leading to transmural lesions. In particular, in the secondary stage of hypereosinophilic syndromes, the LGE pattern is typically subendocardial and circumferential, without correlation with specific coronary distribution. Since LGE can be located both in areas where cardiomyocyte necrosis has occurred and in areas where a fibrotic scar has been created during the healing process, the evaluation of LGE at follow-up does not allow to distinguish subacute or chronic myocarditis, without taking into consideration clinical data.

1.7.3. T1 mapping

T1 mapping is a quantitative technique that measures the longitudinal relaxation time (T1) of myocardial tissue. T1 mapping can detect diffuse myocardial fibrosis, which is a common finding in myocarditis. It can also be used to distinguish acute and chronic myocarditis. In fact, acute myocarditis is associated with increased T1

relaxation time due to the presence of edema, while chronic myocarditis is associated with decreased Tq1 due to the presence of fibrosis (192).

1.7.3.6 T2 mapping

T2 mapping is a quantitative technique that measures the transverse relaxation time (T2) of myocardial tissue. It can detect myocardial edema with high sensitivity and specificity. T2 mapping has been shown to be more accurate than T2 weighted imaging for detecting myocardial edema (193). It may also be used to distinguish acute and chronic myocarditis. In fact, acute myocarditis is associated with an increased T2 relaxation time due to the presence of edema, while chronic myocarditis is associated with T2 reduction due to the presence of fibrosis.

1.7.3.7 Extracellular Volume

ECV is a measure of the signal in T1 before and after contrast agent administration, corrected by the hematocrit (194). ECV is used to quantify the degree of fibrosis in myocardial tissue, which can be caused by inflammation. Therefore, ECV can be used to assess the severity of myocarditis and to monitor response to treatment. Although the prognostic role of ECV in myocarditis is still under investigation, this technique is a promising tool for myocarditis evaluation on CMR.

Importantly, it has to be highlighted that CMR does not provide any information on the etiology of myocarditis. Recently, the LLC diagnostic criteria have been updated to increase CMR accuracy, including T2 and T1 based imaging. The application of CMR in the context of myocardial inflammation is indicated primarily to confirm clinical suspicion, to support the indication to invasive diagnostic procedures such as EMB, possibly to guide EMB procedure and to suggest further examinations. The ultimate prognostic role of traditional LGE techniques and new techniques, including T2 mapping, native T1 and ECV, is still under investigation.

1.7.4 Nuclear medicine

The routine use of nuclear medicine is not indicated in the diagnostic workup of myocarditis, as these methods lack sensitivity and specificity. One possible

exception is sarcoidosis, for whose diagnosis and monitoring gallium-67 scintigraphy or 18-fluorodeoxyglucose PET may be useful (195).

1.7.5 Biomarkers

The diagnosis of myocarditis is complex and, even if there is no specific biomarker, serum biomarkers may be used to better define the clinical scenario. Inflammatory markers such as C-reactive protein (CRP) and erythrocyte sedimentation rate are generally increased in myocarditis, but they do not have much use for its diagnosis. A recent study showed that increased CRP values in infarct-like myocarditis identify patients with clinically suspected myocarditis and less severe clinical features, but do not help in predicting survival (8). Elevation of cardiac troponins, released into the bloodstream as a result of cardiomyocyte damage, is caused by myocardial damage, and although troponin levels are frequently increased during myocarditis, normal values cannot exclude the disease (196). Brain natriuretic peptide (BNP) and its N-terminal fragment (NT-proBNP) are used as HF biomarkers, and, although non-specific (since their levels may rise in every volume overload condition), may used as a complementary test for myocarditis diagnosis and response to therapy, especially in HF presentations. Viral serology should not be routinely used for myocarditis diagnosis, but may be useful in specific cases such as HCV myocarditis, Lyme carditis or HIV patients (197). Serum autoantibodies positivity may indicate autoimmune myocarditis, and the determination of specific autoantibodies for myocarditis/DCM may be useful in identifying patients who could benefit from immunosuppressive/immunomodulatory therapy. In summary, the diagnosis of myocarditis requires the combined analysis of different biomarkers and the evaluation of clinical data.

1.7.6 Endomyocardial biopsy

EMB is an invasive diagnostic technique that consists in the sampling of myocardial tissue for pathological, immunohistochemical and molecular examinations. EMB was first introduced in 1963 (198) and, after the implementation of new diagnostic techniques (especially immunohistochemistry), its diagnostic capacity has been greatly improved. Currently, EMB is the gold standard for the diagnosis of

myocarditis (25,28,199). It is mainly used for the diagnosis of myocarditis and other heart diseases that may be difficult to diagnose by other non-invasive diagnostic techniques. EMB procedure can be performed in the right or left ventricle, at the level of the interventricular septum. Tissue sampling is performed with a catheter that is inserted into the femoral vein or the internal jugular vein for a right ventricular biopsy, or into the femoral artery for left ventricular biopsy. The procedure is performed under fluoroscopic guidance to increase the accuracy and safety of the sampling (1). To increase the sensitivity and minimize sampling errors, the LGE site on CMR can be evaluated before biopsy is performed. A biventricular biopsy can reduce the sampling error and increase diagnostic sensitivity. Moreover, the biventricular approach does not increase periprocedural risk (199). If sampling from a single ventricle is chosen, the left ventricle biopsy is more frequently used, since according to the ESC consent document 2013, there would be no significant advantage of left versus right biopsy, which is less frequently performed (25). Comparing the diagnostic yield between the two ventricles, it was found to be 96.3% for left-sided and 71.4% for right-sided EMBs. This discrepancy further increases when isolated abnormalities of the left ventricle are detected on echocardiography (200).

1.7.6.1 EMB complications

The complication rate is very low when the procedure is performed by experienced operators and is between 0-0.8% (201). Major complications include death, cardiac perforation with cardiac tamponade, pericardial effusion, thrombosis with subsequent pulmonary embolism or stroke, permanent complete atrioventricular block. Minor complications include transient conduction disorders, transient ventricular or supraventricular arrhythmias, and cardiac hematoma. Local complications may also occur at the access site, such as hematoma, local nervous paresis, and arteriovenous fistulas. It is important to note that the risk of complications is higher in patients with severe left ventricular dysfunction and with renal failure (25,201). To minimize the risk of complications, it is important that EMB is performed only in specialized centers and by experienced operators. In addition, the patient should be properly prepared and

monitored during and after the procedure to promptly detect any complications (199).

1.7.6.2 Samples analysis

Once tissue samples are obtained they should be analyzed with immunohistochemical and molecular techniques to confirm or rule out the diagnosis of myocarditis. Immunohistochemistry allows the identification of T and B cells, natural killer cells, dendritic cells, macrophages and other cellular elements present in the myocardial tissue (202). The PCR technique allows the detection of viral DNA/RNA in the myocardial tissue, even in the presence of a low viral load (203). Once tissue samples have been analysed, it is important to store them properly for future analysis. Specimens fixed in formalin can be stored for long periods of time and used for morphological and immunohistochemical analysis. Frozen samples, however, can be used for molecular analysis such as PCR (25).

1.8 Therapy

The main aims of myocarditis therapy is, on the one hand, to support cardiovascular function and treat arrhythmias and, on the other hand, to cure the disease etiology, be it viral or autoimmune. The management of myocarditis depends on the underlying cause and severity of the symptoms and requires a personalized assessment and treatment by an experienced cardiologist.

1.8.1 Cardiological supportive therapy

Supportive cardiological therapy depends on the degree of symptoms, so hemodynamically stable patients with no signs of heart failure or arrhythmias do not require specific therapy. However, they should be kept under observation through ECG assessment, echocardiography and troponin dosage (25). An important precaution in the acute stage of myocarditis is exercise restriction for at least six months after the resolution of the acute phase, to avoid the posible trigger of arrhythmic events and myocarditis relapses (201,202).

1.8.1.1 Therapy of associated pericarditis

In patients with myocarditis and associated pericardial inflammation, the first-line treatment is standard pericarditis therapy according to international guidelines

(184). However, non-steroidal anti-inflammatory drugs such as acetylsalicylic acid, ibuprofen and indomethacin should be used carefully as, in murine models, their use has been found to worsen myocarditis prognosis (19,203,204). At the moment, clinical data on the use of such drugs in humans are still lacking.

1.8.1.2 Therapy of heart failure

In patients with hemodynamically stable heart failure, medical treatment is based on the general recommendations for heart failure management (186). In general, medical therapy includes administration of angiotensin converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARB), Sacubitril-valsartan (ARNI) is lack of response to ACE or ARB, beta blockers, and, if the patient shows signs of congestion, diuretics. If the patient is still symptomatic despite optimal therapy, mineralocorticoid receptor antagonists (MRA) may be added. Advanced therapies for unremitting HF include cardiac resynchronization device (CRT) implantation.

1.8.1.3 Fulminant myocarditis therapy

In patients with a fulminant presentation, i.e. cardiogenic shock and severe reduction of ventricular function, resulting in hemodynamic instability, it is necessary to proceed to support cardio-respiratory function. Circulatory support is initially pharmacological, through the prompt administration of intravenous loop diuretics to reduce congestion and fluid overload, and vasodilators, which reduce both preload and after-load, helping to improve cardiac output, but which are contraindicated in case the systolic blood pressure is less than 90 mmHg. In severe cases, infusion of inotropes, such as dobutamine or dopamine, may be needed to improve contractile function of the heart. These medications have an effect on both cardiac contractility and heart rate and lead to an improved cardiac output and better perfusion. However, inotropes may trigger arrhythmias, and therefore their usage must be carefully monitored. Other options are vasopressors, such as noradrenaline, that should be used in the case of persistence of hypoperfusion despite inotropic therapy (205). If the pharmacological support is not sufficient it is possible to mechanically support the circulation by means of ECMO (extracorporeal membrane oxygenation) or VAD (ventricular assist device)

as a bridge to recovery or to transplantation, or as destination therapy (206). In a minority of cases, heart transplant may be necessary (207).

1.8.1.4 Antiarrhythmic therapy

Treatment of arrhythmias should be in line with the ESC recommendations on arrhythmia management (2,24,208,209).

1.8.1.4.1 Pharmacological therapy

Antiarrhythmic drugs that can be used include bisoprolol, amiodarone or flecainide. Beta-blockers such as bisoprolol can be used in the prevention of arrhythmias following myocarditis: they work by blocking the stimulating effects of adrenergic stimulation on the heart, thereby reducing the heart rate and the likelihood of arrhythmias, particularly ventricular arrhythmias. In addition, beta-blockers can also reduce inflammation of the heart and improve contractile function of the heart muscle (210).

1.8.1.4.2 Implantable cardioverter defibrillator

If the patient does not respond to medical therapy or has life-threatening arrhythmias, the implantation of an implantable cardiac defibrillator (ICD) may be required. This device, which can be implanted subcutaneously in the chest, can automatically detect and stop life-threatening arrhythmias, such as ventricular fibrillation (FV) or sustained ventricular tachycardia (VTS). The ICD is also able to continuously record heart activity, allowing the assessment of the risk of life-threatening arrhythmias over time (2).

1.8.2 Immunomodulatory therapy

Immunomodulating therapy in myocarditis is based on the use of antiviral drugs, high-dose immunoglobulins and immunoadsorption. Despite some data on their efficacy, there is no clear indication for the use of antiviral drugs in virus-positive myocarditis. However, in some cases of fulminant presentation or in EMB-proven adenovirus or enterovirus myocarditis, the use of interferon beta has proven effective in the clearance of viral genome and in the improvement of symptoms (211,212).

1.8.2.1 Intravenous immunoglobulin

Administration of intravenous immunoglobulin (IVIG) has both a pro-inflammatory and anti-inflammatory effect. IVIG are able to activate innate immunity cells and the complement cascade, favoring the opsonization of infectious agents. In addition, IVIG may exert an antiinflammatory effect through increased production of antiinflammatory cytokines, reduction of pro-inflammatory cytokines and modulation of dendritic cell, macrophages and regulatory T lymphocyte activities (213–215). In autoantibody-mediated diseases, IVIGs promote their neutralization and clearance and reduce their production (216,217). Studies have shown that the use of IVIG in adenovirus, PVB19 and CMV myocarditis is helpful, as it is associated with a reduction in viral load with inflammation healing (218–221). However, no recommendation has yet been made for the use of IVIG in the course of myocarditis.

1.8.2.2 Immunoadsorption

Immunoadsorption consists of a therapeutic apheresis that removes antibodies and circulating immune complexes from the patient's plasma. The rationale for the use of such therapy in myocarditis and DCM lies in the presence of autoantibodies directed against cardiac antigens that favor the inflammatory process and the progression towards cardiac dilation. Studies have shown that immunoadsorption in the treatment of myocarditis and autoimmune cardiomyopathy has led to an improvement in the ejection fraction, exercise tolerance and cardiac output, as well as a reduction in heart failure biomarkers (222–228). Despite these results, the mechanism behind such improvements is still unknown, and there has been wide inter-individual variability reports regarding response to this therapy. Pending the results of a large randomized clinical trial, immunoadsorption in myocarditis and dilated cardiomyopathy is not yet routinely indicated.

1.8.3 Immunosuppressive therapy

Immunosuppressive therapy is indicated for the treatment of selected cases of inflammatory virus-negative cardiomyopathy (229). Several studies have shown

that patients treated with the combination of prednisone and azathioprine experienced an improvement in cardiac function, particularly in a recovery of the left ventricular ejection fraction, a reduction in ventricular size and volume, and an improvement in both short- and long-term clinical symptoms and prognosis (230–233). In addition to the diagnosis of virus-negative myocarditis, a diagnostic workup must be performed to minimize the risks associated with therapy, including the search for latent infections such as hepatitis B and C, HIV, tuberculosis, EBV, CMV, and research of malignant tumors such as breast and uterine cervix cancer in women and prostate cancer in men, according to a concept of "safety checklist" in order to identify potential absolute and/or relative contraindications (1). Immunosuppressive therapy is based on the administration of corticosteroids in combination with other immunosuppressive agents such as azathioprine or cyclosporine for a period of six months, although other immunosuppressive drugs such as mycophenolate mofetil, methotrexate and some monoclonal antibodies can be used in combination with steroid (230–232).

1.8.3.1 Corticosteroids

Corticosteroids of choice are prednisone and methylprednisolone, which at a dosage of more than one milligram per kilogram of body weight per day produce a significant immunosuppressive effect by inhibiting immune cell migration and reducing capillary permeability to pro-inflammatory mediators. The daily dose of corticosteroids administered should be gradually reduced during therapy until it is stopped.

1.8.3.2 Azathioprine

Azathioprine is an immunosuppressive drug that acts as an antimetabolite, impairing leukocyte formation in the bone marrow. Azathioprine is a pro-drug that inhibits purine synthesis, which is necessary for white blood cells production. Since purines are needed to produce DNA and RNA, by inhibiting purine synthesis, less DNA and RNA are produced for the synthesis of immune cells, thus causing immunosuppression. Its administration also allows to reduce the corticosteroids' dose, limiting their side-effects. The initial dose is two milligrams of azathioprine per kilogram of body weight per day. The complete pharmacological effect is

reached a few weeks after the start of therapy and, before starting this drug, the search in the peripheral blood of a mutation of the enzyme thiopurine methyltransferase (TPMT), which increases the risk of developing iatrogenic agranulocytosis, is mandatory (9).

1.8.3.3 Ciclosporin A

Cyclosporine A is a calcineurin inhibitor and represents an excellent alternative in cases where the administration of azathioprine is contraindicated. Unlike the latter, however, cyclosporine is less clinically manageable, as it has a higher frequency of side effects and drug interactions, and its plasma drug levels must be monitored, at least initially, to regulate the dose administered within the therapeutic range.

1.8.3.4 Mycophenolate mofetil

Mycophenolate mofetil is a reversible inhibitor of inosine monophosphate dehydrogenase and has been shown to be effective in patients intolerant to azathioprine or when first-line therapy is ineffective (234).

1.8.3.5 Methotrexate

Methotrexate is an antimetabolite that inhibits the synthesis of nucleic acids, is generally well tolerated but can cause side effects, which can be partially reduced by weekly administration of folic acid. Its use is frequent, especially in cases of SID.

1.8.3.6 Monoclonal antibodies

Some monoclonal antibodies such as rituximab (anti CD20) or mepolizumab (anti IL-5) showed promising results when added to the standard immunosuppressive therapy for treatment of both acute myocarditis and associated conditions such as GCM or autoimmune myocarditis (45,235,236).

1.9 Prognosis

In myocarditis, prognosis depends on the etiology, clinical presentation and stage of disease (25,237). In 50% of cases, acute myocarditis resolves between two and four weeks after the onset of symptoms, while 25% of patients develop persistent cardiac dysfunction and 12-25% may have an acute deterioration of cardiac

function which may lead to death or evolve into a chronic form. The prognosis is also determined by the etiological type; for exemple, in GCM the survival rate is six months, in the absence of immunosuppressive therapy. The strongest predictor of death or heart transplantation appears to be the presence of biventricular dysfunction at diagnosis, while other prognostic markers are young age, a NYHA II-IV class and a long duration of symptoms before diagnosis (237).

With respect to virus positivity on EMB, its prognostic significance is still uncertain, as the literature provides conflicting data (29,212,237,238). In particular, some studies report an improvement in ventricular function and prognosis at ten years associated with the clearance of the viral genome (212,238). Kindermann et al. (29) enrolled 181 patients undergoing EMB to identify prognostic indicators, highlighting how a III-IV NYHA class, the presence of immunohistopathological signs of inflammation, and the lack of β-blocker therapy were related to worse prognosis; however, in this study Dallas criteria were not always met and the presence of viral genomes was not always assessed.

Kytö et al. (239) studied a cohort of 1662 patients diagnosed with clinically suspected acute myocarditis to assess the rate of recurrence and to identify predictors. The recurrence rate during the 4.5 year follow-up was 10.3%. Among the predictors of recurrence, they identified: a prolonged hospitalization at the first diagnosis (longer than seven days), indicators of disease severity, ventricular arrhythmias, young age at diagnosis, the presence of chronic inflammatory bowel disease and chronic lung disease.

Finally, the identification of prognostic factors associated with worse prognosis in patients with non-infectious autoimmune myocarditis is crucial because it can help identify those who could clinically benefit from immunosuppression. A recent study of 466 patients with myocarditis, of which 250 were biopsy-proven cases, the female sex, fulminant onset, depressed EF at diagnosis and high-titre organ-specific ANA and AHA were independent predictors of death and heart transplantation. This same study was also the first to identify young age and a previous myocarditis as independent predictors of disease recurrence (8). This study was conducted in patients not treated with immunotherapy (IT) and future

studies are needed to confirm whether these autoimmune characteristics in myocarditis may also represent predictors of favorable response to immunosuppression, that should be used in selected EMB-proven cases, according to the latest recommendations (229).

2. THE ROLE OF GENETICS IN MYOCARDITIS

The etiology of myocarditis has been the subject of extensive research, and recently the interest has shifted towards the genetic susceptibility to the disease. The hypothesis of a link between genetic profile and susceptibility to myocarditis arises from twin studies (240–242), rare cases of family aggregation (140,243,244), rare mendelian transmitted disease that has been associated with myocarditis (1,245–247) and finally epidemiological studies on the genetic background of human populations and coexisting pathogens (248).

Although it has been shown that a polygenic predisposition to myocarditis exists, monogenic genetic causes behind the disease have not yet been identified with certainty, except in very rare cases that are still under investigation.

2.0.1 Polygenic susceptibility to myocarditis

Polygenic susceptibility to myocarditis has been demonstrated in several genetic association studies. For example, a large study conducted on German patients with myocarditis identified various regions of the genome that are associated with the disease (249). Other studies have shown the involvement of genes involved in the regulation of immune response and cytokine production (36,60). In particular, the presence of some *HLA* genetic variants gene has been associated with an increased risk of developing the disease (50).

2.0.2 Mendelian genetic cause of myocarditis

At present, there is no known Mendelian genetic cause of myocarditis. However, there are some very rare cases where genetic mutations associated with the disease. A mutation in the *TNNT2* gene was associated with cases of clinically suspected acute myocarditis and sudden death (250). However, these cases represent only a small percentage of all myocarditis cases and are still under study. Recently the attention of the scientific community has turned towards the role of immunity genes in terms of myocarditis susceptibility, as in patients with primary immunodeficiency diseases (PID)(1), as well as genes related to hereditary cardiomyopathies (251). In particular, the increased genetic susceptibility to infections due to mutations of genes related to the immune system seems to be a

factor increasing the risk of myocarditis (252). The hypothesis of a possible contribution of the known cardiomyopathy genes to the risk of developing myocarditis is based upon the assumption of a genetically determined "fragility" of the myocardium in some patients.

2.1 Genes involved in the immune response

2.1.1 Infectious susceptibility

Individual variability in cardiac inflammatory response, and thus in myocarditis pathogenesis, is a field that still has to be explored; however, preliminary studies have already been carried out on the genetic profile of patients with myocarditis related to infections. As stated above, myocarditis can be associated with infection by numerous pathogens, in particular: Enterovirus, Herpesvirus, Chagas disease.

2.1.1.1 Enteroviruses

Enteroviruses are a viral family associated with various pathologies, including gastrointestinal disorders, acute flaccid paralysis and, in this case, myocarditis. Coxsackievirus, in particular, has been identified as one of the main pathogens involved in viral myocarditis. Enteroviral myocarditis is often caused by an abnormal immune response of the host to infection of the virus. Enteroviruses are a family of viruses associated with various diseases, including gastrointestinal disorders, acute flaccid paralysis and, notably, myocarditis. Coxsackievirus, in particular, has been identified as one of the main pathogens involved in viral myocarditis. Enteroviral myocarditis is often caused by an abnormal immune response of the host to the virus. However, there are also genetic susceptibility factors that affect the onset of the disease. One of these factors is involved in the PI4KB/ACBD3 signal pathway (phosphatidylinositol kinase 4, type 3 beta/acyl-coa-binding domain-containing protein 3) (253). ACBD3 protein is essential for the efficiency of enteroviral virus infection and mediates the interaction between viral protein 3A and cellular protein PI4KB. In addition, studies have shown that genetic polymorphism of the NOD2 receptor (nucleotide-binding oligomerization domain 2) may increase the risk of developing myocarditis from Coxsackie B3 (254). Understanding of genetic susceptibility to enteroviral myocarditis could therefore lead to new opportunities for the development of targeted therapies.

A protein indispensable to the Coxsackie B3 virus to anchor and penetrate the host cell is the CAR transmembrane receptor (Coxsackie and Adenovirus Receptor), encoded by the *CXADR gene* (MIM 602621). Animal studies have demonstrated resistance to Coxsackie B3 virus infection following genetic ablation of *CAR* (162). Increased CAR expression could partially justify the different susceptibility to Coxsackie myocarditis within the population (255). However, no *in vivo* mutations of the *CXADR* gene have been found yet, and its role therefore remains only hypothetical.

2.1.1.2 Human Herpesvirus 6

Herpesviruses are a group of DNA viruses that can cause a wide range of diseases, including infectious mononucleosis, cold sores, chickenpox, and genital herpes. In particular, Human Herpesvirus 6 (HHV-6) has recently been identified as a possible causative agent of myocarditis. The presence of HHV-6 integrated in the host chromosomes (ciHHV-6) has been associated with an increased risk of developing myocarditis and other heart disorders (256). The presence of ciHHV6 affects 0.1-1% of the general population (257). The viral genome integrated into the genetic heritage of host cells (OMIM 604474)(258) may cause an important concentration of viral DNA in the blood of patients. Such alteration may act as a confounding factor in the diagnosis of HHV-6 infection (259) and in its role in myocarditis and DCM (260). Some evidence suggests that ciHHV-6, when integrated into host germ cells, can be inherited vertically according to an autosomal dominant Mendelian model (260). The integrated viral genome would lead to the secretion of viral chemokines and is assumed to interfere with human genes related to inflammatory disease and supports the reactivation of HSV-6A as a source of endogenous infection (257).

2.1.1.3 Trypanosoma Cruzi

Chagas disease is a parasitic infection caused by the protozoan Trypanosoma Cruzi, mainly transmitted by insects of the triatomine subfamily in endemic areas of Latin America. Not all patients infected with T. Cruzi develop CCC, indicating a possible genetic predisposition to disease progression. Numerous genes and biomolecular signal pathways have been associated with CCC, including numerous

genes associated with the immune response, such as *HLA* II genes, cytokines, chemokines and their receptors as well as specific cardiogenic genes, such as those involved in the renin-angiotensin system, ion channels and extracellular matrix proteins. The progression of CCC has been associated with a genetic profile comprising the alleles *HLA-DRB101:01* and *HLA-DQB105:01*, which are related to the activation of autoimmune responses against the heart tissue (261). One study suggested that polymorphisms in the IL-6 and IL-10 genes may modulate the risk of developing CCC (262). Regarding the involvement of chemokines, it has been shown that the *CXCL10* gene is associated with CCC, as well as the increase of parasitic counts and the degree of inflammation (263). In addition, variants in genes encoding cardiac ion channels, such as *SCN5A* and *KCNE1*, have been associated with arrhythmias and sudden cardiac death in CCC patients (264). Finally, genes involved in the renin-angiotensin system, such as *AGTR1* and *ACE*, have been associated with myocardial fibrosis and remodeling in CCC (265). Overall, these results indicate that genetic susceptibility plays a significant role in the progression of Chagas disease to CCC. Identifying the genetic factors involved in CCC can help improve risk stratification and disease management. However, further studies are needed to validate these findings and to clarify the underlying mechanisms of CCC pathogenesis.

2.1.2 Primary immunodeficiency diseases

Primary immunodeficiency diseases (PID) are genetic diseases characterized by a heterogeneous phenotype (266–268), which involve changes in the immune system. Their incidence is relatively low, with an estimate of about 1 case in 5000-10,000 live births, but the impact on patients' health can be significant.

PID can be divided into different categories depending on the type of cells or molecules involved. For example, primitive immunodeficiencies involving a deficiency in B cell function are the most common ones and include X-linked agammaglobulinemia (XLA) syndrome and Common Variable Immunodeficiency (CVID) syndrome. These diseases are characterized by a reduction or absence of circulating antibodies, with an increased risk of bacterial and viral infections. Other PID involve T cells, such as DiGeorge syndrome and Wiskott-Aldrich syndrome,

which are characterized by a deficiency in helper T cell function and may lead to increased susceptibility to bacterial and viral infections, as well as autoimmune complications. Finally, some primitive immunodeficiencies involve phagocytes, such as Chediak-Higashi syndrome and chronic granulomatous disease, which are characterized by a deficit in phagocytic function and increase the risk of bacterial infections. In general, patients with PID have an increased susceptibility to bacterial, viral and fungal infections, but also an increased risk of autoimmune, inflammatory and neoplastic diseases. PID treatment depends on the type of disease and may include substitution therapies (e.g., immunoglobulin or growth factor administration), immunomodulatory therapies or gene therapies.

2.1.2.1 PID-associated myocarditis

The condition of immunodeficiency underlies the predisposition and onset of myocarditis (252). Cases of PID-associated myocarditis are mainly observed in childhood, such as the case of a child with disseminated infection with Mycobacterium Avium carrier of an IL-12Rβ1 deficiency for compound heterozygosity, died of acute heart failure due to Coxsackie myocarditis and poor nutritional status (269). In adults, PID is rarer, but possible. In an adult patient diagnosed with enteroviral myocarditis, a toll-like receptor mutation 3 has been identified (270). The latest classification of such disorders drives genetic and molecular diagnostics of patients with these rare conditions (271,272). The extent of the impact that such diseases have exactly on the cardiovascular system is still to be defined with certainty (1).

2.1.3 Non-infectious susceptibility

The role of a genetic predisposition to myocarditis was postulated years ago (2): an unfavorable genetic predisposition, associated with infectious or non-infectious environmental stimuli, could explain the occurrence of myocarditis in some individuals, as well as the clinical and phenotypic heterogeneity of some cases compared to others.

Distinguishing acquired and inherited forms is clinically difficult, also because hereditary forms are often transmitted to the offspring as recessive traits. Finally,

hereditary immunodeficiencies, syndromes and self-inflammatory diseases may have overlapping causes and phenotypes in which myocarditis may occur.

2.1.3.1 Autoimmune diseases

Although viral infections are considered the main cause of myocarditis, there are several non-viral etiologies of myocarditis, including sarcoidosis, GCM and hypereosinophilic syndrome (HES)(25). It is believed that these non-viral etiologies of myocarditis have a genetic component, in terms of predisposition to develop the disease. Here we will explore the current knowledge on the role of genetics in sarcoidosis, GCM and HES .

2.1.3.1.1 Cardiac sarcoidosis

The exact etiology of sarcoidosis remains unknown, but recent studies have suggested that genetic factors may play a role in the development of the disease. A genomic study (GWAS) identified several single nucleotide polymorphisms (SNPs) associated with sarcoidosis, including SNPs in the *HLA* region and the BTNL2 gene (273). In addition, a recent study found that some *HLA* alleles are associated with cardiac involvement in sarcoidosis patients, again suggesting that genetic factors may influence the development of cardiac sarcoidosis (274). Finally, in sarcoid myocarditis, mutations in the desmosomal genes *DSP* and *DSG2* have been identified, suggesting a potential role of the latter in the pathogenesis of cardiac involvement in sarcoidosis (275).

2.1.3.1.2 Giant cells myocarditis

The etiology of GCM is unknown, but the fundamental etiological mechanism is certainly autoimmune. A genetic predisposition to GCM has been hypothesized and numerous genetic associations are analyzed in the literature, including those with *HLA-DRB1* and *HLA-DQB1* (276). A recent study identified an association between the allele with increased risk of GCM in the *HLA-DRB1* region and myocardial expression of GCM, suggesting that the expression of this allele may contribute to the development of GCM (35).

2.1.3.1.3 Hypereosinophilic syndrome

Hypereosinophilic syndrome (HES) is a rare disease characterized by persistent eosinophilia and multiorgan involvement, including myocarditis. HES has been associated with mutations in the *FIP1L1-PDGFRA* fusion gene and the *GATA2* . The fusion gene *FIP1L1-PDGFRA* is found in about 10% of patients with HES and is associated with a favorable response to therapy with tyrosine kinase inhibitors (277). The *GATA2* gene is associated with familial platelet disorder and predisposition to acute myeloid leukemia (AML) and has been linked to eosinophilic disorders, including HES. Mutations in the *GATA2* gene have been identified in patients with HES who do not have the *FIP1L1-PDGFRA* fusion gene (278).

2.1.3.2 Autoinflammatory syndromes

AIS are a group of rare conditions characterized by recurrent fever, rashes and specific organ inflammation. Among these diseases, adult Still's disease (AOSD) has been associated with myocarditis in a small number of cases. Although the exact mechanism of AOSD-related myocarditis is not yet fully understood, recent studies suggest that genetic factors may play a role within it. *HLA-B*51*, a genetic variant found in a subset of AOSD patients, has been associated with an increased risk of myocarditis in AOSD (279). Additionally, a GWAS identified numerous genetic variants, including rs6871626 and rs1160542, which were significantly associated with AOSD-related myocarditis (280). These findings suggest that there may be a genetic predisposition to AOSD-related myocarditis, but further research is needed to understand the underlying mechanisms and potential therapeutic targets.

2.1.3.3 Autoimmunity in the mouse model

Autoimmune myosin-induced myocarditis is an autoimmune disease extensively studied in the mouse model. In genetically predisposed mice, cardiac myosin has been shown to induce myocarditis. Studies have shown that *HLA-DQ8* plays a crucial role in the development of this disease. Transgenic mice for *HLA-DQ8* and knockout for *NOD* develop autoimmune cardiomyopathy and heart block spontaneously, while mice with normal MHC and non-MHC trim develop myocarditis that mimicked human disease. These findings suggest that genetic

factors, particularly *HLA-DQ8*, may play a role in the development of autoimmune myocarditis (20,281,282).

2.2 Non immune genes

Some studies suggest that myocarditis may have poor prognosis and it evolves towards DCM when genetics and damaging factors act synergistically (283). The genetic basis of myocarditis and DCM is complex and multifactorial. Although there is a strong immune component to the pathogenesis of myocarditis, recent studies have identified possible genetic mutations associated with myocarditis and cardiomyopathy development (1,283,284).

2.2.1 Arrhythmogenic right ventricular cardiomyopathy

ARVC is an inherited disorder that is characterized by progressive fibroadipose replacement of the right ventricular myocardium. Genetic mutations have been identified in a number of genes that are associated with ARVC and may also predispose individuals to myocarditis. Mutations in desmosomal genes, including those for *plakophyllin 2* (PKP2), *desmoplakin* (*DSP*) and *desmoglein 2* (DSG2), are the most common genetic mutations associated with ARVD, and has been hypothesized their role in myocarditis (285,286). In the literature there is a study on a case of autosomal recessive cardiomyopathy presenting as acute myocarditis proven by EMB. The authors identified a homozygous mutation in the *RYR2* gene, which encodes the receptor of cardiac ryanodine, in the affected individual. The RYR2 gene is known to be associated with ARVC and catecholaminergic polymorphic ventricular tachycardia (CPVT). The authors stressed the importance of genetic testing in the diagnosis of myocarditis and cardiomyopathy (284).

2.2.2 Dystrophin associated protein complex

The “dystrophin associated protein complex” is a structure that stabilizes the cell membrane and prevents its rupture during muscle contractions. It is composed primarily of dystrophin, a large structural protein, and numerous other proteins, including sarcoglycan, sarcospan, and syntaxin. Dystrophin acts as a bridge between the extracellular matrix and the cytoskeleton, allowing the transfer of force from the cytoplasm to the extracellular matrix. Mutations in the dystrophin

gene can lead to Duchenne muscular dystrophy, a genetic disease that causes progressive muscle degeneration and weakness (287).

2.2.2.1 Dysferlin

*D*ysferlin deficiency leads to increased susceptibility to coxsackievirus-induced cardiomyopathy in animal models. Dysferlin is a protein involved in membrane repair and encoded by the *DYSF* gene. Mutations in the *DYSF* gene are associated with 2B cingulate muscular dystrophy (LGMD2B). Studies of mouse models have reported that mice deficient in dysferlin were more susceptible to coxsackievirus-induced myocarditis and had more severe myocytic damage than wild-type mice. This suggests that dysferlin deficiency may predispose individuals to viral-induced myocarditis and cardiomyopathy (288).

2.2.2.2 Dystrofin

Other studies supporting this hypothesis have shown that enteroviral protease 2A cleaves dystrophin, leading to cytoskeletal dysfunction in acquired cardiomyopathy (155,289). Damage to cytoskeletal function can play a decisive role in the pathogenesis of infectious and non-infectious cardiomyopathies. Similar to dysphenyllin, this dysfunction can be caused by dystrophin defects in X-related dilated cardiomyopathies (290). In contrast, in enteroviral cardiomyopathy sarcoglycans and the carboxylic term dystrophin dissociate from the sarcolemma (291).

2.2.3 Genes associated with cardiomyopathies

A GWAS study investigated the prevalence of rare variants in genes previously associated with cardiomyopathy, including desmosomal genes, sarcomeric genes, and the titin gene. The authors found a higher prevalence of these variants in patients with familial cardiomyopathy than among the controls. However, they also found a significant amount of these variants in patients with myocarditis, particularly those with a family history of cardiomyopathy. Among the most frequent were missense mutations for *MYBPC3* genes and in *SCN5A* Overall, the study provides evidence of a possible, albeit infrequent, genetic overlap between

acute myocarditis and hereditary cardiomyopathy, suggesting that some cases of myocarditis may have an underlying genetic predisposition (286,292).

In conclusion, although the role of genetics in myocarditis is not yet fully understood, studies suggest that genetic factors may play a predisposing role in its development and may interact with other noxae such as viral infections. The genetic correlation between myocarditis and gene mutations of cardiomyopathies, particularly those involved in ARVD and DCM, requires further investigation to better understand the underlying mechanisms and to develop effective treatments.

3. AIMS

Myocarditis is a syndrome with heterogeneous clinical and instrumental presentations, as well as variable etiology and prognosis. Genetic factors have been hypothesized as clinical-diagnostic predictors in biopsy-proven myocarditis, although the predictive value of genetics remains largely unknown. Similarly, the correlations between genetics and morpho-functional imaging characteristics and autoantibody serological profile have been poorly explored.

The aims of this study are:

1) To determine the prevalence of pathogenic/likely pathogenic (P/LP) variants in a subset of 174 genes associated with cardiomyopathies in a prospective cohort of biopsy-proven myocarditis patients;

2) To investigate the potential diagnostic and prognostic role of genetic analysis in biopsy-proven myocarditis.

4. MATERIALS AND METHODS

4.1 Data collection

The study is based on a prospective analysis of the database containing the data of patients regularly followed at the Cardioimmunology outpatient clinic of the cardiology unit at the University of Padua from January 1993 to May 2023. Consecutive patients with a biopsy-proven diagnosis of myocarditis were selected for genetic analysis starting from July 2020. The diagnosis of histologically proven myocarditis was based on a suggestive clinical presentation, exclusion of coronary artery disease through dedicated instrumental investigations (coronary angiography or coronary CT angiography, depending on the 2020 ESC guidelines (26)), and exclusion of other cardiovascular pathologies that could explain the symptoms (such as valvular diseases, hypertensive heart disease, genetic cardiomyopathies). Additionally, the diagnosis relied on suggestive findings of myocarditis on CMR imaging and the presence of myocarditis on histological, immunohistochemical, and molecular examination of the EMB. Patient data was systematically collected and entered into a database divided into multiple sections. The first section included the patient's demographic data, family and personal history, with particular attention to the presence of systemic immunomediated diseases and cardiovascular pathologies. This was followed by a section dedicated to describing the clinical presentation, its duration, and any changes in symptoms in the period leading up to the diagnosis. Subsequent sections involved entering data from clinical and instrumental examinations performed on the patient, including cardiac troponin levels, C-reactive protein, BNP/NT-proBNP levels, electrocardiography and echocardiography, which were systematically conducted upon hospital admission. Other tests included 24-hour Holter monitoring, CMR, cardiac catheterization or coronary CT angiography, serum testing for AHA and other autoantibodies (such as antinuclear antibodies, anti-mitochondrial antibodies, anti-endothelial cell antibodies, anti-parietal gastric cell antibodies, anti-thyroid antibodies, anti-extractable nuclear antigen antibodies, anti-smooth muscle antibodies, ANCA), EMB with associated PCR to detect viral genome within the myocardium, and concurrent blood sampling to

rule out false positives in the biopsy due to blood contamination. Finally, there was a section dedicated to determining the eligibility for immunosuppressive therapy and the current administration regimen. The same method was used to enter data obtained during subsequent follow-ups.

4.1.1 ECG

The 12-lead resting ECG was evaluated near the clinically suspected diagnosis and during the follow-up. The duration of the waveform intervals was measured using commercial software, with a paper speed of 25 mm/sec. The analyzed ECG parameters included cardiac rhythm (sinus, atrial fibrillation, or other), cardiac axis (normal, right deviation, or left deviation), presence of PR segment depression, presence of pre-excitation, duration of the QRS complex in milliseconds, presence of Q waves and their location (anterior, septal, lateral, inferior), presence of nonspecific intraventricular conduction delay (defined as QRS duration >120 ms in the absence of criteria for right bundle branch block and left bundle branch block), atrioventricular blocks (all degrees of block were grouped as a single variable), bundle branch blocks (right or left), and fascicular blocks (anterior or posterior), presence of fragmented QRS (fQRS), its location (anterior, septal, lateral, inferior), and the number of leads in which it was present, presence of ST segment elevations and their location (anterior, septal, lateral, inferior), presence of early repolarization, presence of ST segment depressions, presence of inverted T waves and their location (anterior, lateral, inferior), presence of QT interval abnormalities (prolonged or shortened) and calculation of the QTc value using the Bazett formula ($QTc=QT/\sqrt{RR}$), Sokolow-Lyon index value (calculated as the sum of the S wave amplitude in lead V1 and the R wave amplitude in lead V6), presence of low voltages, and identification of supraventricular and ventricular ectopic beats, indicating their morphology and axis.

4.1.2 Echocardiography

The resting transthoracic echocardiogram included measurements of wall thickness, systolic diameters (end-systolic diameter, ESD), and diastolic diameters (end-diastolic diameter, EDD) of the cardiac chambers, evaluation of valve function and measurement of transvalvular flows, measurement of indexed

telediastolic (LVEDV, left ventricular end-diastolic volume) and telesystolic volumes (LVESV, left ventricular end-systolic volume) adjusted for body surface area, and assessment of cardiac function expressed as left ventricular ejection fraction (LVEF) and right ventricular fractional area change (FAC). Wall thickness was measured in the parasternal long-axis view, both in one-dimensional and two-dimensional mode. Valve function and transvalvular flow were evaluated using color Doppler imaging, and the severity of valvular regurgitation was classified as minimal, mild, moderate, or severe. For the measurement of chamber volumes and subsequent assessment of cardiac function, the 4-chamber and 2-chamber views acquired from the apical window were utilized. Biplane LVEF (4-chamber and 2-chamber) was calculated as (LVEDV - LVESV) / LVEDV and expressed as a percentage, while FAC was calculated as (telediastolic RV area – telesistolic RV area) / (telesistolic RV area), expressed as a percentage. Lastly, the subcostal window was used to evaluate the presence of pericardial effusion.

4.1.5 CMR

All patients underwent a 1.5T CMR imaging examination, which included the acquisition of long-axis and short-axis cine images, T2-weighted images, and post-contrast images. Long-axis and short-axis cine images were acquired using steady-state free precession (SSFP) sequences, while T2-weighted images for edema evaluation were acquired using short tau inversion recovery (STIR) or T2-weighted turbo spin echo sequences. Early gadolinium enhancement (EGE) images were acquired 1-3 minutes after the administration of gadolinium-based contrast agent (GBCA), while late gadolinium enhancement (LGE) images were acquired 10-15 minutes after administration.

Ventricular volumes, function, and left ventricular mass were measured in end-diastole and end-systole on short-axis projections using dedicated software. The volume and mass values were indexed to body surface area.

Regarding edema assessment, both short-axis and long-axis images were used to determine its presence and potential location. Edema was defined as present only if detectable in two orthogonal planes or if the myocardium-to-skeletal muscle signal ratio was greater than or equal to 2.

For LGE, the presence, location, and distribution pattern were evaluated. It was considered present only if visually detected in two orthogonal projections. The distribution pattern was defined as epicardial stria, intramural stria, epicardial/intramural spots, subendocardial ischemic pattern, diffuse pattern, or transmural ischemic pattern.

The diagnosis of myocarditis was made according to the Lake Louise criteria (174,187).

4.1.3 EMB

The diagnosis was based on histopathological analysis, referring to the Dallas criteria (22,293), and the characterization of the inflammatory infiltrate using immunohistochemistry (25). Furthermore, the results of PCR analysis on the EMB and blood were reported to detect any viral etiology, excluding blood contamination of the tissue sample. The absence of viral genome within the biopsy sample also allowed for determining the patient's eligibility for immunosuppressive therapy.

Histopathological diagnosis involved the use of 4 µm thick sections of myocardium embedded in paraffin. According to the Dallas criteria, the presence of active myocarditis was defined when histological analysis revealed an inflammatory infiltrate and necrosis/degeneration of cardiomyocytes, while borderline myocarditis was defined solely by the presence of the inflammatory infiltrate (22,293).

Immunohistochemical criteria defined the inflammatory infiltrate as abnormal when the number of leukocytes was ≥14/mm^2, including up to 4 monocytes/mm^2, with at least 7 CD3-positive T lymphocytes per mm^2 (25,28–31).

PCR analysis involved the detection of DNA viruses such as Adenovirus, PVB19, HHV6, HSV, EBV, CMV, VZV, and RNA viruses such as Enterovirus, Paramyxovirus, Influenza A and B viruses, HCV, mumps virus, and Rhinovirus A.

4.1.4 Autoantibodies serology

The detection of circulating cardiac autoantibodies such as AHA, AIDA, and AECA was performed using standard indirect immunofluorescence (s-IFI) on 4 µm cryostat sections obtained from atrial and skeletal muscle tissue of a normal subject with blood type O, obtained during cardiac surgery. This was accompanied by evaluating absorption on homogenates of human cardiac and skeletal muscle and mouse liver to determine their cardiac specificity. The definition of autoantibodies was as follows (97):

• Organ-specific: They bound only to cardiac tissue, showing a diffuse cytoplasmic pattern, and not to skeletal muscle tissue.

• Partially organ-specific (also known as type 1 cross-reactive): They bound to cardiac tissue, showing a finely striped pattern, and also yielded weakly positive results on skeletal muscle tissue.

• Type 2 cross-reactive (similar to myasthenia gravis): They exhibited a broad, striated positive pattern on both cardiac and skeletal muscle tissue.

4.1.6 Follow-up

Patients were followed up at the Cardioimmunology outpatient clinic of the Cardiology Department at the University of Padua with visits every 6-12 months, or more frequently if clinically indicated. During each visit, clinical data were collected, and every patient underwent a resting 12-lead ECG and transthoracic color Doppler echocardiography. The surrogate outcome variable was defined as the presence of FEVS<50% and/or NYHA class >I at the last follow-up and/or during subsequent clinical follow-ups after the diagnosis.

4.2 Genetic analysis

Genetic analysis has been performed on whole blood samples. DNA was isolated using MagnaPure Complex instrument (Roche). Quantification and quality control of nucleic acids was performed by fluorimetric (Qubit, ThermoFisher) and capillary electrophoresis (TapeStation 4200, Agilent) methods. Library preparation were carried out following user manual, specifically a targeted panel of 174 genes correlated with cardiovascular diseases (TruSight Cardio Panel-Illumina) in a MiSeq platform (llumina).

Passing filter quality reads were analysed and variant annotation was performed by the Cardiovascular Genetics laboratory pipeline. Only passing filter variants with a Minor Allele Frequency (MAF) lower than 0,0001 were interrogated and classified following American College of Medical Genetics and Genomics rules (ACMG) (294) and ClinGen framework (295). Putative causative variants classified as pathogenic or likely pathogenic were validated by direct sequencing on an ABI3500Dx (Life Technologies) genetic analyser (294).

4.3 Statistical analysis

Continuous variables were reported using median and interquartile range (I–III quartile), and categorical variables with absolute number and percentage (relative frequencies). Baseline characteristics were compared using the Wilcoxon or the Kruskal–Wallis rank sum test for continuous variables and the Chi-square test or the Fisher exact for categorical ones.

Overall survival (OS) distribution in the gene positive and gene negative groups was evaluated using a weighted Kaplan–Meier approach (296). Cox proportional hazards models were estimated for overall survival. The results of these analyses were reported as hazard ratio (HR) and 95% CI.

For all analyses, a two-sided $p<0.05$ was considered to be significant.

Statistical analyses was performed using the R System version 4.1.0 (297) and packages WeightIt (298), cobalt (299), gtsummary (300), survival (301), survminer (302), ggsurvfit (303) and tidycmprsk (304).

5.RESULTS

5.1 Patients' characteristics at diagnosis

5.1.1 Epidemiological and clinical characteristics

Epidemiological and clinical data of the whole patients' cohort at diagnosis are shown in Table III. Eighty-four patients with biopsy-proven myocarditis who underwent genetic screening were included in the study. Sixteen patients (16%), tested positive at the genetic screening, while the other 68 (81%) tested negative. Majority (70%) of patients were male and the median age at diagnosis was 46 years. A non-negligible quote of patients (13%) reported a family history of extra-cardiac immune-mediated diseases (such as Hashimoto's thyroiditis, insulin-dependent diabetes mellitus, inflammatory bowel diseases, psoriatic arthritis), and even more (37%) had a positive family history for cardiovascular diseases, with coronary artery disease as the most represented one (28%) (Table III).

Regarding past medical history, 25% of patients reported an acute viral infection in the six months before diagnosis. About a quarter (24%) suffered from a systemic immune-mediated disease: a plethora of autoimmune conditions was registered throughout the cohort, for example Hashimoto's thyroiditis (7.5%), SS (3%), psoriatic arthritis (3%) and IBD (4.5%). The most frequent clinical presentation was heart failure (44%), followed by infarct-like (36%) and arrhythmic presentation (14%); 8.3% of patients had a fulminant hemodynamic presentation and only 6% was pauci-symptomatic. Half of the patients had functional class NYHA>I at presentation (51%), and 37% complained of symptoms before diagnosis, with a median duration of 4 months. About a third (32%) complained of palpitations, 30% of chest pain and 3% of syncope.

Gene positive and gene negative patients did not present any clinically significant difference in terms of epidemiological or clinical features at diagnosis. In particular, a majority of middle-aged caucasian male was observed in both subgroups. Both groups had a heterogeneous family history and personal past medical history, and the only statistically significant difference was that gene positive patients more frequently had a family history of coronary artery disease

(50% vs 22%, p=0.048). Interestingly, genetic positivity did not influence the type of myocarditis presentation (HF presentation was the prevalent one in both groups), nor NYHA class at diagnosis (in both groups, about half of patients were in NYHA class>II). In addition, gene positive patients had a higher frequency of allergy (21% vs 50% with a p value of 0.026) and of variation of symptoms in the preceding 12 months (31% vs 62% wih a p value of 0.018). (Table III)

Table III. Epidemiological and clinical data of the whole patients' cohort and by genetics results (positive vs. negative).

Diagnostic information		N	Whole cohort	Genetics		P-value
				-	+	
Number of patients, n		84	84	68 (81%)	16 (19%)	
Ethnicity, n (%)	Asian	84	1 (1.2%)	0 (0%)	1 (6.2%)	0.3
	African		1 (1.2%)	1 (1.5%)	0 (0%)	
	Caucasian		82 (98%)	67 (99%)	15 (94%)	
Gender, n (%)	Female	84	34 (30%)	28 (41%)	6 (38%)	0.8
	Male		50 (70%)	40 (59%)	10 (62%)	
Age at diagnosis, median (IQR), years		84	46 (34, 52)	46 (34, 52)	42 (33, 52)	0.8
Family history						
Immune-mediated diseases, n (%)		67	11 (13%)	9 (13%)	2 (12%)	>0.9
Psoriatic arthritis		67	1 (1,5%)	1 (1.9%)	0 (0%)	>0.9

IBD		1 (1.5%)	1 (1.9%)	0 (0%)	
Hashimoto thyroiditis		5 (7.5%)	4 (7.5%)	1 (7.1%)	
IDDM		1 (1.5%)	2 (3.8%)	0 (0%)	
Heart disease, n (%)	83	31 (37%)	20 (30%)	11 (69%)	**0.004**
Coronary heart disease, n (%)	69	19 (28%)	12 (22%)	7 (50%)	**0.048**
Congenital heart disease	68	2 (2.9%)	1 (1.9%)	1 (7.1%)	0.4
Dilated cardiomyopathy	69	4 (5.8%)	2 (3.6%)	2 (14%)	0.2
Myocarditis	69	2 (2.9%)	1 (1.9%)	1 (6.7%)	0.4
Systemic arterial hypertension, n (%)	83	7 (8.4%)	4 (6.0%)	3 (19%)	0.13
Past medical history					
Acute viral infection within six months before diagnosis, n (%)	84	21 (25%)	16 (24%)	5 (31%)	0.5
Immune-mediated diseases, n (%):	84	20 (24%)	16 (24%)	4 (25%)	>0.9
Systemic lupus erythematosus	66	1 (1.5%)	1 (1.9%)	0 (0%)	>0.9
Systemic sclerosis		2 (3%)	2 (3.8%)	0 (0%)	
Mixed connective tissue disease		1 (1.5%)	1 (1.9%)	0 (0%)	

Eosinophilic granulomatosis with polyangiitis		1 (1.5%)	1 (1.9%)	0 (0%)	
Inflammatory myopathies		1 (1.5%)	1 (1.9%)	0 (0%)	
Rheumatoid arthritis		1 (1.5%)	1 (1.9%)	0 (0%)	
Psoriatic arthritis		2 (3%)	2 (3.8%)	0 (0%)	
IBD		3 (4.5%)	3 (5.7%)	0 (0%)	
Celiac disease		1 (1.5%)	1 (1.9%)	0 (0%)	
Multiple sclerosis		1 (1.5%)	0 (0%)	1 (7.7%)	
Basedow disease		1 (1.5%)	1 (1.9%)	0 (0%)	
IDDM		1 (1.5%)	1 (1.9%)	0 (0%)	
Hashimoto thyroiditis	69	5 (7.5%)	3 (5.7%)	2 (15%)	0.2
Allergy, n (%)	84	22 (26%)	14 (21%)	8 (50%)	**0.026**
Asthma, n (%)	84	5 (6%)	4 (5.9%)	1 (6.2%)	>0.9
Systemic arterial hypertension, n (%)	83	12 (14%)	9 (13%)	3 (19%)	0.7
Alcohol abuse, n (%)	84	3 (3.6%)	3 (4.4%)	0 (0%)	>0.9
Myocarditis history, n (%)	84	14 (17%)	11 (16%)	3 (19%)	0.7
Clinical presentation					

Infarct-like, n (%)		84	30 (36%)	24 (35%)	6 (38%)	0.9
Arrhythmic, n (%)			12 (14%)	9 (13%)	3 (19%)	0.7
Heart failure, n (%)			37 (44%)	30 (44%)	7 (44%)	>0.9
Pauci-symptomatic, n (%)			5 (6.0%)	5 (7.4%)	0 (0%)	0.6
Fulminant hemodynamic presentation, n (%)			7 (8.3%)	6 (8.8%)	1 (6.2%)	>0.9
Symptoms before the diagnosis, n (%)		84	53 (63%)	41 (60%)	12 (75%)	0.3
Duration of symptoms, median, (IQR), months		84	4 (1, 12)	4 (1, 9)	4 (2, 12)	0.5
NYHA class, n (%)	I	84	41 (49%)	34 (50%)	7 (44%)	0.2
	II		15 (18%)	10 (15%)	5 (31%)	
	III		18 (21%)	14 (21%)	4 (25%)	
	IV		10 (12%)	10 (15%)	0 (0%)	
Variation of symptoms in the previous 12 months, n (%)		84	31 (37%)	21 (31%)	10 (62%)	**0.018**
Variation of NYHA class in the previous 12 months, n (%)		46	9 (20%)	8 (22%)	1 (10%)	0.7
Chest pain, n (%)		70	21 (30%)	17 (30%)	4 (29%)	>0.9

Palpitations, n (%)	68	22 (32%)	16 (29%)	6 (46%)	0.3

Legend: IQR= Inter Quartile Range; IBD= Inflammar Bowel Disease; IDDM= Insuline Dependent Diabetes Mellitus; NYHA New York Heart Association.

5.1.2 Electrocardiogram at diagnosis

ECG characteristics at diagnosis are illustrated in Table IV. The vast majority (94%) of patients were in sinus rhythm and 5% had atrial fibrillation. The QRS complex was frequently abnormal: 8% patients had a non-specific intraventricular conduction delay, 9% a right branch block, 11% a left branch block. The most commonly reported ECG abnormality was of T wave inversion (TWI): 42% of patients had TWI in the lateral leads, 33% in the inferior leads and finally 31% in the anterior leads. Only a minority (5%) of the investigated patients had lateral STE, and 5% had inferior STE. The QT interval was prolonged in 17% of patients and QTc median duration by Bazett was 420 ms. Finally, in 26% of the ECGs low voltages were found. The ECG alterations were heterogeneous in the whole cohort, but no statistically significant difference has been found between gene positive vs negative patients. (Table IV)

Table IV. ECG findings at diagnosis in the whole cohort and its genetic subgroups. Crea didascalia simile alla tabella precedente

ECG at diagnosis		N	Whole cohort	Genetics		P value
				-	+	
Cardiac Rhythm, n (%)	Sinus rhythm	80	75 (94%)	60 (94%)	15 (94%)	0.2
	Atrial fibrillation		4 (5.0%)	4 (6.2%)	0 (0%)	

	Other		1 (1.3%)	0 (0%)	1 (6.2%)	
ST elevation, n (%)	Lateral	40	2 (5.0%)	2 (5.9%)	0 (0%)	>0.9
	Inferior	39	2 (5.0%)	2 (6.1%)	0 (0%)	>0.9
	NSTE	37	3 (8.1%)	2 (6.5%)	1 (17%)	0.4
T wave inversion, n (%)	Anterior	32	10 (31%)	9 (35%)	1 (17%)	0.6
	Lateral	33	14 (42%)	11 (41%)	3 (50%)	>0.9
	Inferior	33	11 (33%)	9 (33%)	2 (33%)	>0.9
Q waves, n (%)	Anterior	38	1 (2.6%)	1 (3.1%)	0 (0%)	>0.9
	Septal	37	1 (2.6%)	0 (0%)	1 (17%)	0.2
Long QT, n (%)		36	6 (17%)	5 (17%)	1 (17%)	>0.9
Intraventricular conduction delay, n (%)		75	6 (8.0%)	5 (8.5%)	1 (6.2%)	>0.9
Atrioventricular block, n (%)		75	1 (1.3%)	1 (1.7%)	0 (0%)	>0.9
Bundle branch block, n (%)	Right	76	7 (9.2%)	6 (10%)	1 (6.2%)	0.4
	Left		8 (11%)	8 (13%)	0 (0%)	
Electrical axis, n (%)	Right	76	2 (2.6%)	2 (3.3%)	0 (0%)	0.7
	Left		11 (14%)	10 (17%)	1 (6.2%)	

Sokolov index, median (IQR), mm	28	20 (15, 23)	16 (14, 22)	22 (20, 24)	0.10
QRS duration, median (IQR), msec	16	105 (93, 128)	104 (91, 138)	109 (104, 112)	0.8
QTc by Bazett formula, median (IQR), msec	15	420 (394, 478)	425 (402, 483)	395 (394, 430)	0.5
Low voltages, n (%)	23	6 (26%)	5 (26%)	1 (25%)	>0.9

5.1.3 Echocardiogram at diagnosis

Echocardiographic characteristics are shown in Table V. The patients globally had a reduced left ventricular ejection fraction (median LVEF 41%) and a preserved RV function (median FAC 37%, TAPSE 20 mm). Left ventricular volumes were globally mildly increased, while the right ventricle was generally not dilated (LVEDD 56 mm, LVEDV 76 ml/mq, RV telediastolic area 21.5 cmq). Mitral regurgitation was reported as absent or not significant in 40% of cases, in the remaining 60% it was mostly of mild degree, with only 6.7% severe cases. No clinically significant differences were reported for any echocardiographic feature between gene positive vs negative patients; the only significant difference was LV posterior wall thickness that was thinner in the gene-positive group (10 vs 9 mm, p=0.016) (Table V).

Table V. Echocardiographic findings at diagnosis in the whole cohort.

Echocardiogram at diagnosis		N	Whole cohort	Genetics		P value
				-	+	
Left Ventricle Wall Thickness, median (IQR), mm		44	10.00 (9.00, 11.00)	10.00 (9.00, 11.00)	9.00 (8.00, 9.00)	**0.016**
Left Ventricle Diameter, median (IQR), mm	Systolic	11	43 (38, 51)	43 (38, 51)	NA	
	Diastolic	47	56 (50, 61)	56 (50, 60)	56 (49, 72)	0.6
Mitral regurgitation, n (%)	Absent (-)	60	11 (18%)	9 (19%)	2 (15%)	0.6
	Trivial (+/-)		13 (22%)	10 (21%)	3 (23%)	
	Mild (+)		15 (25%)	13 (28%)	2 (15%)	
	Moderate (2+)		17 (28%)	13 (28%)	4 (31%)	

	Severe (4+)		4 (6.7%)	2 (4.3%)	2 (15%)	
Left Atrium Volume, median (IQR), ml/m^2		35	39 (26, 51)	39 (26, 45)	42 (30, 70)	0.4
Left Atrium antero-posterior diameter, median (IQR), mm		35	48 (40, 55)	47 (40, 55)	53 (43, 56)	0.8
Left Ventricle End Diastolic Volume, ml/m^2		57	76 (65, 99)	76 (65, 93)	85 (72, 120)	0.3
Left Ventricle Ejection Fraction, median (IQR)		84	41 (30, 57)	44 (30, 57)	33 (25, 56)	0.5
Right Ventricle Telediastolic Area, median (IQR), cm^2		36	21.5 (18.0, 25.0)	21.0 (18.0, 23.8)	26.0 (18.0, 27.7)	0.4
Fractional Area Change, median (IQR), %		40	37 (30, 50)	36 (30, 50)	40 (35, 42)	>0.9
Tricuspid Annular Plane Systolic Excursion, median (IQR), mm		9	20.0 (18.0, 24.0)	19.0 (16.5, 23.0)	23.0 (21.5, 24.5)	0.4
Myocardial mass, median (IQR), g		9	20.0 (18.0, 24.0)	188 (171, 275)	157 (144, 184)	0.4
Myocardial mass/volume ratio, median (IQR), g/cm^2		9	83 (71, 95)	82 (71, 94)	83 (42, 114)	>0.9

5.1.4 Biomarkers at diagnosis

The results of biochemical analysis and autoantibodies serology are listed in table VI. The value of the troponin I peak was abnormal in 84% of cases, with a median value of 1,422 ng/L (IQR 62, 6,020), while C Reactive Protein (CRP) was abnormal in 54% of patients, with a median value of 6 mg/dl (IQR 3, 58). Gene negative patients had significantly higher values of CRP at diagnosis (CRP was abnormal in 62% vs 14%, median value 15 vs 3 mg/dl, p = 0,036 and 0,008 respectively).

Serum AHA were tested in 80 patients, of whom 50% tested positive. AHA had an Organ-specific pattern in 42% of cases, and a partially organ-specific pattern in 5,5%. Finally, AIDA were positive in 39% of patients and ANA in 5%. No statistically significant difference was observed in terms of antibody immunophenotype between the two patient groups. (Table VI)

Table VI. Blood tests findings at diagnosis in the whole cohort.

Blood tests at diagnosis	N	Whole cohort	Genetics		P value
			-	+	
Abnormal troponin, n (%)	55	46 (84%)	38 (83%)	8 (89%)	>0.9
Troponin levels, median (IQR), ng/L	52	1,422 (62, 6,020)	1,570 (50, 5,598)	1,220 (123, 16,600)	0.5
Abnormal Reactive C Protein, n (%)	41	22 (54%)	21 (62%)	1 (14%)	**0.036**
Reactive C Protein levels, n (%), mg/L	37	6 (3, 58)	15 (3, 63)	3 (3, 3)	**0.008**
Abnormal Brain Natriuretic Peptide, n (%)	8	5 (62%)	5 (71%)	0 (0%)	0.4
Brain Natriuretic Peptide levels, median (IQR), ng/L	6	1,062 (302, 1,566)	1,477 (648, 1,596)	12 (12, 12)	0.3
Anti-Heart Antibodies positive, n (%)	80	40 (50%)	33 (52%)	7 (44%)	0.6

Type of AHA result, n (%)		73	Negative	38 (52%)	29 (49%)	9 (64%)
			Organ-specific	31 (42%)	26 (44%)	5 (36%)
			Partially organ-specific	4 (5.5%)	4 (6.8%)	0 (0%)
Organ specific (OS) AHA pattern, n (%)	Positive	35	10 (29%)	8 (29%)	2 (29%)	>0.9
	Strong positive		5 (14%)	4 (14%)	1 (14%)	
	Weakly positive		20 (57%)	16 (57%)	4 (57%)	
OS+partially organ-specific (PO) titer, n (%)	Negative	51	26 (51%)	22 (52%)	4 (44%)	0.8
	Positive		9 (18%)	7 (17%)	2 (22%)	
	Strong positive		4 (7.8%)	4 (9.5%)	0 (0%)	
	Weakly positive		12 (24%)	9 (21%)	3 (33%)	
Anti-Intercalated Disk Autoantibodies result, n (%)	Negative	79	48 (61%)	39 (61%)	9 (60%)	0.8
	Positive		10 (13%)	9 (14%)	1 (6.7%)	
	Strong positive		2 (2.5%)	2 (3.1%)	0 (0%)	
	Weakly positive		19 (24%)	14 (22%)	5 (33%)	

Anti-Nuclear Antibodies positivity, n (%)	60	5 (8.4%)	5 (10.4%)	0 (0%)	0.7

Legend: TnI= Troponin I; TnT= Troponin T.

5.1.5 Cardiac catheterization at diagnosis

The findings of cardiac catheterization at diagnosis are listed in table VII. Evaluation by cardiac catheterization showed healthy coronaries in 99% of patients. The median LVFE was 33% (IQR 26-46) while LVEDV had a median value of 111 ml (IQR 72, 224). There were no statistically significant differences between the two patient groups. (Table VII)

Table VII. Cardiac catheterization data of the whole patients' cohort and by genetics results (positive vs. negative).

Cardiac catheterization at diagnosis		N	Studied population	Genetics		P
				-	+	
Healthy coronaries, n (%)		77	76 (99%)	60 (98%)	16 (100%)	>0.9
Left Ventricle Pressure, median (IQR), mmHg	End Diastolic	30	15 (6, 22)	15 (9, 22)	5 (0, 10)	0.10
	Systolic	33	120 (110, 140)	120 (110, 140)	105 (92, 129)	0.3
Right Ventricle Pressure, median (IQR), mmHg	End Diastolic	29	5.0 (4.0, 10.0)	5.0 (3.5, 10.2)	5.0 (4.0, 7.0)	0.5
	Systolic	28	35 (30, 50)	35 (26, 50)	30 (30, 35)	0.9
Aortic mean pressure, median (IQR), mmHg		32	85 (74, 96)	85 (75, 99)	78 (71, 84)	0.3
Left Ventricle Ejection Fraction, median (IQR), %		31	33 (26, 46)	33 (25, 45)	34 (29, 50)	>0.9
Left Ventricle Volume, median (IQR), ml	End Diastolic	23	111 (72, 224)	110 (78, 217)	177 (67, 214)	>0.9
	Systolic	19	77 (32, 161)	68 (33, 164)	138 (31, 151)	>0.9

5.1.6 Medical therapy

Among the total cohort of patients, 45% of patients were treated with antiarrhythmic therapy. A minority (12%) of patients received anticoagulants, and 19% ACE inhibitors. More than half (54%) of patients were treated with immunosuppressive therapy, according to international guidelines (229) the main reason being unremitting heart failure despite optimal medical therapy (64%). With respect to immunosuppression, the first-line therapy administered was in 73% of cases a combination of prednisone and azathioprine, and the median duration of immunotherapy was 21 months (IQR of 11, 26). In the second and third line of immunosuppressive therapy the more frequently administered therapeutic combination was prednisone in association with Mycophenolate Mofetil (58% in the second line and 40% in the third line of therapy). No statistically significant differences were observable in terms of HF medical treatment. Gene-positive patients tended to be more frequent on immunosuppressive therapy; the only significant difference was a shorter duration of immunosuppressive therapy in genetic positive patients than genetic negative ones (502 days vs 723 days, p =0.030). (Table VIII)

Table VIII. Administered therapy data of the whole patients' cohort and by genetics results (positive vs. negative).

Therapy		N	Whole cohort	Genetics		P value
				-	+	
Antiarrhythmic therapy, n (%)		75	34 (45%)	25 (42%)	9 (56%)	0.3
Bisoprolol, n (%)		75	8 (11%)	7 (12%)	1 (6.2%)	>0.9
Anticoagulant therapy, n (%)		84	10 (12%)	9 (13%)	1 (6.2%)	0.4
ACE inhibitors therapy, n (%)		84	16 (19%)	22 (32%)	4 (25%)	0.4
Immunosoppressive therapy, n (%)		84	45 (54%)	33 (49%)	12 (75%)	0.056
Reason to treat, n (%)	Asymptomatic TnI release or CMR signs of active disease	45	1 (2.2%)	1 (3.0%)	0 (0%)	0.7
	Malignant arrythmia		4 (8.9%)	3 (9.1%)	1 (8.3%)	

	Mixed pattern		4 (8.9%)	4 (12%)	0 (0%)	
	Other		3 (6.7%)	3 (9.1%)	0 (0%)	
	Recurrent chest pain with TnI release		4 (8.9%)	2 (6.1%)	2 (17%)	
	Worsening/unremitting heart failure		29 (64%)	20 (61%)	9 (75%)	
I line of specific immunotherapy, n (%)	PDN	45	3 (6.7%)	3 (9.1%)	0 (0%)	0.3
	PDN+AZA		33 (73%)	21 (64%)	12 (100%)	
	PDN+MMF		3 (6.7%)	3 (9.1%)	0 (0%)	
	Other		4 (8.9%)	4 (12%)	0 (0%)	
	PDN+MTX		2 (4.4%)	2 (6.1%)	0 (0%)	
Therapy duration, median (IQR), days		37	648 (346, 779)	723 (396, 817)	502 (319, 620)	**0.030**
Response to therapy, n (%)		45	31 (69%)	22 (67%)	9 (75%)	0.7
II line of specific immunotherapy, n (%)	MMF	19	1 (5.3%)	1 (7.1%)	0 (0%)	0.5
	PDN		1 (5.3%)	0 (0%)	1 (20%)	
	PDN+AZA		1 (5.3%)	1 (7.1%)	0 (0%)	
	PDN+MMF		11 (58%)	7 (50%)	4 (80%)	
	PDN+Cya		1 (5.3%)	1 (7.1%)	0 (0%)	
	Other		4 (21%)	4 (29%)	0 (0%)	
Response to therapy, n (%)		19	15 (79%)	11 (79%)	4 (80%)	>0.9
III line of specific immunotherapy, n (%)	AZA	5	1 (20%)	1 (33%)	0 (0%)	0.6
	MMF		1 (20%)	1 (33%)	0 (0%)	
	PDN+MMF		2 (40%)	1 (33%)	1 (50%)	
	PDN+Cya		1 (20%)	0 (0%)	1 (50%)	
Response to therapy, n (%)		5	3 (75%)	1 (50%)	2 (100%)	>0.9

Legend: AHA=Anti Heart Antibodies; CMR=Cardiac Magnetic Resonance; PDN= Prednison; AZA= Azathioprine; MMF= Mycophenolate Mofetil; MTX= Methotrexate; Cya= Cyclosporin A.

5.1.7 CMR at diagnosis

The CMR findings at diagnosis are listed in Table X. Among the 63 available CMRs, 48% showed edema, mainly located in the inferolateral walls site (18%). LGE was present in almost all (78%9 evaluated CMR; the most reported LGE pattern was epicardial stria (36%), followed by epicardial/intramyocardial spots in 24% of cases. In 63% of cases, LGE affected more than one LV segment. With respect to biventricular function, median LVEF was 44% (IQR 23, 55), and RVEF was 50% (IQR 40, 59) respectively. Segmentary and diffuse parietal kinetics were observed in 34% and 54% of patients, respectively. There were no statistically significant differences between the two patient groups, i.e. the genetic results did not affect CMR findings. (Table IX)

Table IX. CMR data of the whole patients' cohort and by genetics results (positive vs. negative).

CMR at diagnosis		N	N (%)	Genetic		p value
				-	+	
Edema, n (%)	Absent	63	30 (48%)	24 (48%)	6 (46%)	0.7
	Present in same site		24 (38%)	17 (34%)	7 (54%)	
	Present in different site		4 (6.3%)	4 (8.0%)	0 (0%)	
	Not executed		2 (3.2%)	2 (4.0%)	0 (0%)	
	Not evaluable		3 (4.8%)	3 (6.0%)	0 (0%)	
Edema site, n (%)	Septum	18	4 (4.8%)	3 (4.4%)	1 (6.2%)	0.8
	Anterior wall		6 (7.1%)	3 (4.4%)	3 (19%)	
	Inferior wall		8 (9.5%)	6 (8.8%)	2 (12%)	
	Lateral wall		8 (9.5%)	6 (8.8%)	2 (12%)	
	Right ventricle		2 (2.4%)	1 (1.5%)	1 (6.2%)	
LGE, n (%)	Absent	64	13 (20%)	10 (20%)	3 (23%)	>0.9
	Present		50 (78%)	40 (78%)	10 (77%)	
	Not executed		1 (1.6%)	1 (2.0%)	0 (0%)	

LGE pattern, n (%)	Epicardial/intramural spots	48	14 (24%)	11 (23%)	3 (25%)	>0.9
	Epicardial stria		21 (36%)	16 (34%)	5 (42%)	
	Intramyocardial stria		9 (15%)	7 (15%)	2 (17%)	
	Subepicardic pattern		4 (3%)			
	Diffused		5 (8.5%)	4 (8.5%)	1 (8.3%)	
	Absent		4 (6.8%)	3 (6.4%)	1 (8.3%)	
	Ischemic transmural pattern		1 (1.7%)	1 (2.1%)	0 (0%)	
	Ischemic subendocardic pattern		4 (3%)	5 (11%)	0 (0%)	
LGE site, n (%)	Septum	52	8 (15%)	4 (10%)	4 (31%)	0.3
	Inferior wall		0 (0%)	0 (0%)	0 (0%)	
	Anterior wall		0 (0%)	0 (0%)	0 (0%)	
	Lateral wall		6 (12%)	6 (15%)	0 (0%)	
	More than one site		33 (63%)	25 (64%)	8 (62%)	
	Absent		3 (5.8%)	2 (5.1%)	1 (7.7%)	
	Diffused		2 (3.8%)	2 (5.1%)	0 (0%)	
CMR diagnosis, n (%)	Acute myocarditis	64	28 (44%)	22 (43%)	6 (46%)	0.8
	Chronic/previous myocarditis		4 (6.2%)	4 (7.8%)	0 (0%)	
	DCM		10 (16%)	6 (12%)	4 (31%)	
	Myopericarditis		1 (1.6%)	1 (2.0%)	0 (0%)	
	Not evaluable		7 (11%)	6 (12%)	1 (7.7%)	
	Other		5 (7.8%)	4 (7.8%)	1 (7.7%)	
	Subacute myocarditis		7 (11%)	6 (12%)	1 (7.7%)	
	Takotsubo		2 (3.1%)	2 (3.9%)	0 (0%)	
Left Ventricle, median, median (IQR)	End Diastolic Volume, ml	35	116 (92, 154)	113 (94, 144)	127 (89, 158)	0.7

	End Systolic Volume, ml	32	78 (40, 118)	78 (40, 117)	87 (40, 125)	0.7
	Stroke volume, ml/m2	33	63 (52, 78)	63 (53, 80)	68 (45, 78)	0.9
	Ejection Fraction, %	36	44 (23, 55)	44 (23, 56)	44 (18, 53)	0.4
	Mass, g/m2	34	73 (56, 88)	74 (58, 84)	72 (54, 90)	>0.9
Right Ventricle, median, median (IQR)	End Diastolic Volume, ml	33	86 (68, 96)	86 (69, 96)	80 (66, 97)	>0.9
	End Systolic Volume, ml	31	44 (31, 52)	44 (31, 52)	44 (32, 54)	0.8
	Stroke volume, ml/m2	33	68 (49, 86)	64 (49, 88)	69 (60, 74)	>0.9
	Ejection Fraction, %	33	50 (40, 59)	50 (40, 60)	48 (33, 55)	0.4
Pericardial effusion, n (%)		39	11 (28%)	9 (29%)	2 (25%)	>0.9
Pleural effusion, n (%)		39	8 (21%)	7 (23%)	1 (12%)	>0.9
Regional wall motion abnormalities, n (%)		38	13 (34%)	11 (37%)	2 (25%)	0.7
Diffuse wall motion abnormalities, n (%)		39	21 (54%)	16 (52%)	5 (62%)	0.7
Transmural edema, n (%)		15	4 (27%)	3 (27%)	1 (25%)	>0.9
Transmural LGE, n (%)		25	9 (36%)	8 (42%)	1 (17%)	0.4
LGE mass, median (IQR), g		22	4.2 (2.2, 6.4)	3.9 (2.2, 6.3)	5.9 (3.3, 8.7)	0.4
LGE mass, median (IQR), %		22	3.9 (1.1, 6.3)	3.8 (1.0, 6.1)	6.3 (1.8, 8.1)	0.3

Legend: EGE= Early Gadolinium Enhancement; LGE= Late Gadolinium Enhancement.

5.1.8 Endomyocardial biopsy

EMB features at diagnosis are listed in Table X. Among the entire cohort of patients, all with an EMB-based diagnosis of myocarditis, 74% were diagnosed with active lymphocytic myocarditis, 12% with borderline lymphocytic myocarditis, 5% with eosinophilic myocarditis and 2.4% with GCM. On viral PCR, diagnosis of viral lymphocytic myocarditis was made in only 14 patients (18%); PVB19 was the most frequently found causative agent (13%). Therefore, 82% of patients were diagnosed with autoimmune myocarditis (i.e. virus-negative myocarditis). (Table X)

Table X. Bioptical data of the whole patients' cohort and by genetics results (positive vs. negative).

Endomyocardial biopsy		N	Whole cohort	Genetics -	Genetics +	p value
Diagnosis according to Dallas criteria, n (%)	Active myocarditis	84	67 (80%)	56 (82%)	11 (69%)	0.3
	Borderline myocarditis		17 (20%)	12 (18%)	5 (31%)	
Histological type, n (%)	Lymphocytic/active	84	62 (74%)	53 (78%)	9 (56%)	0.14
	Borderline		12 (14%)	8 (12%)	4 (25%)	
	Giant cells		2 (2.4%)	2 (2.9%)	0 (0%)	
	Polimorph		1 (1.2%)	0 (0%)	1 (6.2%)	
	Eosinophilic		4 (4.8%)	3 (4.4%)	1 (6.2%)	
	Other		3 (3.6%)	2 (2.9%)	1 (6.2%)	
PCR results, n (%)	Positive n (%)	80	15 (22%)	12 (18%)	3 (18%)	>0.9
Identified viral genome, n (%)	CMV	69	1 (1.4%)	0 (0%)	1 (7.7%)	0.3
	HHV6	68	3 (4.4%)	3 (5.5%)	0 (0%)	>0.9
	Influenzavirus A	69	1 (1.4%)	1 (1.8%)	0 (0%)	>0.9
	Parvovirus B19	68	9 (13%)	8 (15%)	1 (7.7%)	0.8
	Rhinovirus A	68	1 (1.5%)	0 (0%)	1 (7.7%)	0.2

Legend: CMV= Citomegalovirus; HHV-6= Human Herpesvirus 6; PCR= Polymerase Chain Reaction; PVB19= Parvovirus B19.

5.1.9 Genetic characterization

In Table XII are listed all the genetic mutations registered within the study and their prevalence in the studied population. All 84 subjects of the study underwent genetic testing and mutation in 7 different genes were found, the most frequent (50% of cases) was the titin (*TTN*), gene which transcripts into the titin protein which has a structural role in heart muscle cells. (Table XI)

Table XI. Genetic characterization of the whole cohort.

Genetics		Genetic +
Muted genes	*TTN*	8 (50%)
	DSP	3 (19%)
	FLNC	1 (6%)
	MYH7	1 (6%)
	TNNT2	1 (6%)
	DES	1 (6%)
	MYBPC3	1 (6%)

Legend: DSP= Desmoplakin; DES= Desmin; FLNC=Filamin C; MYH7= MutY Homolog7; TNNT= Troponin T2; TTN= Titin; MYBPC3= Myosin Binding Protein C3

5.2 Patients' characteristics at follow-up

5.2.1 Clinical characteristics

The follow-up features of the entire cohort are shown in Table XII. The median duration of follow-up was 64 months (IQR 34, 103) and none of the patients died or underwent cardiac transplantation. During follow up, 3 patients (3.6%), all genetically negative, had a recurrence of myocarditis. No patient had a NYHA class>II, and most of pateints were classified as NYHA I (93%). No statistically significant difference in terms of clinical characteristics at follow-up between the two patient groups.

Table XII. Clinical data of the whole patients' cohort and by genetics results (positive vs. negative).

Clinical characteristics at follow-up		N	Whole cohort	Genetics		p value
				-	+	
Duration of follow up, median (IQR), months		84	64 (34, 103)	64 (33, 103)	66 (52, 109)	>0.9
Death or heart transplant, n (%)		84	1 (1,2%)	0 (0%)	1 (6%)	>0.9
NYHA Class, n (%)	I	84	78 (93%)	64 (94%)	14 (88%)	0.3
	II		6 (7.1%)	4 (5.9%)	2 (12%)	
	III		0 (0%)	0 (0%)	0 (0%)	
	IV		0 (0%)	0 (0%)	0 (0%)	
Relapse, n (%)		84	3 (3.6%)	3 (4.4%)	0 (0%)	>0.9

Legend: NYHA= New York Heart Association.

5.2.2 ECG at follow-up

ECG characteristics at diagnosis are illustrated in Table IV. The vast majority (94%) of patients were in sinus rhythm and 5% had atrial fibrillation. The QRS complex was frequently abnormal: 18% patients had a non-specific intraventricular conduction delay, 12% a right branch block, 8% a left branch block. The most commonly reported ECG abnormality was of T wave inversion (TWI): 15% of patients had TWI in the lateral leads, 13% in the inferior leads and finally 11% in the anterior leads. The QT interval was prolonged in 8% of patients and QTc median duration by Bazett was 432 ms (IQR 416, 450). Finally, in 18% of the ECGs low voltages were found. The ECG alterations were heterogeneous in the whole cohort, but no statistically significant difference has been found between gene positive vs negative patients. (Table XIII)

Table XIII. ECG data of the whole patients' cohort and by genetics results (positive vs. negative).

ECG at follow-up		N	Whole cohort	Genetics		p value
				-	+	
Cardiac Rhythm, n (%)	Sinus rythm	84	73 (87%)	57 (84%)	16 (100%)	0.4
	Atrial fibrillation		5 (6.0%)	5 (7.4%)	0 (0%)	
	Other		6 (7.1%)	6 (8.8%)	0 (0%)	
T wave inversion, n (%)	Anterior	84	9 (11%)	7 (10%)	2 (12%)	0.7
	Lateral		13 (15%)	9 (13%)	4 (25%)	0.3

	Inferior		11 (13%)	9 (13%)	2 (12%)	>0.9
Long QT, n (%)		84	7 (8.3%)	6 (8.8%)	1 (6.2%)	>0.9
Intraventricular conduction delay, n (%)		84	15 (18%)	14 (21%)	1 (6.2%)	0.3
Atrioventricular block, n (%)		84	6 (7.1%)	4 (5.9%)	2 (12%)	0.3
Bundle branch block, n (%)	Right	84	10 (12%)	8 (12%)	2 (12%)	>0.9
	Left		7 (8.3%)	6 (8.8%)	1 (6.2%)	
Electrical axis, n (%)	Right	84	2 (2.4%)	1 (1.5%)	1 (6.2%)	0.2
	Left		12 (14%)	11 (16%)	1 (6.2%)	
Sokolov index, median (IQR), mm		84	15 (11, 20)	15 (11, 20)	14 (11, 19)	0.4
QRS duration, median (IQR), msec		83	106 (94, 130)	106 (94, 134)	104 (94, 109)	0.4
QTc by Bazett formula, median (IQR), msec		83	432 (416, 450)	433 (416, 451)	428 (417, 444)	0.6
Low voltages, n (%)		83	15 (18%)	11 (16%)	4 (25%)	0.5

5.2.3 Echocardiogram at follow-up

Echocardiographic characteristics are shown in Table XIV. The patients globally had a normal left ventricular ejection fraction (median LVEF%) and a preserved RV function (median FAC 40%, TAPSE 21.5 mm). Left ventricular volumes were globally mildly increased, while the right ventricle was generally not dilated (LVEDD 53 mm, LVEDV 65 ml/mq, RV telediastolic area 19 cmq). Mitral regurgitation was reported as absent or not significant in 63% of cases, in the remaining 37% it was mostly of mild degree, with no severe cases. No clinically significant differences were reported for any echocardiographic feature between gene positive vs negative patients; the only significant difference was LV diastolic diameter that was greater in the gene-positive group (52 vs 57 mm, p=0.014). The results at follow up were on average better at follow up than at diagnosis, in particular the median LVEF increased from 41% to 55%. (Table XIV)

Table XIV. Echocardiogram data of the whole patients' cohort and by genetics results (positive vs. negative).

Echocardiogram at follow-up		N	Whole cohort	Genetics		P value
				-	+	
Left Ventricle Wall Thickness, median (IQR), mm		81	8.00 (8.00, 9.00)	8.00 (8.00, 9.00)	9.00 (7.50, 9.50)	0.5
Left Ventricle Diameter, median (IQR), mm	Systolic	68	36 (30, 47)	36 (30, 45)	40 (30, 48)	0.3
	Diastolic	81	53 (48, 57)	52 (47, 57)	57 (50, 62)	**0.014**
Mitral insufficiency, n (%)	Absent (-)	80	20 (25%)	16 (24%)	4 (31%)	0.7
	Trivial (+/-)		30 (38%)	25 (37%)	5 (38%)	

	Mild (+)		22 (28%)	20 (30%)	2 (15%)	
	Moderate (2+)		8 (10%)	6 (9.0%)	2 (15%)	
	Severe (4+)		0 (0%)	0 (0%)	0 (0%)	
Left Atrium Volume, median (IQR), ml/m^2		69	36 (28, 44)	36 (28, 45)	38 (29, 42)	0.8
Left Atrium, median (IQR), mm		32	38 (33, 42)	37 (33, 41)	38 (32, 43)	>0.9
Left Ventricle End Diastolic Volume, ml/m^2		82	65 (55, 82)	68 (58, 82)	62 (53, 92)	0.5
Left Ventricle Ejection Fraction, median (IQR), %		83	55 (47, 60)	55 (49, 60)	54 (45, 59)	0.4
Right Ventricle Telediastolic Area, median (IQR), cm^2		80	19.0 (17.0, 23.0)	19.0 (17.0, 23.0)	20.5 (15.0, 22.5)	>0.9
Fractional Area Change, median (IQR), %		80	40 (37, 45)	41 (37, 45)	40 (36, 46)	0.9
Tricuspid Annular Plane Systolic Excursion, median (IQR), mm		76	21.5 (18.0, 25.0)	22.0 (18.8, 25.0)	19.5 (18.0, 22.2)	0.3
Heart mass, median (IQR), g		80	160 (132, 206)	155 (132, 199)	183 (144, 229)	0.11
Heart mass/volume ratio, median (IQR), g/cm^2		80	86 (75, 110)	85 (75, 104)	107 (80, 115)	0.14

5.2.4 Biomarkers findings at follow-up

The results of biochemical analysis and autoantibodies serology are listed in table XV. The value of the troponin I peak was abnormal in 15% of cases, with a median value of 24 ng/L (IQR 18, 38), while C Reactive Protein (CRP) was abnormal in only one patient (6%) with a concentration of 6.9 mg/dl. The laboratory findings were heterogeneous between the two genetically divided subgroups but no statistically significant difference was found between them. (Table XV)

Table XV. Blood tests data of the whole patients' cohort and by genetics results (positive vs. negative).

Blood tests at follow up	N	Whole cohort	Genetics		p value
			-	+	
Abnormal troponin, n (%)	60	9 (15%)	7 (14%)	2 (18%)	0.7
Troponin levels, median (IQR), ng/L	9	24 (18, 38)	21 (16, 26)	60 (49, 70)	0.2
Abnormal Reactive C Protein, n (%)	16	1 (6.2%)	1 (6.7%)	0 (0%)	>0.9
Abnormal Brain Natriuretic Peptide, n (%)	22	6 (27%)	5 (29%)	1 (20%)	>0.9
Abnormal NT-proBNP, n (%)	29	18 (62%)	15 (65%)	3 (50%)	0.6
NT-proBNP levels, median (IQR), pg/L	19	334 (260, 1,080)	393 (276, 1,404)	218 (137, 494)	0.2

Legend: TnI= Troponin I; TnT= Troponin T.

5.2.5 Therapy at follow-up

Among the total cohort of patients at follow-up, 86% of patients were treated with antiarrhythmic therapy the most widely administered antiarrythmic drug being bisoprolol (46%). A minority (14%) of patients received anticoagulants, and nearly half (46%) ACE inhibitors. 18% of patients were treated with immunosuppressive therapy, according to international guidelines (229) the main reason being unremitting heart failure despite optimal medical therapy (80%). With respect to immunosuppression, the first-line therapy administered was in 54% of cases a combination of prednisone and azathioprine, and the median duration of immunotherapy was 4.5 months (IQR of 1, 10). In the second and third lines of immunosuppressive therapy the more frequently administered therapeutic combination was prednisone in association with Mycophenolate Mofetil (58% in the second line and 40% in the third line of therapy). No statistically significant differences were observable in terms of HF medical treatment neither immunsuppressive therapy; the only significant difference was a shorter duration of immunosuppressive therapy in genetic positive patients than genetic negative ones (502 days vs 723 days, p =0.030). (Table XVI)

Table XVI. Administered therapy data of the whole patients' cohort and by genetics results (positive vs. negative).

Therapy at follow up		N	Whole cohort	Genetics		p value
				-	+	
Antiarrhythmic therapy, n (%)		75	72 (86%)	57 (84%)	15 (94%)	0.4
Type of antiarrhythmic drug, n (%)	Bisoprolol	75	39 (46%)	32 (47%)	7 (44%)	0.8
	Carvedilolo		10 (12%)	8 (12%)	2 (12%)	>0.9
	Metoprolol		9 (11%)	6 (8.8%)	3 (19%)	0.4
	Sotalol		5 (6.0%)	3 (4.4%)	2 (12%)	0.2
	Atenolol		3 (3.6%)	3 (4.4%)	0 (0%)	>0.9
	Nadolol		1 (1.2%)	1 (1.5%)	0 (0%)	>0.9
	Ivabradine		3 (3.6%)	3 (4.4%)	2 (12%)	0.2

	Amiodarone		6 (7.1%)	6 (8.8%)	0 (0%)	0.4
Anticoagulant therapy, n (%)		84	12 (14%)	11 (16%)	1 (6.2%)	0.4
ACE inhibitors therapy, n (%)		84	39 (46%)	30 (44%)	9 (56%)	0.4
Sartans therapy, n (%)			19 (23%)	14 (21%)	5 (31%)	0.3
Immunosoppressive therapy, n (%)		84	15 (18%)	13 (19%)	2 (12%)	0.7
I line of specific immunotherapy, n (%)	PDN	13	1 (7.7%)	1 (9.1%)	0 (0%)	>0.9
	PDN+AZA		7 (54%)	5 (45%)	2 (100%)	
	PDN+MMF		1 (7.7%)	1 (9.1%)	0 (0%)	
	Other		3 (23%)	3 (23%)	0 (0%)	
	PDN+MTX		1 (7.7%)	1 (9.1%)	0 (0%)	
Therapy duration, median (IQR), days		4	134 (35, 302)	220 (110, 384)	47 (47, 47)	>0.9
II line of specific immunotherapy, n (%)	MMF	7	0 (0%)	0 (0%)	0 (0%)	>0.9
	PDN		0 (0%)	0 (0%)	0 (0%)	
	PDN+AZA		1 (14%)	1 (17%)	0 (0%)	
	PDN+MMF		3 (43%)	2 (33%)	1 (100%)	
	PDN+Cya		0 (0%)	0 (0%)	0 (0%)	
	Other		3 (43%)	3 (50%)	0 (0%)	
Response to therapy, n (%)		6	5 (100%)	1 (100%)	4 (80%)	>0.9
III line of specific immunotherapy, n (%)	AZA	2	1 (50%)	1 (50%)	0 (0%)	0.6
	MMF		0 (0%)	0 (0%)	0 (0%)	
	PDN+MMF		1 (50%)	1 (50%)	0 (0%)	
	PDN+Cya		0 (0%)	0 (0%)	0 (0%)	
Response to therapy, n (%)		2	2 (100%)	2 (100%)	0 (0%)	>0.9

Legend: ACE= Angiotensin Converting Enzyme; AHA=Anti Heart Antibodies; CMR=Cardiac Magnetic Resonance; PDN= Prednison; AZA= Azathioprine; MMF= Mycophenolate Mofetil; MTX= Methotrexate; Cya= Cyclosporin A.

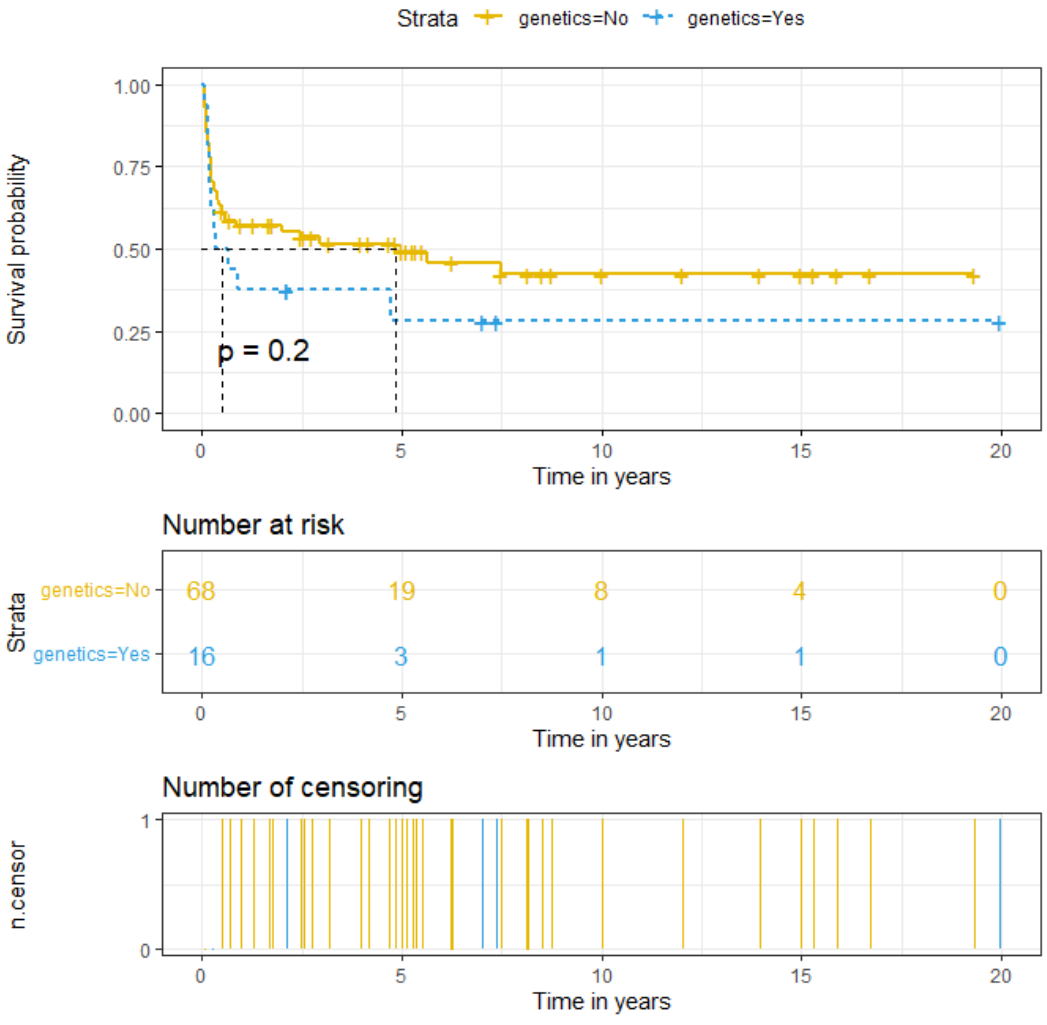
5.3 Statistical outcomes

The primary outcome was death or heart transplant, however, as it is shown in table XIII only one patient included in the study reached the primary outcome. For this reason, the statistical analysis utilized a surrogate outcome of clinical or instrumental disease progression and disease severity, which was the presence of a NYHA class>I or FE<50% calculated during echocardiography at follow-up.

5.3.1 Surrogate outcome at any follow-up

Figure 1 consider the secondary outcome in the two genetically subdivided cohort at any follow-up. The Kaplan Meier curves shows that both groups reach a plateau around 5 years from the start of follow up and the gap between the two groups is not statistically significant (p value of 0.2). This is coherent with the data collected during follow up reported in tables XIII-XVII.

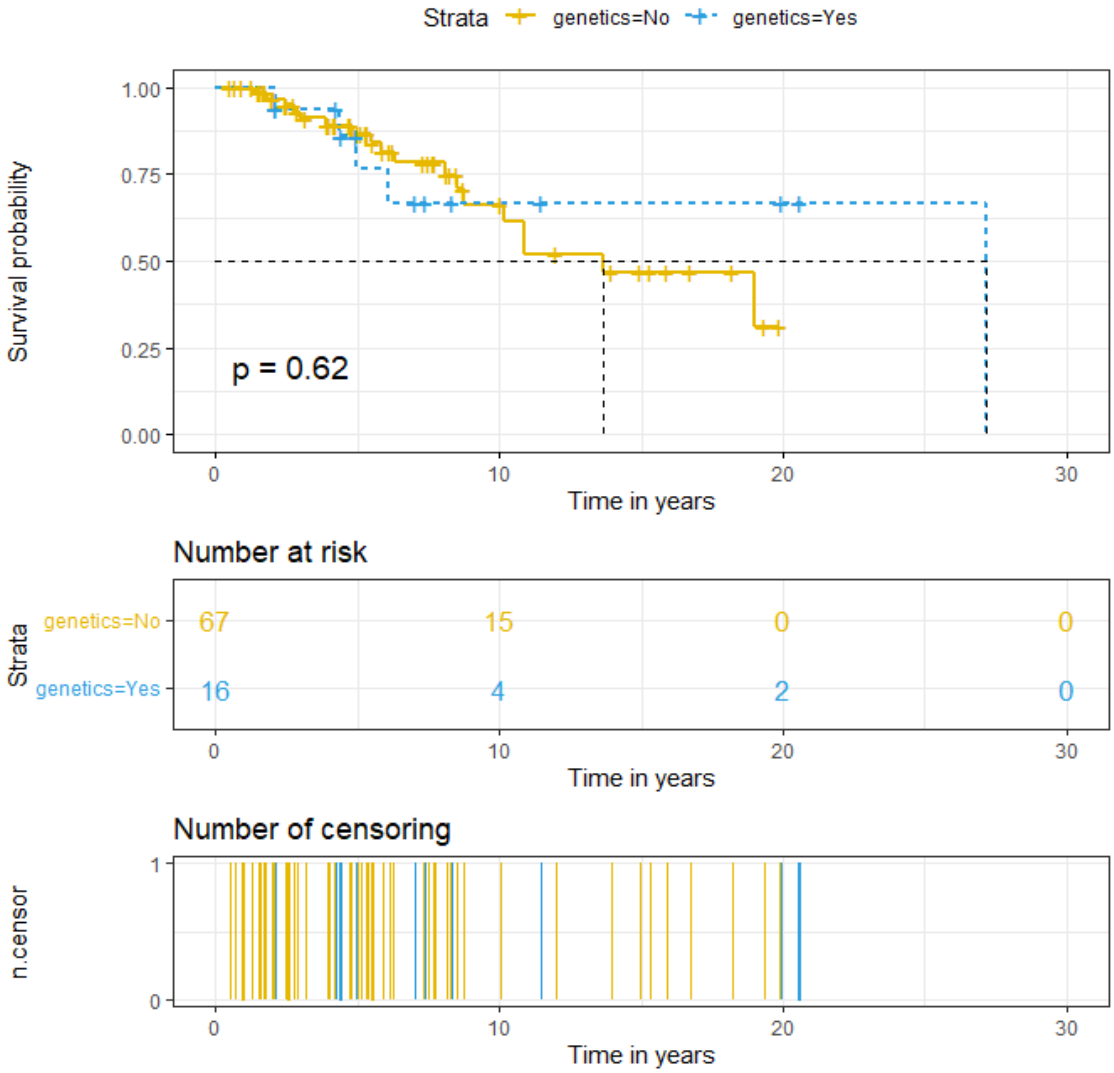
Figure 1. Outcome: NYHA > I or %EF < 50 at any follow-up



5.3.2 Surrogate outcome at last follow-up

Figure 2 considers the surrogate outcome only at the last follow-up. The Kaplan Meier curves intercept each other showing even more than in figure one the absence of significant difference between the two groups (with a higher p value of 0.62).

Figure 2. Outcome: NYHA > I or %EF < 50 at last follow-up



6.DISCUSSION

For the first time, a systematic, exhaustive genetic characterization of a prospective cohort of biopsy-proven myocarditis patients has been performed. In the present study, mutations in cardiomyopathy-associated genes did not show any correlation with the clinical (i.e. type or severity symptoms, fulminant presentation), imaging (i.e. biventricular function assessed on multimodality imaging) or histological (i.e. myocarditis type, viral genome assessed on PCR) features of myocarditis at diagnosis, nor at follow up.

Notably, all the patients included in the study have a histological diagnosis of myocarditis on EMB. This is crucial, with respect to previously published studies (286,305,306), since it ensures that a definite diagnosis of myocarditis was achieved in all cases. These data do not come from registries and patients were followed up in a single third-level centre, accounting for homogeneity in data assessment. Moreover, a detailed analysis of EMB, in keeping with the 2013 ESC recommendations (25), was performed, allowing a complete characterization of the disease in terms not only of histological, but also of immunohistochemical and microbiological aspects. In addition, among the pre-specified wide panel of variants included in the genetic analysis, only pathogenic variants (PV) were investigated, ensuring a precise genotype-phenotype correlation in positive cases.

A positivity for genetic mutations of cardiomyopathy-related genes was found in 16 of the 84 EMB-proven myocarditis patients (19%). This prevalence is similar to that reported in previous studies: in their work, Kontorovich et al report a frequency of genetic variants in 19/117 of predominantly clinically suspected acute myocarditis cases (16%) (306), while Lota et al found positive genetic variants in 8% of their 336 again mainly clinically suspected acute myocarditis patients cohort (286). In the present study, the majority of mutations were found in the *TTN* genes (8/16, 50%), but a variety of genetic mutations was present, *DSP* being the second more prevalent (3/19, 19%). Mutations in the FLN, *DES*, *MYH7*, *TNNT2* and *MYBPC3* were found in one patient each. This is in line with the literature: in fact, previous studies also reported a relatively higher prevalence of *TTN* (286,306) and *DSP* (305) mutations among gene positive cases. In the present

study, the family history of patients was also carefully investigated. A relevant proportion of gene positive patients (11/16, 69%) had a positive family history for heart disease, but in most cases for coronary artery disease (7/11, 50%). Only two of these patients has a positive family history of DCM and one for myocarditis.

The 84 EMB-proven myocarditis patients included in the study constitute a heterogeneous cohort, reflecting the wide, complex clinical spectrum of myocarditis presentation, clinical features and prognosis. A relevant quote of patients presented symptoms of HF at presentation (44%), but also infarct-like chest pain with normal coronary arteries (36%) and arrhythmias (14%) were frequent clinical presentations of the disease. In 7 cases, myocarditis had a fulminant hemodynamic onset, which is known to be a marker of dismal prognosis both at short and long term follow up (8). On ECG at diagnosis, a variety of abnormalities was observed, ranging from alterations in the cardiac rhythm (6%), to ST segment elevation (10%), to T wave inversion (being the lateral leads the most involved, 44%), to presence of BBB (right BBB in 9%, left BBB in 11%). This confirms the absence of a pathognomonic ECG pattern in myocarditis (307). On echocardiography, left ventricular function was overall reduced (median LVEF 41%), and despite gene positive patients having a lower LVEF at presentation (33% vs 44%), this difference was not statistically significant (p=0.5). The LVEF reduction was consistently observed also on left ventricular catheterization (mean LVEF 33%). Conversely, right ventricular function was generally preserved in the whole patients' cohort (median FAC 40% on echocardiography). Moderate or severe mitral regurgitation, a marker of adverse left ventricular remodelling and worse prognosis in HF (308), was present in more than a third (34.7%) of cases. CMR evaluation was performed in 64 patients, showing a generally preserved RV function (median RVEF in the whole cohort 50%), and a reduced LVEF (median LVEF in the whole cohort 44%). On CMR, edema on T2-weighted sequences was present in 48% of cases, and LGE on T1-weighted sequences was observed in 78%. In 16% of cases, CMR gave a diagnosis of DCM instead of myocarditis; this is in keeping with literature, since CMR sensitivity in myocarditis has already been proven particularly low in HF presentation (167). With respect to the antibody immune-phenotype, AHA positivity was observed in 50% of patients, and in the

majority of cases with an organ-specific pattern (42%), in keeping with previously reported data (309).

The analysis of EMB revealed a variety of histological types, being lymphocytic myocarditis the most represented type (74%), in keeping with literature (237). The second most prevalent histological type was borderline lymphocytic (14%). Four patients (4.8%) had a histological diagnosis of eosinophilic myocarditis, two of GCM (2.4%), and one of polymorphic myocarditis (1.2%). Fifteen patients had a diagnosis of viral myocarditis; viral PCR revealed a positivity for PVB19 in the majority of cases (9/14), and HHV-6 was also present in a relevant quote of cases (3/14). PVB19 was considered as pathogenic if a viral load of >500 copies/mm3 was found on a quantitative assessment of EMB, according to literature (60).

More than half (54%) of the whole patients' cohort were treated with immunosuppressive therapy (IT), in keeping with international recommendations (25,229), especially because of unremitting HF despite optimal medical therapy (64%). Another possible indication for IT was the presence of a systemic immune-mediated disease (SID) requiring specific medical therapy; indeed, according to international recommendations, the presence of myocarditis in the context of a SID is an indication to start, or intensify, IT regimen, being this a marker of poor prognosis (120). Among gene positive patients, IT was used in 12/16 (75%) of cases. IT was administered in a long-term fashion (median 22, IQR 12-26), only after exclusion of contraindications, according to the concept of a "safety check-list" (310), and involved mainly an association of prednisone and azathioprine, which are extensively described as the most efficacious and safe drugs in this setting (232,311). In case of lack of response to first line IT, or adverse drug reactions, a second line therapy was used, involving mainly mycophenolate mofetil and prednisone (58%), which is a drug association already proved to be useful in myocarditis (234). A third line IT was deemed necessary in a minority of cases (5 patients). All patients treated with IT were classified as responders at the end of the IT treatment, with no difference between gene positive vs negative patients. This could suggest that genetic positivity does not influence the response

to IT; nevertheless, studies specifically addressing possible factors influencing the response to IT in EMB-proven myocarditis are currently lacking.

The clinical curse of the disease was free from major cardiovascular adverse events (MACE) for the majority of patients during long-term follow up, which had a duration of 64 months (IQR 34-103). Only one patient underwent heart transplantation because of end-stage HF during the observation period, who was a carrier of a pathogenic mutation in the *TTN* gene. At last follow-up, the vast majority of patients (93%) showed no symptoms, with only a minority (7.1%) being in NYHA II functional class. A low rate of myocarditis relapse was observed (only 3 patients experienced myocarditis recurrence), and exclusively among gene negative patients. On echocardiography, LVEF was overall normalized in the overall population, with no statistically significant difference between gene positive (54%) vs gene negative (55%) patients (p=0.4). LVEDD was slightly higher in gene positive patients (57 vs 52 mm, p=0.014), but LVEDVs were not different (62 vs 68 ml/mq, p= 0.5). Due to the low incidence of MACE during the follow up period of the study, two composite clinical outcomes were identified: presence of NYHA functional class >=2 and/or LVEF < 50% on echocardiography during any of the follow ups after diagnosis, or only al last follow up (Figure 2 and 1). The probability of survival from both of these surrogate outcomes was not different between gene positive vs gene negative patients (p=0.2 and p=0.62, respectively), indicating a lack of the role of genetic mutations in the disease clinical course and prognosis.

Our results partially differ from previously published studies. This may be explained by differences in the design and in the selected population of our study, with respect to other cohorts.

In 2021, Kontorovich et al were among the first to conduct a retrospective study on human patients with acute myocarditis and genetic mutation in genes classically associated with cardiomyopathy or neuromuscular disorders with cardiac involvement (306). They included paediatric and adult patients with both EMB-proven and clinically suspected myocarditis from three different registries, and viral PCR on EMB was available only for those belonging to two of such

registries. By investigating the rates of putatively damaging genetic variants between patients and healthy control, the Authors found no statistically significant differences in histology, viral positivity assessed on viral PCR or outcomes between gene positive vs negative cases. This is perfectly in line with our findings. The Authors concluded that such "cardiomyopathic" genes were candidates for genetic susceptibility to myocarditis, and hypothesised that mutations in proteins of the sarcomere or cytoskeleton could lead to an increased risk for myocarditis after viral infection. Conversely, in our study only 2/16 (13%) of gene positive patients have a molecular diagnosis of viral myocarditis on EMB. This seems to be in contrast with the hypothesis that genetic positivity confers a susceptibility to viral myocarditis. In fact, the majority of gene positive patients in our cohort developed autoimmune/immune-mediated (i.e. virus-negative) myocarditis, suggesting that similarly to other immune-mediated disease (i.e. autoimmune thyroiditis, SLE, etc), a polygenic predisposing background may account for the development of myocarditis and its progression to DCM (295,312–316). In addition, to date no data on the geographical distribution of such gene variants has been described, leading to difficulties in comparing studies conducted in different geographic areas (e.g. Southern vs Northern Europe).

In 2022, Lota et al conducted a study on a population-based cohort of 336 patients with acute myocarditis. Only a small minority (12 out 336) underwent EMB. The study involved two retrospective cohorts: the first one was predominantly made up by young individuals with infarct-like chest pain myocarditis presentation, troponin rise and preserved LVEF, while the second by slightly older patients with HF and reduced LVEF at diagnosis. The Authors found an enrichment of *DSP* mutations in the first cohort (3.1% vs 0.4% of healthy controls), and of *TTN* mutations in the latter (7% vs 1% of healthy controls). Mortality at 5 years was relatively low (5.4%, and not in all cases for cardiovascular causes), in line with our findings. The Authors concluded that genetic mutation carriers might remain phenotypically silent until the occurrence of an exogenous trigger. These data are in line with the prevalence of diseased genes that we also found in our study population (i.e. relatively higher prevalence of *TTN* and DPS mutations), but some important points should be highlighted. Firstly, the "second hit" hypothesis

postulated by the Authors is not sufficiently supported by the evidence; in particular it does not explain why gene negative patients develop the disease in a way that is completely similar to gene positive patients. Secondly, the lack of a definitive diagnosis of myocarditis was not reached in all cases (i.e. EMB was available in only 12 of the 336 patients), and one could argue that these patients were affected by other diseases (i.e. arrhythmogenic cardiomyopathy or DCM). Thirdly, the "second hit" event is not sufficiently described: even if such a role for a viral infection can be hypothesised, the lack of histological evidence of viral genome in the myocardium does not allow further speculations.

Furthermore, Ammirati et al investigated the prognostic role of PV or likely pathogenic variants of desmosomal genes in a retrospective multicentre cohort of 97 myocarditis patients, of which 36 did not undergo genetic testing. Histology data were available only in 36.1% of the 36 gene positive patients, and among these 12 patients not all matched he diagnostic criteria for myocarditis. Histological diagnosis of myocarditis was reached in just 9 genetic positive patients; EMB was also performed only in 28% in gene negative patients, and 27.8% in those without genetic testing. The vast majority of mutations (88.9%) in the 36 gene positive patients were *DSP* variants. The composite outcome of death, VT/VF, acute HF and myocarditis recurrence was met in 62.3% at 5 years in the gene positive patients. The Authors concluded that patients with acute myocarditis and desmosomal gene variants are at higher risk for myocarditis relapse and ventricular arrhythmias, and present more frequently a family history of myocarditis, NSVTs on telemetry, and septal or ring-like LGE pattern on CMR. The Authors also state that potential triggers of myocarditis in gene positive patients remain to be defined. Again, the lack of a precise histological characterization of the cohort does not allow to achieve robust conclusions (i.e. 23% of gene positive patients presented imaging criteria for arrhythmogenic cardiomyopathy on follow-up CMR), and the fact that 36/97 patients did not undergo genetic assessment may have altered the study results.

Besides, rare cases of familial cases of myocarditis, especially in paediatric age, have been sporadically reported (317,318), but these remain very uncommon and

seem to be the “exception that proves the rule”. Moreover, a clear physiopathological explanation of the possible link between the presence of genetic PV and myocarditis onset has not been defined yet, and it is clear that the incidental finding of an association does not imply causality. In addition, in some of the cases reported in the literature a definitive diagnosis of myocarditis on EMB was either not reached or available (317), while in other cases the relative abundance in genetic PV could have been caused by the study of already diagnosed familial cases (318). To date, international recommendations do not support genetic assessment as a routine testing in myocarditis, in all ages (25,283,319). However, it has been suggested that a “genetic basis” of myocarditis may be suspected when specific “red flags” are present in the patients’ personal or family medical history (320), the clinical examination (e.g. neurosensory disorders, keratoderma, woolly hair, skeletal muscle involvement), or biomarker (e.g. CK), ECG (e.g. epsilon wave), or CMR assessment (e.g. adipose infiltration or other ARVC criteria). Except from the extremely rare cases of familial genetic-determined myocarditis, the hypothesised genetic background is complex, likely and supported by few human studies (283). In fact, the majority of available evidence on genes predisposing to myocarditis development after viral infections and/or progression to DCM is based on *in vitro* or animal studies (321–323).

7. CONCLUSIONS

In our study, even if 19% of biopsy-proven myocarditis patients were found to have a genetic P/LP variant in cardiomyopathy-related genes, pathogenic mutations did not seem to exert any influence on the disease phenotype. In fact, the presence of genetic mutations did not lead to any difference in clinical, imaging or histological features at diagnosis or at follow up compared with gene-negative cases.

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