UNIVERSITÁ DEGLI STUDI DI UDINE

Department of Medical and Biological Sciences
PhD Course in Clinical Sciences and Technologies
XXVI Cycle (2011-2013)
PhD Research Thesis

Inflammasome and pro-inflammatory cytokines in the pathophysiology of idiopathic dilated cardiomyopathy before and after ventricular assist device insertion

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Academic Year 2014 - 2015

A Giovanni e Cinzia…

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Abstract

Inflammasome and pro-inflammatory cytokines in the pathophysiology of idiopathic dilated cardiomyopathy before and after ventricular assist device (VAD) insertion

Dilated cardiomyopathy (DCM) represents the most common cardiomyopathy worldwide. The familial type, named "idiopathic", account for 20-48% of all cases.

An accumulating body of literature indicates that the pathophysiologic mechanism of genetic DCM may be much more complex than expected involving the finely tuned mechanism of protein homeostasis. Intracellular proteins aggregation seems to be at the base of the activation of the inflammasome bringing to the release of inflammatory cytokines, such as IL1ß, IL 4 and IL 6 and may be involved in the clinical deterioration of patients affected by idiopathic DCM.

For this purpose the cytokine levels of surgical patients affected by idiopathic terminal DCM were compared with healthy controls to assess the hypothesis that pro inflammatory status plays a role on the natural history of the DCM and to see if the VAD insertion was related to a reduction of the inflammasome activation.

10 idiopathic DCM affected patients who underwent VAD insertion were admitted to the study. The mean follow up was 377 days. The analysis of the circulatory biomarkers showed statistically significant higher concentrations of IL1ß, IL4, IL6 and of IL1ß/ IL1RA ratio in the DCM group.

The follow up analysis of the circulatory BNP levels showed that after the VAD insertion there was a BNP non-significant augmentation during the first post-operatory week and then a reduction during the long-term follow-up while IL 1β and the IL4 levels did not show significant variation.

The correlation analysis between the different biomarkers found that BNP significantly correlate in a negative way to IL1ß (P=0.0462; Spearman r = -0.4292), IL4 (P=0.004; Speerman r = -0,692), and Leptin (P=0.026; Speerman r = -0,462) and in a positive way

to IL 6 (P=0.026; Speerman r = 0,475).

Taken together, these results suggest that in end stage heart failure idiopathic DCM patients there is a higher concentration of pro inflammatory cytokines that can play a role on the progression of the disease.

The reduction of the volume overload done by the VAD does not reverse the mechanisms of chronic inflammation.

I. Introduction

1- The Failing Heart

1.1 Heart Failure: definition

In the American Heart Association (AHA)/American College of Cardiology Foundation (ACCF) guidelines, Heart failure (HF) is defined as a complex clinical syndrome that can result from any structural or functional cardiac disorder that impairs the ability of the ventricle to fill or eject blood [1].

HF may be associated with a wide spectrum of left ventricular (LV) functional abnormalities, which may range from patients with normal LV size to those with severe dilatation and/or markedly reduced ejection fraction (EF). In most patients, abnormalities of systolic and diastolic dysfunction coexist [2].

The ACCF/AHA defines HF in two groups on the basis of the left ventricular ejection fraction[2]:

1. Systolic HF, characterized by a reduced ejection fraction (EF≤40%), and
2. Diastolic HF, when the ejection fraction is preserved (EF≥50%).

1.2 Classification

Both the AHA/ACCF and the New York Heart Association (NYHA) proposed a model in which HF is classified on the basis of functional parameters that emphasize its evolution, severity and progression.
The ACCF/AHA stages of HF emphasize the progressive nature of this disease and can be used to describe individuals and populations, whereas the NYHA classes are merely symptomatic [2].

The ACCF/AHA stages of HF (table 1.1) recognize that both risk factors and abnormalities of cardiac structure are associated with HF. The stages are progressive and inviolate; once a patient moves to a higher stage, regression to an earlier stage of HF is not observed [2].

ACCF/AHA Stages of HF	
Stage A	*At high risk for HF but without structural heart disease or symptoms of HF*
Stage B	*Structural heart disease but without signs or symptoms of HF*
Stage C	*Structural heart disease with prior or current symptoms of HF*
Stage D	*Refractory HF requiring specialized interventions*

Table 1.1 *ACCF/AHA classification of Heart Failure* [2; 3]

The NYHA functional classification (table 1.2) gauges the severity of symptoms in patients with structural heart disease, primarily stages C and D; it represents an independent predictor of mortality. It is widely used in clinical practice and research and for determining the eligibility of patients for healthcare services [2]. In contrast with ACCF/AHA model, HYHA classes are reversible, and a patient with NYHA class IV symptoms might have improvements to class III with diuretic therapy alone[3].

NYHA Functional Classification of HF	
Class I	*Patients with cardiac disease but without resulting limitation of physical activity. Ordinary physical activity does not cause undue fatigue, palpitations, dyspnea or anginal pain.*
Class II	*Patients with cardiac disease resulting in slight limitation of physical activity. They are comfortable at rest. Ordinary physical activity results in fatigue, palpitation, dyspnea or anginal pain.*
Class III	*Patients with cardiac disease resulting in marked limitation of physical activity. They are comfortable at rest. Less than ordinary physical activity causes fatigue, palpitation, dyspnea or anginal pain.*
Class IV	*Patients with cardiac disease resulting in inability to carry on any physical activity without discomfort. Symptoms of HF or the anginal syndrome may be present even at rest. If any physical activity is undertaken, discomfort is increased.*

Table 1.2 *NYHA classification of Heart Failure* [2; 4]

1.3 Epidemiology

HF is a burgeoning problem worldwide, with more than 23 million people affected. In the United States, prevalent cases of HF exceed 5,8 million and each year >550,000 new cases are diagnosed. The overall prevalence of HF in the adult population in developed countries ranges from 1% to 12% based on available data from the United States and Europe [5].

HF prevalence follows an exponential pattern, rising with age, and affects 6-10% of people over age 65. Although the relative incidence of HF is lower in women than in men, women constitute at least one-half the cases of HF because of their longer life expectancy. In North America and Europe, the lifetime risk of developing HF is approximately one in five for a 40-years-old. The overall prevalence of HF is thought to be increasing, in part because current therapies for cardiac disorders, such as myocardial infarction (MI), valvular heart disease and arrhythmias are allowing patients to survive longer [4].

1.4 Etiology

Any condition that leads to an alteration in LV structure or function can predispose a patient to developing HF. Among the risk factors for HF development, the most relevant are hypertension, diabetes mellitus, metabolic syndrome and atherosclerotic disease [2]. Although the etiology of HF in patients with a preserved EF differs from that of patients with reduced EF, there is considerable overlap between the etiologies of these two conditions [4].

A major cause of systolic HF is represented by coronary artery disease (CAD) with

antecedent myocardial infarction. The prevalence of this form of HF is approximately 50%. Hypertension remains the most important cause of diastolic HF, with a prevalence of 60% to 89% from large controlled trials, epidemiological studies and HF registries, although obesity, CAD, diabetes mellitus, atrial fibrillation and hyperlipidemia represent other associated cardiovascular risk factors.

In 20-30% of the cases of HF with a reduced EF, the exact etiology is not known. These patients are referred to as having non-ischemic, idiopathic cardiomyopathy if the cause is unknown. A large number of cases of cardiomyopathies are secondary to specific genetic defects, most notably those in the cytoskeleton (mutations of genes encoding for ß-myosin heavy chain, myosin-binding protein C, cardiac troponin T, desmin, and vinculin), in the nuclear membrane proteins (i.e. lamins) or ion channels [4].

1.5 Prognosis

Despite many recent advances in the evaluation and management of HF, the development of symptomatic HF still carries a poor prognosis, with 30-40% of patients die within 1 year of diagnosis [4] and 50% mortality rate of 5 years, mainly from worsening HF or as a sudden event (e.g.ventricular arrhythmia) [2]. Although it is difficult to predict prognosis in an individual, patients with symptoms at rest (NYHA class IV) have a 30-70% annual mortality rate, whereas patients with symptoms with moderate activity (NYHA class II) have an annual mortality rate of 5-10%. Thus, functional status is an important predictor of patient outcome [4].

1.6 Pathogenesis

HF may be viewed as a progressive disorder that is initiated after an index event either damages the heart muscle, with a resultant loss of functioning cardiac myocytes, or, alternatively, disrupts the ability of the myocardium to generate force, thereby preventing the heart from contracting normally. This index event may have an abrupt onset, as in the case of

a myocardial infarction (MI); it may have a gradual or insidious onset, as in the case of hemodynamic pressure or volume overloading; or it may be hereditary, as in the case of many of the genetic cardiomyopathies. Regardless of the nature of the inciting event, the feature that is common to each of these index events is that they all in some manner produce a decline in the pumping capacity of the heart (fig. 1.1)[4; 6].

In most cases, patients remain asymptomatic or minimally symptomatic after the initial decline in pumping capacity of the heart or develop symptoms only after the dysfunction has been present for some time.

One potential explanation is that a number of compensatory mechanisms become activated in the presence of cardiac injury and/or LV dysfunction allowing patients to sustain and modulate LV function for a period of months to years.

These compensatory mechanisms include:

1. Activation of the renin-angiotensin-aldosterone (RAA) and adrenergic nervous systems, which are responsible for maintaining cardiac output through increased retention of salt and water;
2. Increased myocardial contractility.
3. Activation of a family of vasodilatory molecules (atrial and brain natriuretic peptides - ANP and BNP -, prostaglandins - PGE2 and PGI2 -, and nitric oxide - NO) that offset the excessive peripheral vascular vasoconstriction[4].

The transition to symptomatic HF is accompanied by increasing activation of neurohormonal, adrenergic and cytokine systems that lead to a series of maladaptive changes within the myocardium, collectively referred to as LV remodeling[4].

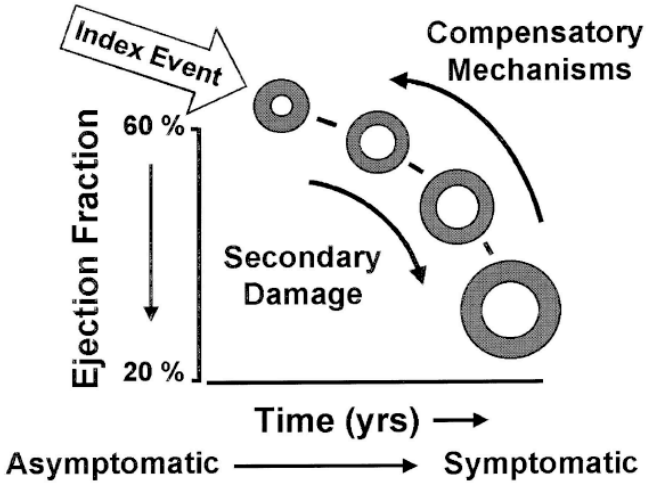


Figure 1.1 Pathogenesis of Heart Failure.
HF begins after an index event produces an initial decline in pumping capacity of the heart. A variety of compensatory mechanisms are activated. In the short term, cardiovascular function is restored to a homeostatic range. However, with time, the sustained activation of these systems can lead to secondary end-organ damage within the ventricle, with LV remodeling and cardiac decompensation. As a result, asymptomatic HF becomes symptomatic.
Taken from (Mann et al., 1999)

1.7 Basic mechanisms of left ventricular remodeling

LV remodeling develops in response to a series of complex events that occur at the cellular and molecular levels. It is defined as the process by which mechanical, neurohormonal and possibly genetic factors alter ventricular size, shape and function.
Remodeling occurs in several clinical conditions, including myocardial infarction, cardiomyopathy, hypertension and valvular heart disease; its hallmarks include hypertrophy, loss of myocytes and increased interstitial fibrosis[3].

1.7.1 Myocardial infarction

Myocardial infarction (MI) is characterized by an acute loss of blood flow to a region of myocardium that results in myocyte necrosis, myofibroblast proliferation, cytokines release from injured myocytes and infiltration of circulating inflammatory cells. Much of this early inflammatory response results in reabsorption of necrotic tissue and the promotion of fibrotic scar formation prior to acute tissue remodeling[7]. In ischemic cardiomyopathy, changes in the ventricular architecture interest both the infarcted and noninfarcted tissue and remodeling is typically divided into acute and chronic stages ranging from hours to days and days to years[7].
The acute loss of myocardial cells results in abnormal loading conditions which induce

dilatation and the change of the ventricular shape, rendering it more spherical, as well as causing hypertrophy (fig. 1.2)[3].

In its compensated stage, chamber dilation is produced by myocytes lengthening, whereas the ventricular number of cells is not altered. Importantly, myocyte diameter also increases, so that a modest absolute increase in wall thickness occurs and the ratio of wall thickness-to-chamber radius remains constant, keeping constant the cardiac wall stress, that follows Laplace law. In addition, the proportion between ventricular mass and chamber volume does not vary. When these relations are not preserved, decompensated concentric and eccentric hypertrophy develop, thus increasing cardiac wall stress[8]. Remodeling continues for months after the initial insult and the eventual change in the shape of the ventricle becomes detrimental to the overall function of the heart as a pump[3].

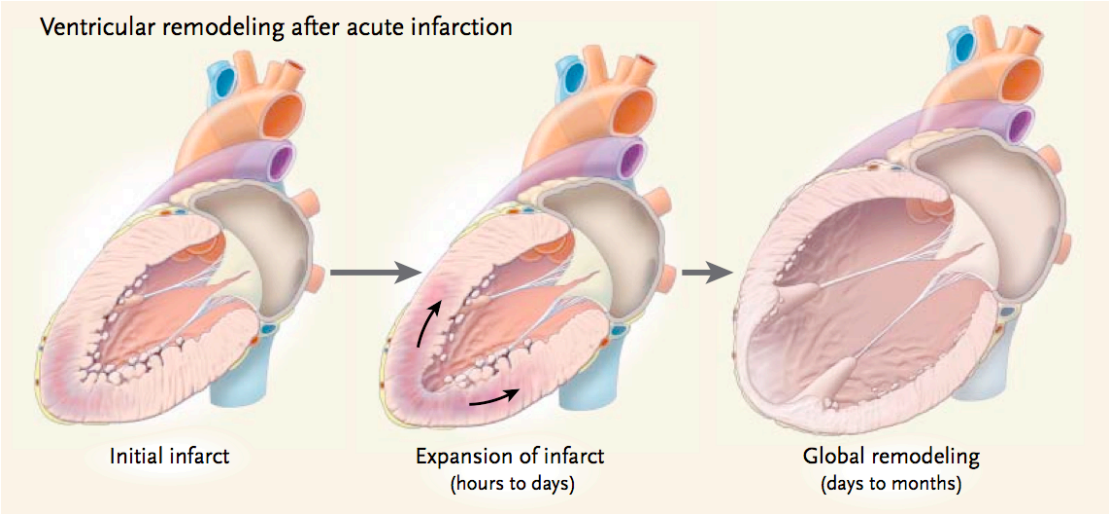


Figure 1.2 Ventricular remodeling after infarction. Few hours-days after an acute myocardial infarction the area of myocardium affected by the infarction begins to expand and becomes thinner; within days to months, global remodeling can occur, resulting in overall ventricular dilatation, decreased systolic function and mitral valve dysfunction.
Adapted from (Jessup et al., 2003)

1.8 Treatment

Once patients have developed structural heart disease, their therapy depends on their classification (fig. 1.3). For patients who have developed LV systolic dysfunction but remain asymptomatic (class NYHA I, ACCF/AHA stage A), the goal should be to slow disease progression by blocking neurohormonal system, reducing cardiac remodeling[3; 4]. For patients with heart failure and a low ejection fraction (Class NYHA II-IV, ACCF/AHA stage B-D) the primary goal is to alleviate symptoms, lessen disability, minimize risk factors and reduce the progression of disease, improving survival[3; 4]. These goals generally require a strategy that combines diuretics (to control salt and fluid retention) with neurohormonal interventions (to minimize cardiac remodeling)[4].

One third of patients with reduced EF and symptomatic HF (NYHA class III-IV) manifest abnormal inter- or intraventricular conduction with dyssynchronous ventricular contraction; the consequences of this latter include suboptimal ventricular filling, a reduction in LV contractility and prolonged duration of mitral regurgitation. Biventricular pacing, also termed cardiac resynchronization therapy (CRT), stimulates both ventricles nearly simultaneously, improving the coordination of ventricular contraction and reducing the severity of mitral regurgitation[4].

Implantation of implantable cardiac defibrillators (ICDs) in patients with mild to moderate HF (NYHA class II-III) and EF≤35% has been shown to reduce the incidence of sudden cardiac death in patients with ischemic or nonischemic cardiomyopathy. An ICD may also be combined with a biventricular pacemaker in patients with NYHA class III-IV HF[4].

Instead, for the management of patients with HF and preserved EF, there are no proven and/or approved pharmacologic or device therapies[4].

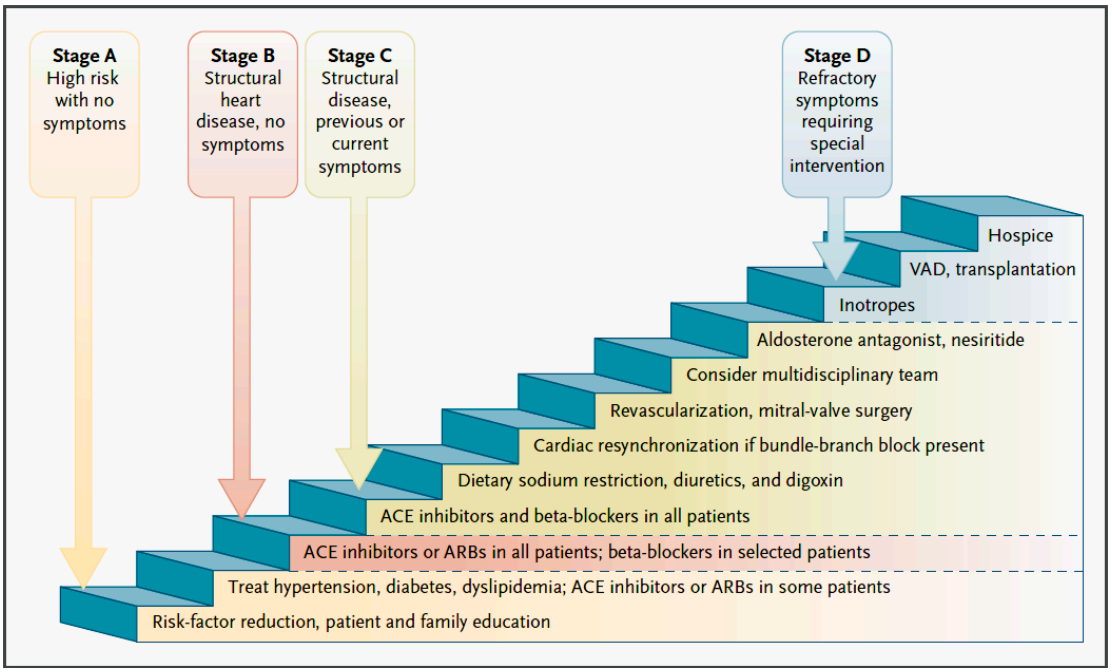


Figure 1.3 Stages of Heart Failure and treatment options for systolic Heart Failure. Taken from (Jessup et al, 2003)

The final treatment for end-stage HF is represented by cardiac transplantation or prolonged assisted circulation. In the last years, a variety of extracorporeal pumps to provide circulatory support for variable periods of time have been developed. Although conceived of initially as alternatives to biologic replacement of the heart, left ventricular assisted devices (LVADs) were introduced as, and are still employed primarily as, temporary "bridges" to heart transplantation in patients who begin to fail medical therapy before a donor heart becomes available. However, improvements in this field have been so dramatic that now they can be regarded as destination therapy for a subset of patients that are not eligible for heart transplantation. Unfortunately, these long-term devices are not totally implantable yet and, because of the need of transcutaneous connections, all share a common problem with infectious complications. They also all share some tendency to thromboembolic complications as well as the expected possibility of mechanical device failure common to any machine. Last, a subset of patients with stage D or refractory end-stage HF who are younger and without significant comorbidities, can be considered as candidates for heart transplantation[4].

1.9 Looking at the future

Pharmacologic and interventional strategies have dramatically improved the outcomes of cardiac disease, but fail to adequately prevent disease progression. Current therapeutic options for end-stage HF patients are limited to cardiac transplantation (with the option of mechanical cardiac assistance as a "bridge" to transplantation) or the option of permanent mechanical assistance of the circulation. Heart transplantation is limited by donor availability (increasing imbalance between supply and demand) and lifelong immunosuppression for transplant recipients; it suffers also of ethical problems concerning the transplantation of another person's organ[4; 9].

In the future, it is possible that genetic modulation of ventricular function or cell-based cardiac repair will be options for such patients. At the present, both approaches are considered to be experimental[4].

Current therapeutic approaches are palliative in the sense that they do not address the underlying problem of the loss of the cardiac tissue; the progression of the regenerative medicine is allowing the development of stem-cell based therapies that have the potential to transform the treatment of HF by achieving myocardial regeneration[10]. Regenerative medicine is defined as "the reconstruction of diseased or injured tissues by activation of endogenous cells or by cell transplantation"[11]. The role and contribution of stem cells in modern medicine is of paramount importance, both for their broad use in basic research and for the opportunities they give us to develop new therapeutic strategies in clinical practice. In addition, stem cells may be able to replace damaged tissues or even regenerate organs[12].

Numerous preclinical and clinical studies have been performed that support the ability of various stem cell populations to improve cardiac function and reduce infarct size in both ischemic and nonischemic cardiomyopathy[10].

2- Cardiomyopathies

2.1 Definition

Cardiomyopathies are defined as myocardial disorders in which the myocardium has abnormal structure and/or function leading to deterioration of myocardial performance often resulting in the development of clinical heart failure.

2.2 Classification

Cardiomyopathies are classified traditionally according to morphological and functional criteria into four categories:
Dilated cardiomyopathy (DCM),
Hypertrophic cardiomyopathy (HCM),
Restrictive cardiomyopathy (RCM),
Arrhythmogenic right ventricular (RV) cardiomyopathy/dysplasia (ARVC/D).

DCM is the most common form of heart muscle disease, comprising approximately 60% of all cardiomyopathies and characterized by left ventricular (LV) dilation and systolic dysfunction. The dilated cardiomyopathy is often assumed as a common pathway of several cardiovascular pathologies.

Cardiomyopathies are also classified by the American Heart Association (AHA) into two major groups based on predominant organ involvement.
Primary cardiomyopathies (genetic, nongenetic, or acquired): solely or predominantly confined to heart muscle.
Secondary cardiomyopathies: present pathological myocardial involvement as part of a several number of systemic pathologies [11,12].

Primary cardiomyopathies	Genetic	HCM/ ARVC/ LVNC/ Conduction defects/ Mitochondrial myopathies/ ion channel disorders
	Mixed	DCM/ restrictive
	Acquired	InÁammatory/ Tako-Tsubo/ Peripartum/ Tachycardia induced/ Infants of IDDM mothers
Secondary cardiomyopathies	InÀltrative	Amyloidosis, Gauchers, Hurler's, Hunter's
	Storage	Fabry's, Glycogen storage disease, Niemann-Pick disease, haemochromatosis
	Toxicity	Drugs, heavy metals
	Endomyocardial	EMF, LoefÁer's endocarditis
	InÁammatory	Sarcoidosis
	Endocrine	Diabetes, hyperthyroidism, hypothyroidism, hyperparathyroidism
	Cardiofacial	Noonan's, lentiginosis
	Neuromuscular	Friedreich's ataxia, Duchenne-Becker muscular dystrophy, myotonic dystrophy
	Nutritional	Beriberi, scurvy, selenium
	Autoimmune	SLE, dermatomyositis, scleroderma
	Consequence of cancer therapy	Anthracyclines, radiation, cyclophosphamide,

ARVC: Arrhythmogenic right ventricular cardiomyopathy/ dysplasia; DCM: Dilated cardiomyopathy; HCM: Hypertrophic cardiomyopathy; LVNC: Left ventricular non-compaction; EMF: Endomyocardial Àbrosis.

In 2013 a new classification, the MOGE(S) classification, was proposed by the World Heart Federation for a phenotype-genotype nomenclature of cardiomyopathies[13].

This classification suggests a nosology that addresses five characteristics of cardiomyopathic disorders:

- morphofunctional state (M),
- organ involvement (O),
- genetic inheritance (G),
- etiologic annotation (E),
- functional state (S) according to ACC/AHA A-D stage and New York Heart Association (NYHA)I-IV functional class.

The MOGE(S) classification has several advantages with regard to simultaneous maximal description of disease from clinical and genetic points. However, this classification does not fulfil the diagnostic criteria of cardiomyopathies in several clinical situations and may not be always applied in clinical practice, because of the lack of genetic testing in many clinical centres. On the other hand, the classification based on systematically genetic testing and monitoring may cause overdiagnostic states without clinically evident signs of cardiomyopathies and absence of clinical phenotype. Further genetic research and development of multicenter registries are needed to clarify the clinical advantages and to make more practical of MOGE(S) classification of cardiomyopathies.

3 Dilated cardiomyopathy

3.1 Definition

Dilated cardiomyopathy (DCM) represents the most common cardiomyopathy worldwide, accounting for approximately 55% of cardiomyopathies [14].

It is a heart muscle disorder defined by the presence of a dilated and poorly functioning left or both ventricles.

Clinical manifestations include heart failure (HF), thromboembolism, and sudden cardiac death (SCD). The age at onset includes newborn through late adulthood, although most patients are diagnosed between 20–50 years of age [15].

3.2 Etiology

It can be primary (genetic, mixed or predominantly familial nongenetic, or acquired) or secondary (inflammatory, autoimmune, or thyrotoxic).

This disease can be diagnosed in association with recognized cardiovascular disease; however, to be qualified as DCM, the extent of myocardial dysfunction cannot be explained exclusively by abnormal loading conditions (hypertension or valve disease) or ischemic heart disease.

A large number of cardiac and systemic diseases can cause systolic dysfunction and LV dilatation, but in the majority of cases no definite cause is found. This has led to the common terminology idiopathic dilated cardiomyopathy (IDC).

3.3 Epidemiology

Prevalence in the general population remains undefined.

This disorder develops at any age, in either sex, and in people of any ethnic origin.

In adults, DCM arises more commonly in men than in women.

In children, the yearly incidence is 0.57 cases per 100000, but is higher in boys than in girls (0.66 vs 0.47 cases per 100000, $P < 0.006$).

In adults, the prevalence is 1 in 2500 individuals, with an incidence of 7 per 100000 per year (but it could be underdiagnosed). The prevalence of DCM in the United States (adjusted for age) is 36 per 100000 of the population [16,17,18].

In many cases, the disease is inherited, and is called familial dilated cardiomyopathy (FDC).

4. FAMILIAL DILATED CARDIOMYOPATHY

Familial DCM refers to DCM that is inherited as a single gene disorder in a Mendelian pattern.

The primary mode of inheritance for familial DCM is autosomal dominant, with X-linked autosomal recessive and mitochondrial inheritance being less frequent.

About 20%-48% of DCM has been reported as familial [19].

There are sufficient data that with new diagnosis of IDC the clinical screening of first-degree family members will reveal familial (genetic) DCM in 20%-35% of those family members.

Recent guidelines recommend that genetic testing should be provided in families in whom familial DCM is suspected for early diagnosis of cardiomyopathy in family members.

4.1 Pathophysiology

Towbin and colleagues proposed more than a decade ago that the development of hereditary cardiac diseases with similar phenotypes was associated with the mutation of genes encoding proteins with similar functions.

After this first suggestion it has been demonstrated by many other authors that genetic DCM are typically associated with mutations involving genes encoding proteins of the sarcomere, cytoskeleton, sarcoplasmic reticulum, T-tubules and others proteins that may perturb force generation [21].

Despite the many confirmations of the Tobin theory, the genotype / phenotype relationship observed in DCM is not straightforward, and mutations of the same gene (e.g. those affecting lamin A/C) may have completely different clinical outcomes [22].

This phenotypical heterogeneity may be explained by different disruption of specific protein binding partners, the presence of comorbidities, differences in penetrance, or genetic resistance to adverse remodelling [20].

In addition to the above mentioned variables involved on the DCM pathogenesis an accumulating body of literature is indicating that the pathophysiologic mechanism of genetic DCM may be much more complex with an involvement of the finely tuned mechanism of protein synthesis and degradation that maintains cell homeostasis and mechanism of sterile chronic inflammation [23].

4.1.1 Genetic mutations in DCM

Table 1
Cardiomyopathy genes

Gene	Gene name	Cardiomyopathy subtype(s)
ABCC9	ATP-binding cassette, sub-family C, member 9	DCM
ACTC1	Actin, α, cardiac muscle 1	DCM, HCM, LVNC
ACTN2	Actinin, α2	DCM, HCM
ANKRD1	Ankyrin repeat domain 1 (cardiac muscle)	DCM, HCM
BAG3	BCL2-associated athanogene 3	DCM
CASQ2	Calsequestrin 2 (cardiac muscle)	LVNC, CPVT
CAV3	Caveolin 3	HCM
COX15	COX15 homolog, cytochrome c oxidase assembly protein	HCM
CRYAB	Crystallin α B	HCM
CSRP3	Cysteine and glycine-rich protein 3	DCM, HCM
CTF1	Cardiotrophin 1	DCM
DES	Desmin	DCM
DMD	Dystrophin	DCM
DNAJC19	DnaJ (Hsp40) homolog, subfamily C, member 19	DCM
DSC2	Desmocollin 2	DCM, ARVC
DSG2	Desmoglein 2	ARVC
DSP	Desmoplakin	DCM, ARVC
DTNA	Dystrobrevin, α	LVNC
EMD	Emerin	DCM
EYA4	Eyes absent homolog 4	DCM
FHL2	Four and a half LIM domains 2	DCM
FKTN	Fukutin	DCM
FOXD4	Forkhead box D4	DCM
GLA	Galactosidase, α	HCM
JUP	Junction plakoglobin	ARVC
LAMA4	Laminin, α4	DCM
LAMP2	Lysosomal-associated membrane protein 2	DCM, HCM
LDB3	LIM domain binding 3	DCM, LVNC
LMNA	Lamin A/C	DCM, LVNC
MYBPC3	Myosin binding protein C, cardiac	DCM, HCM, LVNC
MYH6	Myosin, heavy chain 6, cardiac muscle, α	DCM, HCM
MYH7	Myosin, heavy chain 7, cardiac muscle, α	DCM, HCM, LVNC
MYL2	Myosin, light chain 2, regulatory, cardiac, slow	HCM
MYL3	Myosin, light chain 3, alkali; ventricular, skeletal, slow	HCM
MYLK2	Myosin light chain kinase 2	HCM
MYOZ2	Myozenin 2	HCM
NEXN	Nexilin (F actin binding protein)	DCM, HCM
PKP2	Plakophilin 2	ARVC
PLN	Phospholamban	DCM, HCM
PRKAG2	Protein kinase, AMP-activated, γ 2, non-catalytic subunit	HCM
PSEN1	Presenilin 1	DCM
PSEN2	Presenilin 2	DCM
RBM20	RNA binding motif protein 20	DCM
RYR2	Ryanodine receptor 2 (cardiac)	CPVT
SCN5A	Sodium channel, voltage-gated, type V, α subunit	DCM
SDHA	Succinate dehydrogenase complex, subunit A, flavoprotein	DCM
SGCD	Sarcoglycan, δ	DCM
SYNE1	Spectrin repeat containing, nuclear envelope 1	DCM
SYNE2	Spectrin repeat containing, nuclear envelope 2	DCM
TAZ	Tafazzin	DCM, LVNC
TCAP	Titin-cap (telethonin)	DCM
TMEM43	Transmembrane protein 43	ARVC
TMPO	Thymopoietin	DCM
TNNC1	Troponin C type 1 (slow)	DCM, HCM
TNNI3	Troponin I type 3 (cardiac)	DCM, HCM
TNNT2	Troponin T type 2 (cardiac)	DCM, HCM, LVNC
TPM1	Tropomyosin 1 (α)	DCM, HCM
TTN	Titin	DCM, HCM, ARVC
TTR	Transthyretin	HCM
VCL	Vinculin	DCM, HCM

CPVT, catecholaminergic polymorphic ventricular tachycardia; LVNC, LV noncompaction.

At least 50 single genes have been identified as linked to familial DCM [6, 7].

Some of the genes linked to DCM encode proteins of the sarcomere, costamere, Z band, and nuclear membrane, while others have functions distinct from these broad cell biological classifications (Table 1).

Sarcomere DCM genes.

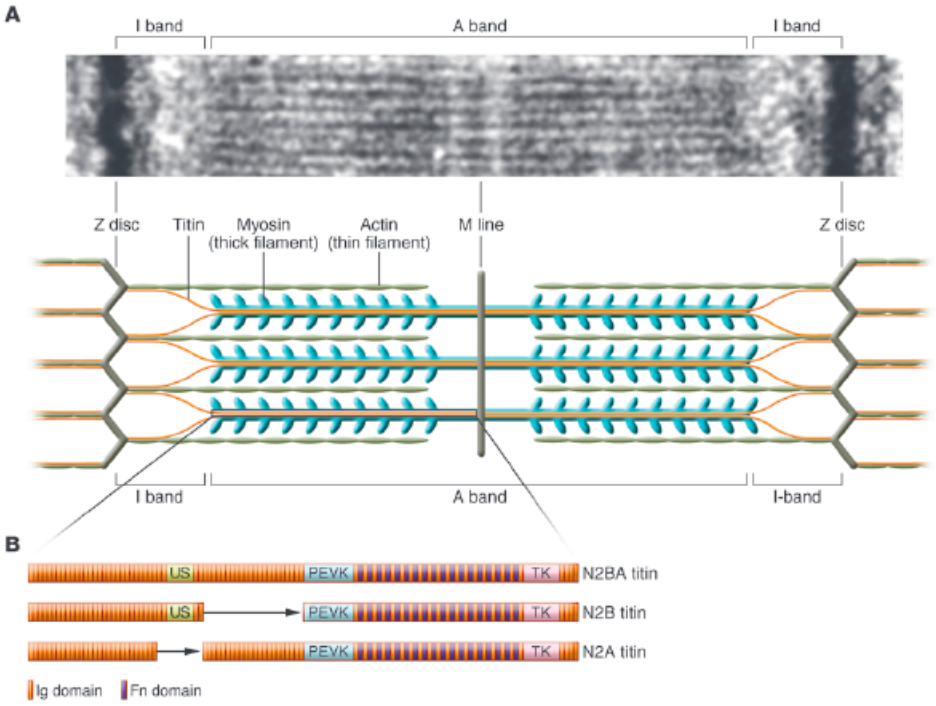


Figure 1: The sarcomere is a target for cardiomyopathy mutations. (**A**) The sarcomere from Z disc to Z disc. Electron micrograph of a sarcomere from a human heart. The TTN gene encoding the giant protein titin is mutated in DCM. Titin's amino terminus anchors in the Z band, and its carboxy terminus ends in the M band. The titin kinase (TK) domain is found at the carboxy terminus and, when mutated, results in impaired stretch sensing and signaling. Titin interacts with both the thin and thick filaments. The thick-filament proteins (grey) are encoded by MYH7 and MYBPC3, two genes commonly linked to HCM. (**B**) Isoforms of titin. Alternative splicing in the region of titin that encodes the I band gives rise to isoforms with varying spring properties. The N2B isoform is found exclusively in cardiac muscle and the N2A isoform in skeletal muscle. The N2BA isoform is also found in cardiac muscle and contains features found in both N2B and N2A titin. N2BA titin has a longer extensible I band region than N2B titin, making it more compliant. US, unique sequence; Fn, fibronectin domains; PEVK, repeating units of amino acids (proline, glutamic acid, valine, and lysine).

The sarcomere is the unit of contraction for striated muscle (Figure 1).

- It is included between two neighbouring Z-lines (or Z-discs, or Z bodies). In electron micrographs of cross-striated muscle, the **Z-line** (from German "Zwischenscheibe", *the disc in between* the I bands) appears as a series of dark lines.
- Surrounding the Z-line is the region of the **I-band** (for isotropic), the zone of thin filaments that is not superimposed by thick filaments.

- Following the I-band is the **A-band** (for anisotropic) that contains the entire length of a single thick filament.
- Within the A-band is a paler region called the **H-zone** (from the German "heller", *brighter*), the zone of the thick filaments that is not superimposed by the thin filaments.
- Inside the H-zone is a thin **M-line** (from the German "Mittelscheibe", the disc in the *middle* of the sarcomere) formed of cross-connecting elements of the cytoskeleton.

The relationship between the proteins and the regions of the sarcomere are as follows:

- *Actin* filaments (gene name ACTC1), the thin filaments, are the major component of the I-band and extend into the A-band.
- *Myosin* filaments, the thick filaments, are bipolar and extend throughout the A-band. They are crosslinked at the centre by the M-band.
- The giant protein titin (connectin) extends from the Z-line of the sarcomere, where it binds to the thick filament (myosin) system, to the M-band, where it is thought to interact with the thick filaments. Titin (and its splice isoforms) is the biggest single highly elasticated protein found in nature. It provides binding sites for numerous proteins and is thought to play an important role as sarcomeric ruler and as blueprint for the assembly of the sarcomere.
- Another giant protein, nebulin, is hypothesised to extend along the thin filaments and the entire I-Band. Similar to titin, it is thought to act as a molecular ruler along for thin filament assembly.
- Several proteins important for the stability of the sarcomeric structure are found in the Z-line as well as in the M-band of the sarcomere.
- Actin filaments and titin molecules are cross-linked in the Z-disc via the Z-line protein alpha-actinin.
- The M-band proteins myomesin as well as C-protein crosslink the thick filament system (myosins) and the M-band part of titin (the elastic filaments).
- The interaction between actin and myosin filaments in the A-band of the sarcomere is responsible for the muscle contraction (sliding filament model).

An estimated 35%–40% of genetic DCMs may result from sarcomere gene mutations [24]. Dominant mutations in the genes encoding these sarcomeric proteins cause DCM. The mechanism of action is for some sarcomeric DCM mutations is dominant negative.

Most recently, mutations in the giant protein titin were estimated to be responsible for approximately 25% of DCM [24]. The titin protein includes more than 35,000 amino acids,

containing many repeating fibronectin-like and Ig-like domains. Titin is considered a molecular ruler that regulates and transmits information about sarcomere length to the sarcomere and the cardiomyocyte [27]. Consistent with this role, the carboxy terminus titin kinase domain is stimulated by activity and stretch [28]. A dilated heart may be even more dependent on signaling through titin, and frameshifting mutations that disrupt this intramolecular signaling pathway would be expected to adversely affect the heart, as it regularly contends with repetitive and dynamic load. In the study by Herman et al., 54 of 312 DCM patients were found to have truncating TTN mutations, which the authors defined as frameshifts, nonsense, or predicted splicesite mutations [24]. The protein-disrupting TTN variants were nonrandomly distributed along the length of titin, with a preference for the A band region of titin. Most TTN variants mapped in regions common to both the N2B and N2BA forms, and nearly all TTN mutations were heterozygous. A subset of subjects had documented familial disease, thus providing strong support for TTN as a dominant DCM gene. In addition to these protein-truncating TTN mutations, a large number of TTN coding variants of unknown significance were found. This type of protein-altering variation may contribute to DCM complexity, as some variants are predicted to be pathogenic.

Given this frequency, TTN mutations in combination with other forms of heart disease would be expected to enhance the severity of cardiomyopathy expression. For example, TTN variants in combination with valve defects, hypertension, or myocardial infarction would be expected to result in more significant cardiomyopathy and congestive heart failure. A larger heart, such as that dilated from regurgitant valve disease, may be more susceptible to impaired elastic recoil or a reduction in titin signaling from mutations ablating the titin kinase domain. It also follows that TTN mutations in combination with other pathogenic genetic variants would enhance the severity of DCM. It is notable that HCM subjects had a strikingly lower percentage of TTN frameshifting mutations in the Herman study, possibly supporting the idea that HCM sarcomere mutations along with TTN frameshifting mutations predispose to DCM. More comprehensive genetic testing is required to explore this possibility, and these observations strongly favor the inclusion of TTN in genetic screening panels.

Tropomyosin is a thin filament protein that can also be mutated in DCM. For example, the TPM1 D230N mutation was described in two large, unrelated families with DCM . Some children presented with cardiomyopathy, while other family members with the identical gene mutation remained largely asymptomatic, with mildly increased LV diameter and mildly reduced systolic function until later life. The D230N TMP1 DCM mutation was modeled in vitro, where a decreased calcium sensitivity of actin-activated myosin ATPase activity was seen [25].

DCM mutations also occur in the sarcomere's thick filament β-myosin heavy chain [26]. Two MYH7 mutations, S532P and F764L map within the S1 head of myosin that harbour the actinbinding, ATPase, and force-producing domains. The S532P mutation occurs within the actinbinding domain, and the F764L mutation occurs within the converter domain. Actin-activated ATPase is reduced, as is in vitro sliding motility of actin. Force production is not decreased, but rather a reduction in myosin step size is observed. Since ATP hydrolysis produces less displacement of myosin, this is a less energetically efficient sarcomere.

Z band proteins and the costamere.

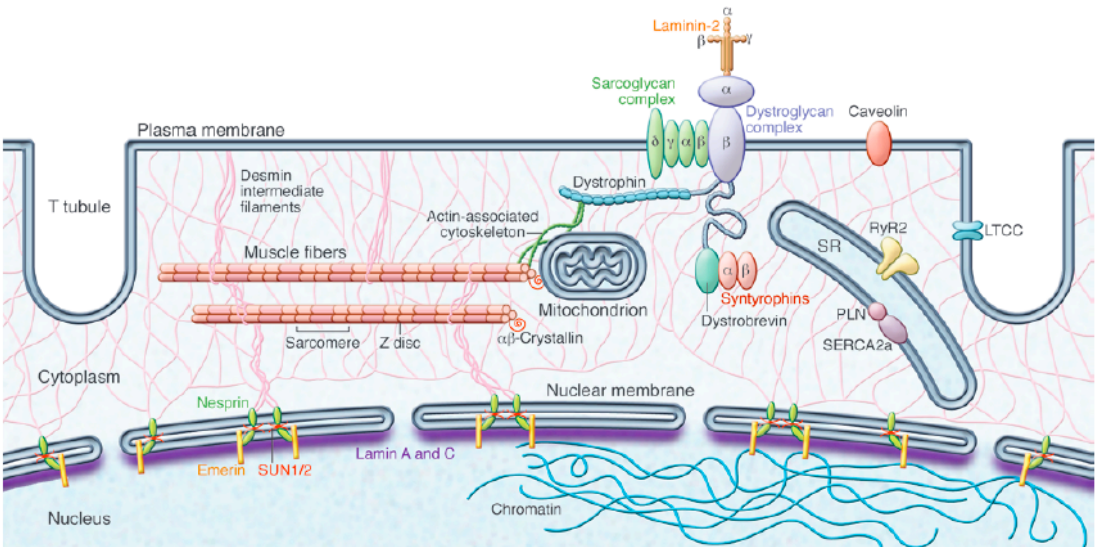


Figure 2: A view of the cardiomyocyte. The cytoplasm of the cardiomyocyte contains sarcomeres, which contain thin and thick filaments. Mutations in sarcomere genes lead to HCM and DCM. Plasma membrane–associated proteins such as dystrophin and its associated proteins, the sarcoglycans, are mutated in inherited DCM associated with skeletal muscle disease. Mutations of genes encoding nuclear membrane proteins such as lamins A and C, emerin, and nesprins lead to inherited DCM due to an inappropriate transcriptional response to mechanical stress. Many of these nuclear membrane genes also induce cardiac conduction system disease.

The Z disc or Z band is an electron-dense structure into which the thin filaments and titin anchor. Gene mutations that have been identified in DCM patients that affect multiple Z band proteins include muscle LIM protein (MLP), cardiac ankyrin repeat protein (CARP), myopallidin, α-actinin 2, TCAP, and nexilin [34].

TCAP and MLP modulate the elastic properties of titin. Phosphorylation of Z band proteins, or even nuclear translocation of Z band elements, are just two mechanisms by which Z band elements signal within the cardiomyocyte.

At the plasma membrane, with a periodicity that matches Z discs, is the costamere, a rib-like structure of cytoplasmic and transmembrane proteins that links the cytoskeleton to the plasma membrane and the extracellular matrix (Figure 3). Dystrophin and its associated proteins, sarcoglycans and dystroglycan, enrich at the costamere. Linked to the cytoplasm alongside

talin, vinculin, and integrin, the dystrophin complex protects against contraction-induced injury in both cardiac and skeletal muscle [35]. The integrity of the dystrophin complex is critical for mechanic-transduction because disruption of this complex from loss-of-function mutations renders cardiomyocytes susceptible to damage elicited from contraction [36]. Mutations in dystrophin and the sarcoglycan produce skeletal muscle disease and cardiomyopathy, and so heart failure in these patients is further compromised by hypoventilation from respiratory muscle weakness.

Because of the narrow window in which DCM develops in patients with Duchenne muscular dystrophy (DMD), this population has been ideal for testing whether medications for cardiomyopathy can prevent or slow progression of DCM. DCM prevention was tested by giving the angiotensin converting enzyme perindopril to boys with DMD who began treatment beginning at age 9.5 years, before the development of reduced LV function. This early treatment was associated with reduced progression of cardiomyopathy after five years and also showed a benefit in 10-year mortality [37, 38]. The addition of β-adrenergic blockade may be expected to further slow progression of disease.

Nuclear membrane defects in genetic DCM.

Lamins A and C, two alternatively spliced products of the LMNA gene, are expressed in all somatic cells, but mutations in LMNA strike in a very tissue-specific manner, with the majority causing cardiomyopathy with variable skeletal muscle involvement. More than 200 different LMNA mutations are associated with inherited cardiomyopathy, primarily DCM that may be associated with conduction system disease prior to the evidence of ventricular dilation. LMNA mutations that cause DCM are primarily inherited in an autosomal-dominant manner, although autosomal-recessive cases have been reported. In a study of patients with familial DCM and prominent conduction block, 33% had LMNA mutations [39].

Lamins A and C are intermediate filament proteins that are present at the nuclear membrane and in the nucleoplasm. Lamins A and C, along with the B-type lamins and other lamina-associated proteins, comprise the nuclear lamina, a fibrous structure that provides support to the nuclear membrane. The lamina proteins are part of the LINC complex that LInks the Nucleus to the Cytoplasm. In addition to lamins A and C, mutations in other members of the LINC complex cause cardiomyopathy, including emerin and nesprins-1 and -2 [40–42]. Stress signals in the cytoplasm are hypothesized to action the LINC complex to affect gene expression in the nucleus.

Both LMNA and emerin-null fibroblasts have altered expression of mechanosensitive genes in response to mechanical stress [43, 44]. LMNA mutations variably affect skeletal muscle, and skeletal muscle from both LMNA mouse models and human patients with LMNA mutations

reveal aberrant expression of electrical activity–dependent genes and epigenetic chromatin modifications, indicating that components of the LINC complex are crucial for an appropriate transcriptional response to mechanical stress [45]. To further understand how the LINC complex affects gene expression, recent studies have examined the role the nuclear lamina plays in chromatin organization. The organization of chromatin in the nucleus is not random and is thought to play a role in transcriptional regulation. Whole chromosomes or portions of chromosomes reorganize in the nucleus, coincident with changes in gene expression. The nuclear lamina participates in the scaffolding of the chromatin through interactions between chromatin and lamins A and C. Fibroblasts from patients with LMNA-induced cardiomyopathy have changes in the three-dimensional location of chromatin compared with normal dermal fibroblasts. This chromatin mislocalization occurs concomitant with changes in gene expression, suggesting that the spatial organization of chromatin may be important for disease pathogenesis [46, 47]. Together, these data indicate that a functional LINC complex is critical for the heart to properly respond to constant mechanical stress, potentially by regulating gene expression or other nuclear functions.

4.1.2 Protein homeostasis impairment in DCM

In mammalian cells, most of the proteins are in a dynamic state of flux. The balance of protein synthesis and degradation in each cell is highly regulated and occurs in a specific manner to maintain cellular homeostasis.

However, under the circumstances of cardiac remodeling during heart disease this balance can be altered leading to accumulation of potentially toxic proteins. To ensure that these misfolded or aberrant proteins are either repaired or removed, a set of molecular mechanisms works in collaboration or separately as a quality control of the cell. This quality control consists of molecular chaperones and co-chaperones, the autophagy-lysosomal pathway (ALP), and the ubiquitin-proteasome system (UPS).

In the past years, the functional significance of the UPS in cardiovascular physiology and disease has become evident. Particularly, UPS alteration is rapidly gaining recognition as a major player in the pathogenesis of several cardiac disorders, including inherited cardiomyopathies.

The ubiquitin-proteasome system

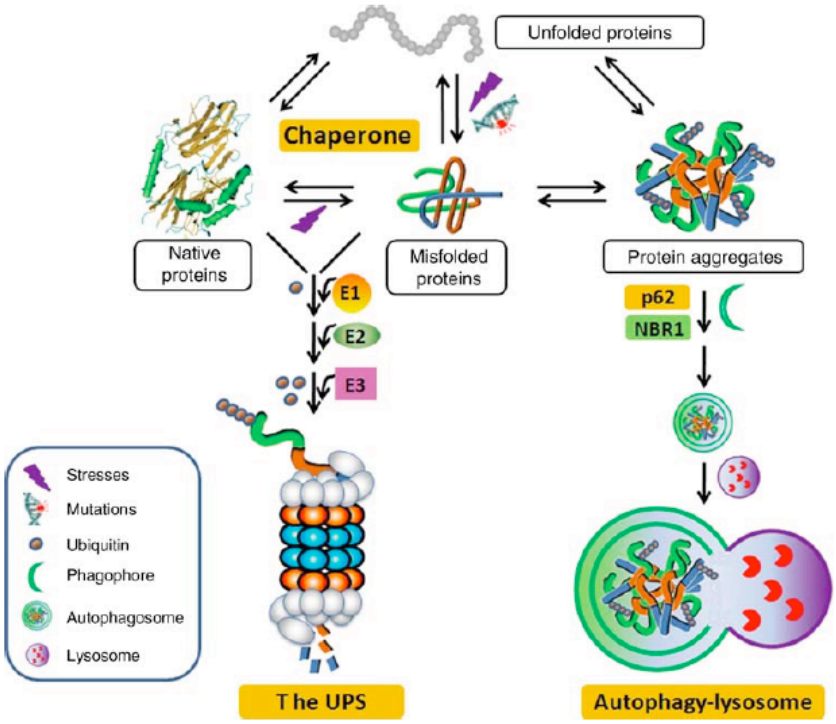


Figure 3: **A schematic illustration of protein quality control in the cell.** Chaperones facilitate the folding of nascent polypeptides and the unfolding/refolding of misfolded proteins, prevent the misfolded proteins from aggregating, and escort terminally misfolded proteins for degradation by the UPS. The UPS degrades both misfolded/damaged proteins and most unneeded native proteins in the cell. This process involves two steps: first, covalent attachment of ubiquitin to a target protein by a cascade of chemical reactions catalysed by the ubiquitin-activating enzyme (E1), ubiquitinconjugating enzymes (E2), and ubiquitin ligase (E3); and then the degradation of the target protein by the proteasome. The autophagy-lysosomal pathway participates in PQC by helping remove protein aggregates formed by the misfolded proteins that have escaped from the surveillance of chaperones and the UPS. Protein aggregates or defective organelles are first segregated by an isolated double membrane (phagophore) to form autophagosomes, which later fuse with lysosomes to form autophagolysosomes, where the segregated content is degraded by lysosomal hydrolases. p62/SQSTM1 and NBR1 (neighbour of BRCA1 gene 1) mediate the activation of autophagy by aggregated ubiquitinated proteins.

The UPS is indispensable for the selective degradation of most intracellular cytosolic, nuclear, and myofibrillar proteins and its major function is to prevent accumulation of damaged, misfolded and mutant proteins [48].

Degradation of proteins by the UPS is an ATP-dependent multistage process that requires first ubiquitination of the target protein prior to its degradation by the 26S proteasome [49, 50].

Ubiquitination of the target protein is achieved via an enzymatic cascade involving the concerted action of E1 (ubiquitin-activating), E2 (ubiquitin-conjugating) and E3 (ubiquitin ligase) enzymes. The process of ubiquitination occurs with spatial, temporal and substrate specificity, which is dictated by the E3 ubiquitin ligases. The E3 ubiquitin ligases have been broadly classified into 2 main categories based on structural similarities: the RING (really interesting new gene) finger domain-containing proteins including the RING-related E3s such as the U-box proteins, and the HECT (homologous to E6-AP carboxy-terminus) domain-containing proteins [51, 52].

The eukaryotic 26S proteasome is a large, multicatalytic protein complex composed of two subcomplexes: the 20S core particle capped by either one or two 19S regulatory particles [53]. The function of the 19S regulatory particle is to recognize, deubiquitinate, and unfold target proteins, and then to translocate them into the 20S core particle, which houses the proteolytic activities within its central chamber. Three distinct proteolytic activities exist, namely the chymotrypsin-like, trypsin-like and caspase-like activities, and each cleaves preferentially after particular amino acid residues [54].

The ubiquitin-proteasome system in dilated cardiomyopathy

UPS impairment might play a role in human or experimental models of familial DCM.

Mutations in CRYAB or DES resulted in accumulation of mutant proteins and severe DCM in desmin-related cardiomyopathy (DRM) [55].

A mouse model of DRM, obtained by overexpression of the R120G mutant CRYAB (CryABR120G) recapitulated the human phenotype [56] and exhibited marked UPS impairment as revealed by GFPdgn-based UPS reporter mice before the development of hypertrophy and heart failure [57]. Similar observations were made in mutant Des-D7 transgenic mice [58]. In both DRM mouse models, UPS impairment started before the cardiac phenotype and seems to concern the delivery of ubiquitinated proteins into the 20S proteasome [59].

Other genes associated with DCM are also subject to UPS-mediated regulation. This is the case for LMNA encoding lamins A/C, which are proteins of the nuclear envelope.

Potential mechanisms leading to UPS impairment in cardiomyopathies

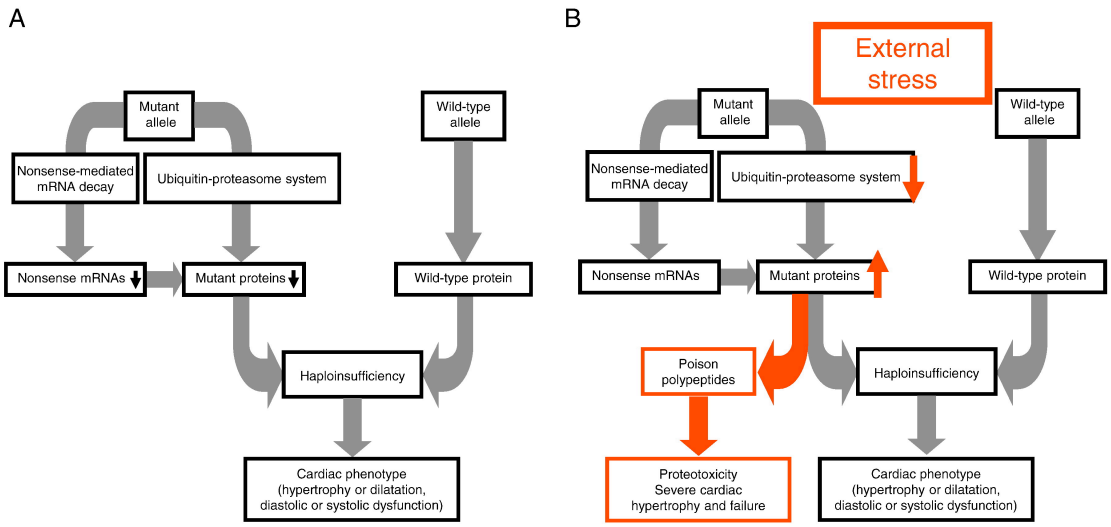


Figure 4: Potentialmechanisms leading to UPS impairment in genetic forms of cardiomyopathies. A) The nonsense-mediatedmRNA decay at themRNA level, and the ubiquitin-proteasome system(UPS) at the protein level regulate the expression of themutant allele, leading to reduced amount ofmutant proteins that could be toxic for cardiac myocytes. Together with the (normal) expression of the wild-type allele, this results in protein haploinsufficiency and the development of the cardiac phenotype (hypertrophy or dilatation, diastolic or systolic dysfunction). B) External stress, e.g. adrenergic stress or aging, could induce impairment of the UPS in cardiomyopathies. Although the exactmechanismis not known, onemay suggest that adrenergic stress induces desensitization of the β-adrenoceptors leading to decreased activities of PKA and therefore reduced phosphorylation of proteasome components, known to be associatedwith decreased proteasome activity. Impaired UPS leads to accumulation ofmutant proteins, which could act as poison polypeptides and further impair the phenotype (severe form of cardiac hypertrophy and heart failure).

The mechanisms by which gene mutations lead to UPS impairment are not fully elucidated. In the absence of external stress, the expression of the mutation is regulated at several levels by quality control mechanisms in order to reduce as much as possible the amount of misfolded or aberrant poison polypeptides, which could induce damage in cardiac myocytes. Missense mutations are expected to produce stable full-length mutant mRNAs and proteins. Misfolded mutant proteins are recognized by chaperones (such as Hsp70, Hsp90) and co-chaperones (such as CHIP, Bag1, Bag3) that will make the decisions about refolding or degrading them by the UPS and/or the ALP. Therefore, in some cases, the expression of missense mutations may be tightly regulated by the protein quality control systems, resulting in low level of full-length mutant proteins. In the specific case of frameshift or nonsense mutations, an additional quality control takes place at the mRNA level, which is the NMD [80]. Low levels (or absence) of mutant proteins and the assumed 50% of wild-type proteins, as expected for autosomal-dominant disease such as cardiomyopathies, result in protein haploinsufficiency, which leads to the cardiac phenotype. In most cases, expression of the wild-type allele partially compensates for protein deficiency.

Several mechanisms could lead separately or in combination to UPS impairment.

First, it has been shown that the continuous degradation of mutant proteins by the UPS saturates this system [60].

Second, the combination of external stress and overwhelmed UPS could precipitate the system into impairment. This is the case in some HCM and DCM disease mouse models, in which the UPS continuously degraded mutant proteins in young adult mice carrying Mybpc3 mutation and became saturated or impaired only after adrenergic stress or aging [61].

Third, misfolded proteins escaping the surveillance of chaperones and UPS tend to form aggregates, which are potentially toxic to the cell. Supporting this, the group of Robbins showed that intracellular amyloidosis was highly prevalent in cardiac myocytes derived from human HCM or DCM hearts [62]. Furthermore, protein aggregation itself impaired proteasome function in cardiac myocytes [63], forming a vicious cycle.

Fourth, increased oxidative stress also results in protein aggregation. In the case of aging, this could be due to increased free radicals production by damaged aging mitochondria [64]. Oxidative stress induced protein oxidation and aggregation of oxidized proteins, which bind to the 20S proteasome and irreversibly inhibit its activity [65]. This could cause a vicious cycle and also lead to accumulation of oxidized proteins, which are normally degraded by the proteasome system.

Fifth, an altered assembly of the proteasome or a switch in the distribution of proteasomal subpopulations [66] could lead to UPS impairment. A recent study demonstrated an impaired docking of the 19S to the 20S in human end-stage heart failure [67], which could affect the degradation capacity of the proteasome and may explain the diminished proteasomal activity found in human failing hearts [68].

Finally, regulation of the proteasome system involves post-translational modifications of proteasomal subunits, such as phosphorylation, acetylation or oxidation.

Potential consequences of UPS impairment

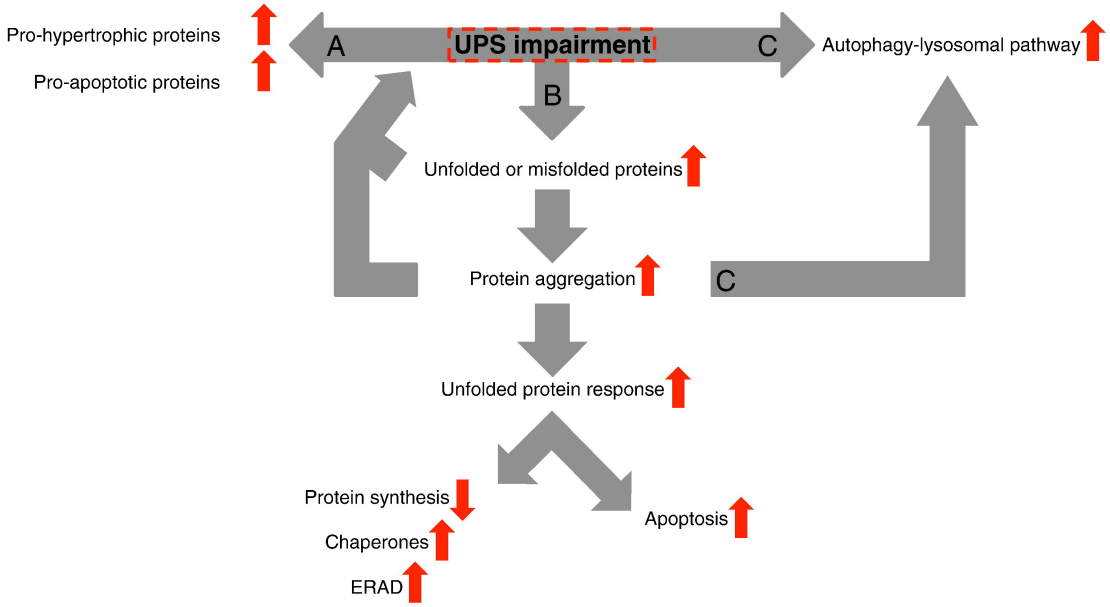


Figure 5: Potential consequences of UPS impairment in cardiacmyocytes. UPS impairment could lead to: A) Accumulation of proteins involved in hypertrophic signaling (e.g. calcineurin) or apoptotic pathway (e.g. p53), which are normally degraded by the UPS. B) Accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER), which leads to protein aggregation. This ER stress activates the unfolded protein response, characterized by attenuation of protein synthesis, up regulation of chaperones genes (e.g. GRP78) and ERAD(ER-associated protein degradation). Prolonged stress will lead to apoptosis. Accumulation and/or aggregation ofmisfolded proteins could itself force UPS impairment. C) Activation of the autophagy-lysosomal pathway directly or indirectly (by protein aggregation) in order to contribute to protein degradation.

UPS impairment could have several consequences in cardiac myocytes. A number of key proteins involved in cardiac apoptosis pathways are targets or components of the UPS.

For instance, p53 is a target of the E3 ubiquitin ligase MDM2 [69]. Therefore, proteasomal impairment could result in increased levels of pro-apoptotic factors.

It has been shown that UPS impairment activated the calcineurin-NFAT pathway and promoted maladaptive remodeling in cardiac myocytes [70]. Furthermore, reduced proteasomal activities were associated with increased levels of pro-apoptotic p53 in human HCM and failing hearts [68].

UPS impairment could also lead to accumulation of unfolded or misfolded proteins and aggregation of proteins. This might result in ER stress, leading to an adaptive response, which is known as the unfolded protein response (UPR). The UPR promotes attenuation of protein synthesis, transcriptional activation of chaperone genes and activation of ER-associated degradation (ERAD) in order to reduce the load of misfolded proteins [71]. If the attempts to resolve the ER stress fail or the UPR is prolonged, UPR-mediated signaling pathways that lead to apoptosis are initiated. An inadequately working UPS (or in this case ERAD) probably

stimulates the switch from adaptive to pro-apoptotic response. This hypothesis is supported by the demonstration that proteasome inhibition induced ER-initiated cardiac myocyte death via CHOP-dependent pathways [72]. Of note, accumulation and/or aggregation of misfolded proteins could itself force UPS impairment, forming thereby a detrimental feedback loop.

Whereas the UPS usually degrades the majority of proteins, the ALP is the other proteolytic system which is primarily responsible for degradation of (generally) long-lived or aggregated proteins and cellular organelles [73]. The ALP engulfs proteins or organelles into autophagosomes, which subsequently fuse with lysosomes to form auto(phago)lysosomes, in which lysosomal proteases degrade autophagosomal content [74]. Although autophagy is generally considered to be independent of the UPS, growing lines of evidence indicate that the UPS and ALP act as a consortium in the removal of misfolded proteins. Another potential consequence of UPS inhibition is the ALP activation. Several proteins such as p62, NBR1 and HDAC6 seem to play a major role in the interplay between the UPS and ALP . Proteasome inhibition activated autophagy in vitro and in vivo, likely as a compensatory mechanism to alleviate proteotoxic stress [84-89].

4.1.3 Role of the immune system and the inflammasome in the DCM

The innate immune system has a number of signalling receptors that recognize foreign molecular structures as well as self-molecules that are altered or that became too abundant like the case of the unfolded or malfolded proteine aggregates.

Tissue damage, cellular stress and infection are sensed by the innate immune system through pattern recognition receptors (PRRs), which, upon activation, initiate defense and repair programs [81,82].

If the cellular or tissue damage is extensive, these receptors trigger an acute inflammatory response that, in the vast majority of cases removes the injurious stimuli and repairs the damaged tissue within days of its initiation.

In some situations, however, the source of tissue or cellular stress cannot be effectively resolved. Continued death of damaged cells leads to progressive destruction of the tissue, while the immune system initiates futile attempts to repair the damage. This simultaneous destruction and healing of tissue is characteristic of chronic inflammation, and is seen in a number of degenerative disease and in chronic pathologies like chronic heart failure [83].

In this function of detection of pathogenic microorganisms and sterile stressor a very important role is played by the inflammasomes, key signalling platforms that activate the highly pro-inflammatory cytokines interleukin-1β (IL1β) and IL-18.

The inflammasomes are a group of multimeric protein complexes that consist of an inflammasome sensor molecule, the adaptor protein ASC and caspase 1. Inflammasome formation is triggered by a range of substances that emerge during infections, tissue damage or metabolic imbalances. Once the protein complexes have formed, the inflammasomes activate caspase 1, which proteolytically activates the pro-inflammatory cytokines IL1β and IL-18.

In addition, inflammasome activation causes a rapid, proinflammatory form of cell death called pyroptosis [84].

Inflammasome components

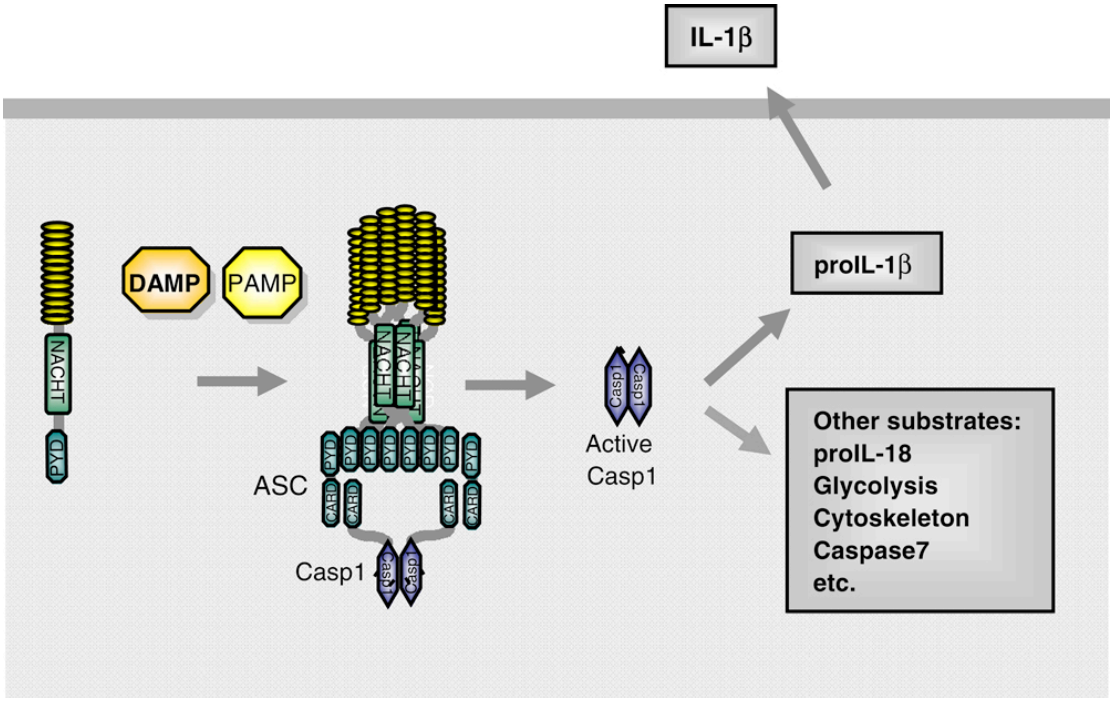
At first glance, the inflammasomes are organized in a very simple manner: inflammasome sensor molecules connect to caspase 1 via ASC, which is an adaptor protein encoded by PYCARD that is common to all inflammasomes.

ASC interacts with the upstream inflammasome sensor molecules triggering the assembly of monomers of pro-caspase 1 into close proximity, which initiates caspase 1 self-cleavage and the formation of the active heterotetrameric caspase 1. Active caspase 1 proteolytically activates a number of proteins including pro-IL1β and pro-IL18, and induces their release via a non-classical secretion pathway [85-90]. The transcription of pro-IL1β is induced by the activation of the transcription factor nuclear factor-κB (NF-κB), whereas pro-IL18 is constitutively expressed and its expression is increased after cellular activation. Therefore, these potent pro-inflammatory cytokines are controlled by two checkpoints: transcription as well as maturation and release.

Caspase 1-mediated activation of members of the IL1β cytokine family leads to the recruitment and the activation of other immune cells, such as neutrophils, at the site of infection and/or tissue damage.

Several inflammasome sensor molecules can trigger the formation of inflammasomes through the interaction with particular inflammasome receptors.

Most of the inflammasomes that have been described to date contain a NOD-like receptor (NLR) sensor molecule, a family of cytoplasmatic PPRs that have a central role in the initiation of the inflammatory responses.



NLRs typically have a tripartite architecture, consisting of a central and defining nucleotide binding and oligomerization (NACHT) domain, C-terminal leucine-rich repeats (LRRs) and an N-terminal effector domain (Fig.). There are 22 known human NLR family members, which can be subgrouped in NLRP (NALP), NOD and IPAF clans.[91-92]

NLRs continuously monitor the cytosol for abnormal conditions. Upon activation, some NLR family members form inflammasomes that serve as platforms for caspase-1 activation and subsequent proteolytic maturation of the potent pro-inflammatory cytokine IL-1β (Fig.). So far, the NLR family members NLRP1, IPAF and NLRP3 and the HIN200 family member AIM2 have been demonstrated to nucleate inflammasomes.

Due to its association with numerous inflammatory diseases, the NLRP3 inflammasome has drawn the most attention. Upon detecting cellular stress, NLRP3 oligomerizes and exposes its effector domain for interaction with the adaptor protein ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), which in turn recruits pro-caspase-1. Pro-caspase-1 clustering leads to its activation via autoprocessing, and active caspase-1 proteolytically cleaves a variety of cytoplasmic targets, including IL-1.

Activation of the NLRP3 inflammasome

A wide variety of danger signals activate the NLRP3 inflammasome (94). These include exogenous danger signals, such as pathogen-associated molecular patterns (PAMPs), as well

as endogenous danger signals or damage-associated molecular patterns (DAMPs) such as anomalous protein aggregates.

The mechanisms by which these activators trigger NLRP3 inflammasome activation are poorly understood.

In light of the sheer number of NLRP3 inflammasome activators and their chemical and structural diversity, it seems plausible that all agonists (or signals downstream of them) influence a common cellular structure or organelle that integrates the various activation signals and consequently initiates a signaling pathway that leads to inflammasome activation.

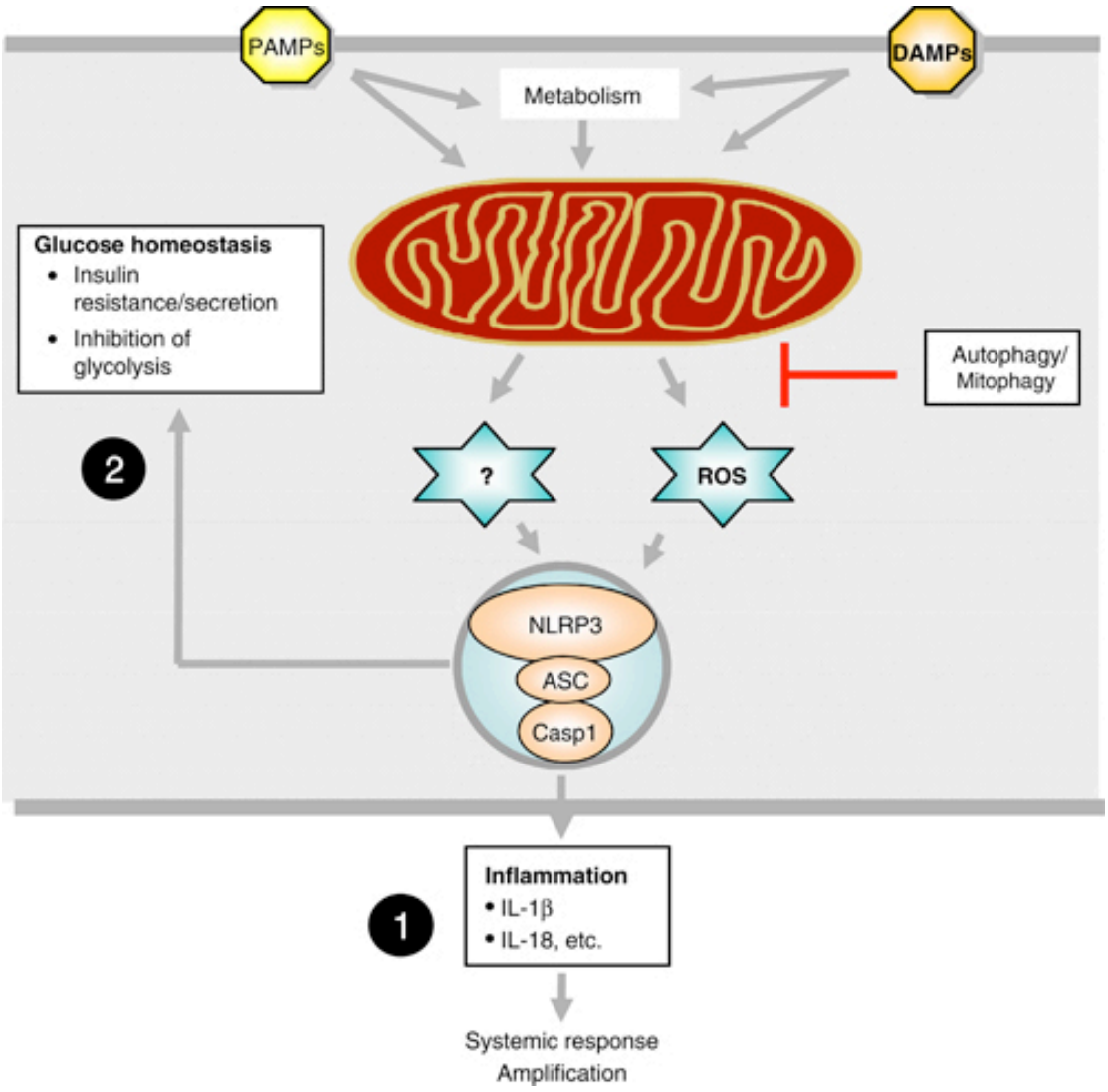
The first evidence for such an integrative organelle-mediated activation mechanism came from Latz and colleagues [95]. Their proposal was that inefficient clearance of the particle following phagocytosis leads to lysosomal destabilization and rupture. The ensuing release of the lysosomal protease cathepsin B into the cytosol then triggers NLRP3 inflammasome activation either by direct cleavage of NLRP3, or by an uncharacterized pathway. However, macrophages from cathepsin B-deficient mice show minimal reduction of inflammasome activation [96], this suggests that the other lysosomal cathepsins are redundant with cathepsin for inflammasome activation [97], or that lysosome-independent inflammasome activation mechanisms exist. The latter view is supported by the fact that lysosome rupture is not required for nonparticulate/small molecule NLRP3 activators such as nigericin and ATP.

The central role of mitochondria in NLRP3 inflammasome activation

All NLRP3 activators examined, cause the generation of short-lived reactive oxygen species (ROS) [99], that sims to be essential for NLRP3 activation. The source of ROS was initially thought to be phagosome-associated NADPH oxidases, which are activated upon the engulfment of inflammasome-activating particulate agonists. However, macrophages deficient in critical subunits of four (out of seven) NADPH oxidases complexes (NOX1, NOX2, NOX3 and NOX4) respond normally or have even slightly elevated inflammasome activity [100]. This suggests compensation by the remaining NADPH oxidase members, or the existence of an entirely different cellular source of inflammasome-activating ROS.

Indeed, recent evidence from two groups suggests that mitochondria are the main source of inflammasome-activating ROS, and as such may constitute the signal-integrating organelle for NLRP3 inflammasome activation [101, 102] (fig 2). NLRP3 inflammasome activation is highly impaired in macrophages in which mitochondrial activity is dampened by depletion of mitochondrial DNA or by inactivation of the mitochondrial outer membrane protein VDAC (voltage dependent anion channel). VDACs are the major channels for the exchange of metabolites and ions between the mitochondria and other cellular compartments including the endoplasmic reticulum (ER) [103]. As such, they are important regulators of mitochondrial

metabolic activity, which is required for ROS production via the mitochondrial electron transport chain. In cells with diminished VDAC expression, caspase-1 activation is considerably impaired upon addition of all NLRP3 inflammasome activators tested. On the contrary, VDAC and thus mitochondria are not essential for the activation of the IPAF or the AIM2 inflammasomes. VDAC activity is regulated by Bcl-2 family members. Overexpression of Bcl-2 leads to partial VDAC closure and a concomitant decrease of mitochondrial Ca2+ levels and ROS production. Consistent with this, IL-1β levels are decreased in macrophages from *bcl2*-transgenic mice.



A link between NLRP3 and mitochondria is also suggested by the subcellular localization of NLRP3. Under resting conditions, most overexpressed and endogenous NLRP3 protein is associated with the ER. Upon inflammasome stimulation, NLRP3 relocates to the perinuclear space and colocalizes with structures that stain positively for both the ER and the

mitochondria. ASC, which is present in the cytoplasm of resting cells, also relocates to these perinuclear areas upon NLRP3 activation.

ER membranes can tightly associate with mitochondria forming mitochondria-associated ER membranes (MAMs). Among other functions, MAMs are important for the transfer of lipids and Ca2+ from the ER to the mitochondria [104]. Subcellular fractionation techniques reveal that the NLRP3-associated ER/mitochondria staining most likely corresponds to MAMs. Thus, by virtue of its ER/mitochondrial localization upon activation, the NLRP3 inflammasome is strategically located to receive signals emanating from mitochondria.

Two other observations provide support for a pivotal role of mitochondria in NLRP3 inflammasome activation. ROS production can be induced specifically in mitochondria by blocking key enzymes of the electron transport chain. Complex I is one of the main sites at which electrons can leak to oxygen [105]. Addition of the Complex I inhibitor rotenone results in the partial loss of mitochondrial membrane potential and strong ROS production. Complex III inhibition by antimycin A has a similar effect, and indeed both drugs lead to NLRP3 inflammasome activation [102]. To avoid cellular damage, ROS-generating mitochondria are constantly removed by a specialized form of autophagy termed mitophagy [96]. Inhibition of mitophagy/autophagy leads to the prolonged presence of ROS-producing damaged mitochondria, and, as a consequence, to the activation of the NLRP3 inflammasome. Taken together, these observations provide good evidence that ROS produced by dysfunctional mitochondria causes NLRP3 inflammasome activation.

Signals released from mitochondria that cause inflammasome activation

It is rather unlikely that the mere increase of ROS from mitochondria directly induces NLRP3 inflammasome activation (for example, by causing a conformational change in NLRP3).

Nonetheless, increased ROS levels induce the association of NLRP3 with a nonmitochondrial protein, thioredoxin-interacting protein (TXNIP). Treatment with NLRP3 agonists triggers the interaction of NLRP3 with TXNIP in an ROS-dependent manner. In unstimulated cells, TXNIP is constitutively bound to the oxidoreductase thioredoxin 1 (TRX1). Upon an increase in cellular ROS concentration, this complex dissociates and TXNIP binds to NLRP3 translocates from the cytoplasm to the mitochondria. Although the role of mitochondria-associated TXNIP is not clear at this point, there is evidence that it regulates mitochondrial events. In mitochondria, TXNIP binds to and oxidizes mitochondrial TRX2, thereby precluding association of TRX2 with apoptosis signal-regulated kinase 1 (ASK1), and allowing for ASK1 phosphorylation and activation. ASK1 activation can lead to cytochrome

c release and the induction of apoptosis [109]. Consistent with these findings, TXNIP deficiency protects pancreatic β cells against apoptosis [110].

In addition to elevated ROS, a decrease in cytoplasmic K+ levels is an obligatory event for NLRP3 inflammasome activation. Under resting conditions, high cytoplasmic K+ concentrations (150 mM) prevent inflammasome activation. ATP, a potent activator of the NLRP3 inflammasome, reduces intracellular K+ concentration by approximately 50% [111].

The bacterial toxin and K+ ionophore nigericin similarly activates the NLRP3 inflammasome [112] In keeping with this; a drop in cytoplasmic K+ concentration to less than 70 mM is required for inflammasome activity in vitro 8. Inhibition of K+ efflux by high extracellular K+ concentration, or by addition of the K+ channel inhibitor glibenclamide [113-115] blocks inflammasome activation in response to all NLRP3 activators tested. Interestingly, low cytoplasmic K+ levels are also required for the activation of the apoptosome, which indicates that "normal" intracellular concentrations of K+ safeguard the cell against inappropriate formation of caspase-containing stress-responsive complexes [116]. Although the mechanism by which cytoplasmic K+ concentration modulates NLRP3 inflammasome activation is unknown, it is possible that its effect is via the mitochondria. These organelles possess several K+ channels that are important for their functioning [117].

Role of the cytokines

Cytokines and chemokines released during inflammation recruit activated inflammatory cells, particularly monocytes, from circulation into the cardiac tissue.

Once inside the cardiac tissue, monocytes differentiate into macrophages and promote inflammation, tissue injury, and fibrosis of myocardial tissue. Activated macrophages produce and secrete several inflammatory mediators, such as monocyte chemotactic protein-1 (MCP1) and TNF-alpha (TNF-α), and fibrogenic activators, such as TGF-β, and in this way support pro-inflammatory and pro-fibrotic processes [118-121,123, 124].

Another very important pyrogenic cytokine secreted by blood monocytes is the Interleukin-1β (IL1β). It has tremendous effects on immunological function at very low concentrations. Within the cardiovascular system, cardiac-derived IL1β impairs contractility by inducing calcium leak from the sarcoplasmic reticulum and ultimately promotes cell death and tissue remodeling in a nitric-oxide-dependent pathway [131, 132, 133, 134].

Activated macrophages also secrete galectin-3, which may induce cardiac fibroblast proliferation, collagen deposition, and ventricular dysfunction [125, 127].

In the table below is possible to see the role covered by the most important cytokines and chemokines involved in cardiovascular disease pathogenesis.

Some biochemical and physiological characteristics of TNF superfamily cytokines and other pro-inflammatory and regulatory cytokines suggested as biomarkers for heart failure.

Biomarker	MW (kDa)[a]	Biochemical structure	Biological characteristics
TNF superfamily			The TNF superfamily currently consists of 19 ligands and 29 receptors in humans. Most TNF ligands are type II transmembrane proteins whose extracellular domains can be cleaved by specific metalloproteinases to generate soluble cytokines. TNF superfamily ligands and receptors play a role in normal developmental processes, apoptosis, regulation of immune cell functions, and also in cancer and autoimmune diseases.
TNFα	About 17 kDa (recombinant, mouse)	156 aa protein (recombinant mouse)	Adipokine/cytokine involved in systemic inflammation and the acute phase reaction
TWEAK (*TNFSF12*)	About 17 kDa soluble protein (recombinant, human)	249 aa membrane protein, 156 aa soluble protein	Transmembrane and soluble (cytokine) protein of the TNF ligand superfamily.
FasL (*TNFSF6* or *CD95L*)	About 40 kDa as a tramsmenbrane protein (human) About 18 kDa as a soluble protein (recombinant, human)	157 aa soluble protein (recombinant, human)	Transmembrane and soluble protein of the TNF ligand superfamily
LIGHT (*TNFSF14* or *CD258*)	About 23 kDa (recombinant, human)	183 aa (recombinant, human)	Member of the TNF ligand superfamily, which acts as a ligand for TNFRSF14
Pro-inflammatory and regulatory cytokines			A pro-inflammatory cytokines are agents promoting systemic inflammation (such as IL-1 and TNF). Regulatory cytokines include IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21, which play a role in the maturation of lymphocytes.
IL-1β	17.4 kDa (recombinant, mouse) 17.4 kDa (recombinant, human)	152 aa (recombinant, mouse) 153 aa (recombinant, human)	IL-1β is a member of the interleukin 1 family of cytokines, which is an important mediator of the inflammatory response, and is involved in a variety of cellular activities (such as cell proliferation, differentiation, and apoptosis).
IL-2	15.3 kDa (recombinant, human)	About 134 aa	IL-2 is a regulatory cytokine necessary for the growth, proliferation, and differentiation of thymic-derived lymphocytes (T cells) to become 'effector' T cells.
IL-6	23.7 kDa (human)	212 aa (human)	IL-6 is a pro-inflammatory cytokine, secreted by macrophages and T cells to stimulate immune response during infection, trauma, and burns.
IL-18	18 kDa (recombinant, human)	157 aa (recombinant, human)	IL-18, also known as interferon-gamma inducing factor, is a proinflammatory cytokine of the IL-1 superfamily.
IL-33	18 kDa (recombinant, human)	159 aa (recombinant, human)	IL-33 is a proinflammatory cytokine expressed on a wide variety of cell types, including fibroblasts, mast cells, dendritic cells, macrophages, osteoblasts, endothelial cells, and epithelial cells.

FasL: Fas ligand; *LIGHT*, an acronym derived from: homologous to Lymphotoxins, Inducible expression, competes with HSV Glycoprotein D for HVEM, a receptor expressed on T-lymphocytes; *IL-1β*: interleukin-1β; *IL-2*: interleukin-2; *IL-6*: interleukin-6; *IL-18*: interleukin-18; *IL-33*: interleukin-33; *TNF*: tumor necrosis factor; *TNFSF*: tumor necrosis factor superfamily; *TWEAK*: TNF-like weak inducer of apoptosis.

[a] The values of MW reported in the table are only indicative because several circulating and tissue isoforms of the same protein are present in humans.

II. Rationale and aim of the study

Protein homeostasis impairment and/or mitochondrial impairment can act as a trigger for the induction of a local and systemic inflammatory response, called sterile inflammation, by the formation and activation of the inflammasome.

Inflammasome occupies a central role in cardiac remodeling and cardiomyocytes apoptosis through the release of pro-inflammatory cytokines such as IL1β.

Circulating levels of inflammatory cytokines are increasingly higher on the blood of HF patients [127] and the levels seems to be proportional to the severity of the NYHA functional class [128] and to the augmentation of the mortality rate [121,122, 130].

In the present study we tested the hypothesis that in patients affected by idiopathic DCM there was a higher concentration of pro inflammatory cytokines probably involved in the clinical evolution of the heart disease.

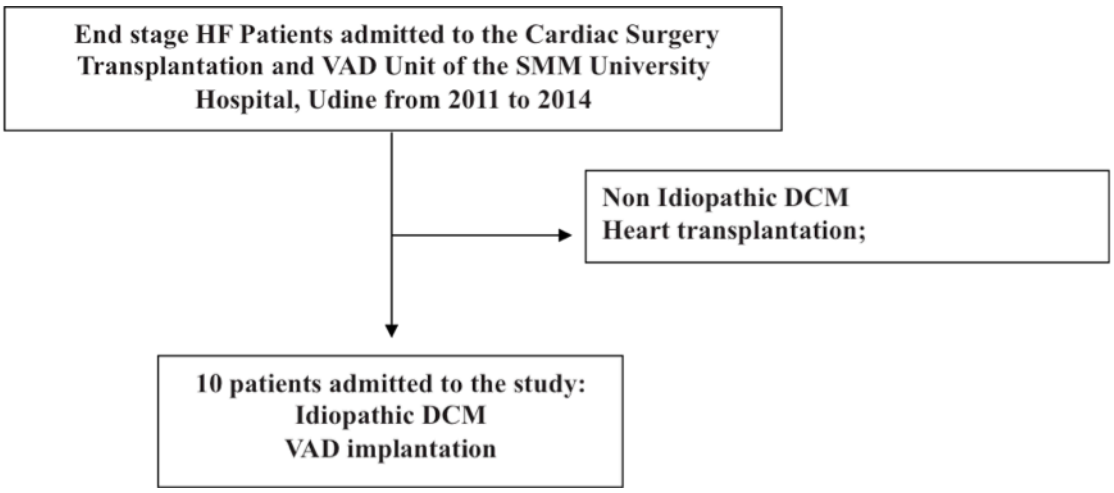
We also tested the hypothesis that the reduction of the ventricular overload stress through the insertion of a ventricular assist device in idiopathic DCM was coupled to a reduction of the inflammasome activation leading to a reduction of the cytokines levels.

To assess the first hypothesis the BNP concentration and the circulatory levels of several cytokines of healthy patients were compared to the BNP and cytokines levels of patients affected by idiopathic DCM in an advanced heart failure stage.

Furthermore we tested the second hypothesis comparing the cytokines levels before and after the ventricular assist device insertion.

III. Materials and Methods

1. Patient enrollment and Ethics



The study was in accordance with the Declaration of Helsinki, was approved by the Udine Ethics Committee (2 August 2011, reference number 47831), written informed consent was obtained from each patient.

We enrolled patients affected by idiopathic dilated cardiomyopathy that underwent Ventricular Assist Device (VAD) implantation at the Cardiac Surgery Unit of the AOUD Santa Maria della Misericordia (Udine) as bridge to heart transplantation in the 80 % of the cases or as destination therapy (20 %).

As controls we employed patients without cardiac abnormalities and good cardiac function matched for age and sex to the patients of the study group.

Patients underwent an in-depth evaluation of their clinical status, which included: coronary angiography, cardiopulmonary exercise test, pulmonary function test, echocardiography, cardiac catheterization, assessment of pulmonary vascular resistance, electrocardiography and tests aimed at excluding the presence of malignancies and other vascular diseases.

2. Analysis of circulating biomarkers

Every patient enrolled in this study was asked to donate 5mL of peripheral blood before ventricular assistance device implantation.

Samples were collected in tubes containing EDTA as anticoagulant, were centrifuged to obtain plasma. This latter was stored at -80°C until analyses were performed.

Plasma levels of IL1β, IL1RA, IL4, IL6, IL10, Leptin, and Ghrelin were analyzed employing a customized plate and a multiplex system (Bioplex, Biorad, USA). Brain Natriuretic Peptide (BNP) and Neutrophil Gelatinase-Associated Lipocalin (NGAL) were analyzed employing a Point-of-Care system (Cardiorenal, Alere, UK). Galectin 3 and IL1 receptor-like 1 (IL1RL1 or ST2) were evaluated employing a solid-phase sandwich ELISA (R&D systems, USA).

3. Anaesthetic and surgical techniques

3.1 Anesthesia

The VAD implantation was performed under general anesthesia; induction was commonly achieved with sodium thiopental (midazolam and/or ketamine could be used alternatively), fentanyl and rocuronium, meanwhile as maintaining drugs were used propofol and sufentanil.

3.2 Positioning and preparation

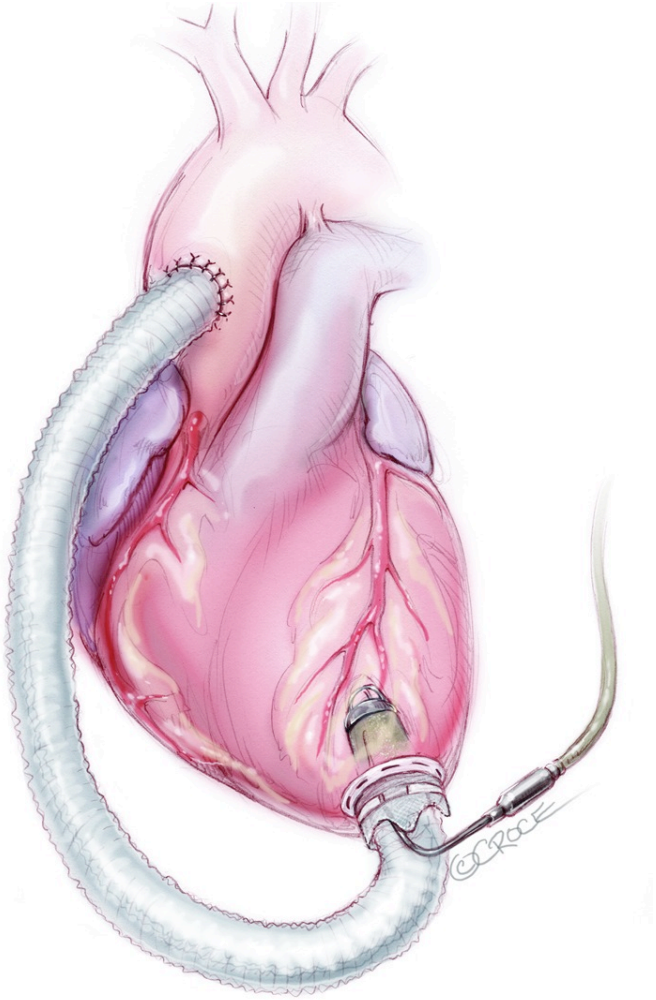
The operative room were set up as for a conventional cardiac surgery, with cardio pulmonary bypass.

Under general anesthesia (as mentioned before), the patients were intubated with a single lumen endotracheal tube; both central and peripheral venous lines were established (large volume infusion catheters are needed in case of significant haemorrhage) as well as an arterial line, followed by the insertion of a transesophageal echocardiography (TEE) probe.

3.3

3.3.1 Jarvik 2000

The Jarvik-2000 (Jarvik Heart, Inc.; New York, NY, USA) is a valveless electrically powered non-pulsatile axial-flow left ventricular assist device (LVAD) that has been developed and refined over the last 25 years.



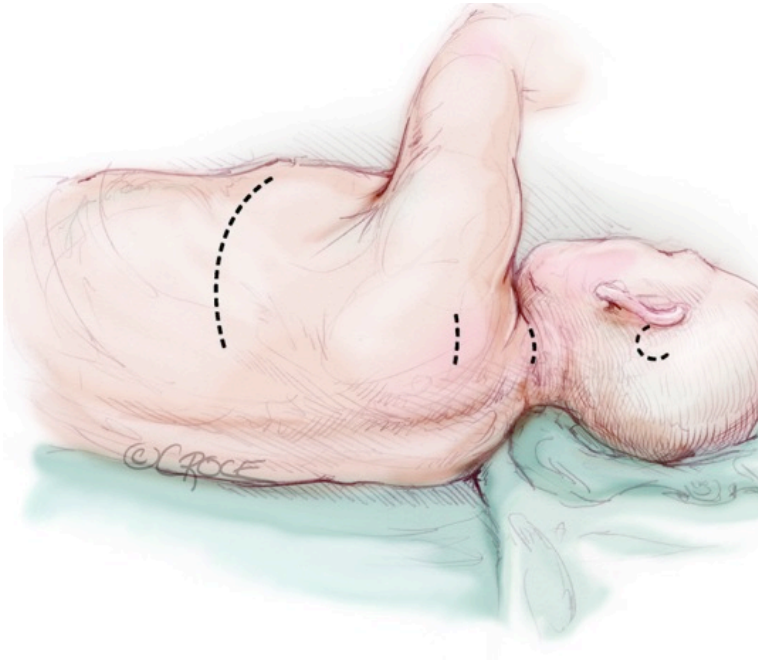
It consists of a miniaturized intraventricular blood pump that is placed on the cardiac apex and creates a blood flow that can be directed either towards the ascending or descending aorta through its outflow conduit.

The Jarvik-2000 is 2.5 cm wide, 5.5 cm long and weighs 85 grams. The pump has one moving part, an impeller that is a neodymium-iron-boron magnet and which is housed inside a welded titanium shell. It is supported by ceramic bearings and spins blood to generate an average flow rate of 5 L/min (up to 7 L/min) with a rotation speed of 8,000-12,000 rpm (4).

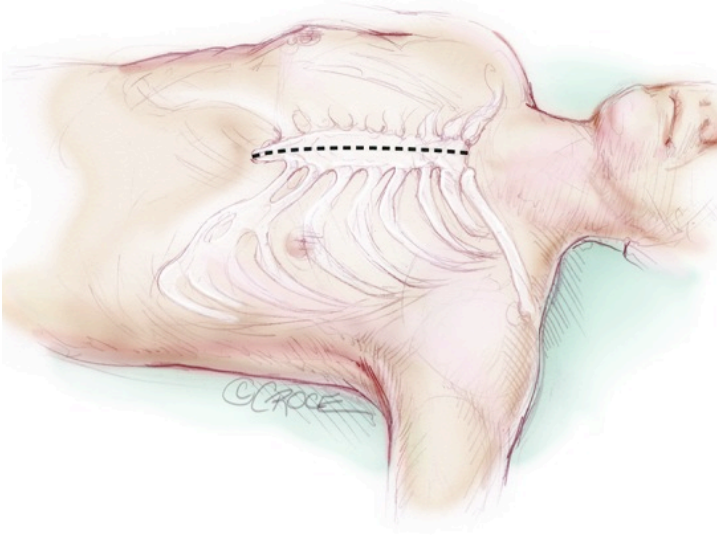
The device is connected to an external controller via a tunneled driveline that delivers power to the impeller. This controller permits manual adjustments of the pump speed and also visualizes the battery charge level.

Nowadays, it can be employed as a bridge to transplant or for permanent use (destination therapy) in case of untreatable end-stage heart failure (1-3).

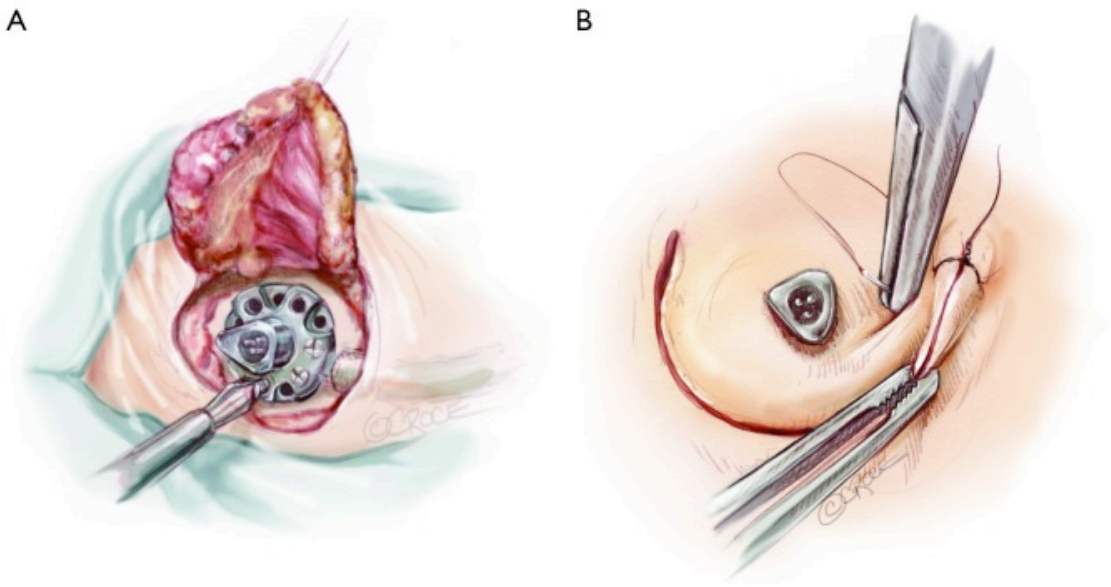
Implantation strategies



Our surgical technique required the cardiopulmonary bypass (CPB) support in 9 patients and the ECMO support in 1 case, while as access we chose a median sternotomy or a single left lateral thoracotomy.



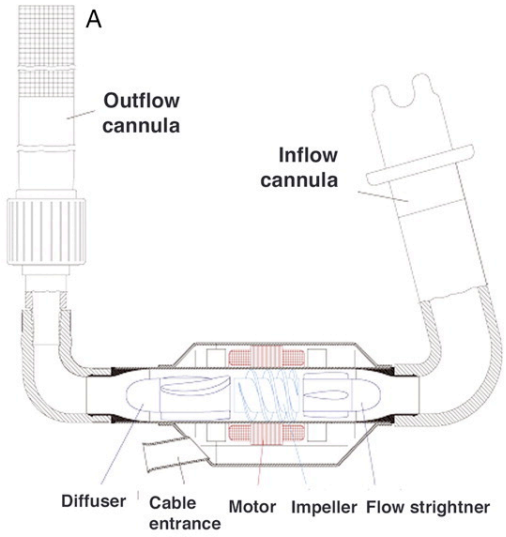
In all cases was created a subcutaneous tunnel to bring the power cable either to the abdominal region or the retro-auricular region where the external cable can be connected to the controller (8-10). The masthoid region is usually chosen to house the skull-pedestal for the power cable skin exit, as it is associated with minimal infective risk.



In the figure above is possible to see the retro auricular implantation of the power cable

3.3.2 Berlin Heart INCOR

The Berlin Heart INCOR system [Berlin Heart AG, Berlin, Germany] is a left ventricle assist device (LVAD),.

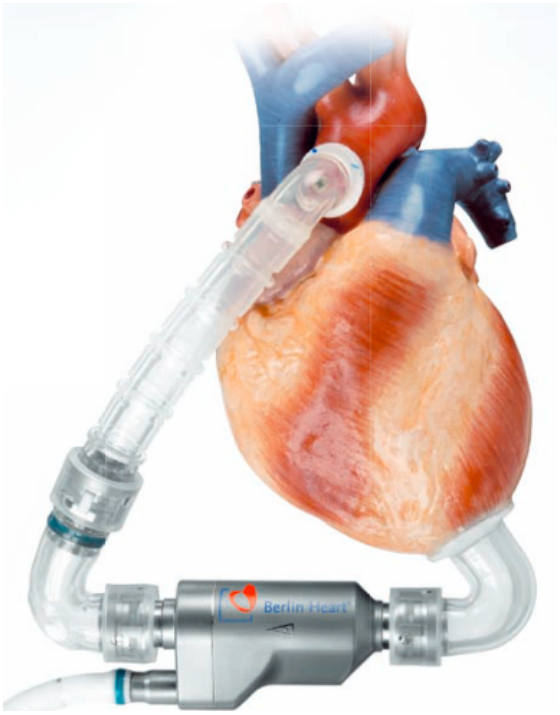


Is an axial flow pump for support of the left ventricle and is able to provide a laminar blood flow of up to 6 l/min against a pressure of 100 mmHg.

It was designed with the aims of minimum wear, low energy consumption and reduced damage to the blood components.

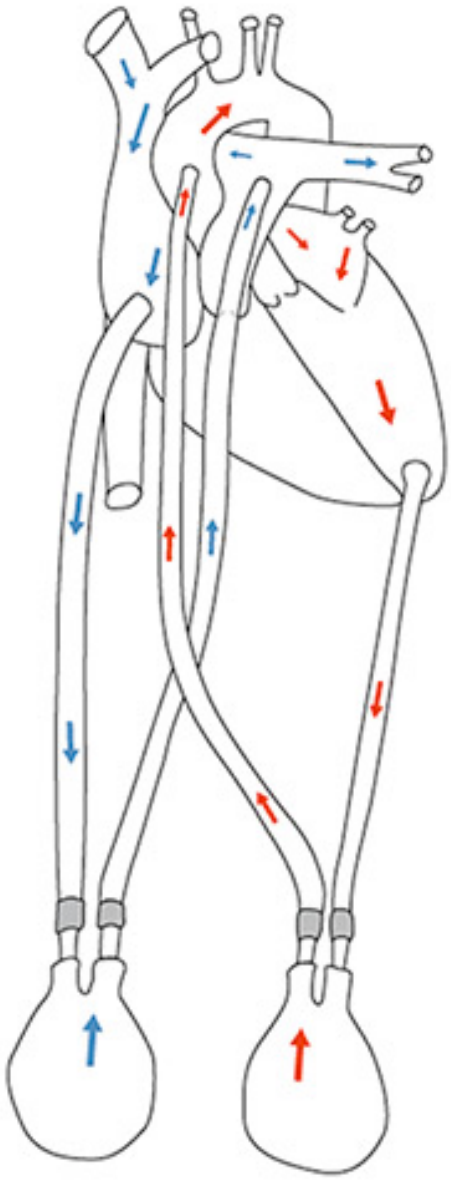
Blood is evacuated from the left ventricle into the pump through an inflow cannula, which is inserted into the apex of the heart.

The blood stream is then directed to the impeller, which is the only moving part of the pump. Magnetic suspension bearings stabilize the impeller actively in the axial direction and passively in the radial direction.



The impeller is driven by an 'air-gap' motor and rotates at a speed between 5000 and 10,000 rpm.

The blood leaving the pump is then transported to the aorta through the outflow cannula.



3.3.3 Berlin Heart EXCOR

The Berlin Heart EXCOR is a mechanical, pulsatile system for short- to long-term support of left or/and right ventricular pumping function. It is indicated for life-threatening illness in adults with severe heart failure after exhausting conservative therapy options.

EXCOR is often used as a bridge until heart transplantation is performed or the heart has recovered but has also been used as a destination therapy alternative to a heart transplant in some patients.

The Berlin Heart VAD consists of one or two chambers that sit outside of the body. It has a blood filled side and an air filled side, separated by a flexible membrane. The blood filled side of the pump is connected directly to the left ventricle and to the aorta in the case of the Left VAD or to the right atrium and main pulmonary artery in the case of a Right VAD.

The air filled side of the pump is connected via a driveline to a driving unit that drives air into and out of the Berlin Heart pump pulling blood from the heart into the pump and then back into the aorta or into the pulmonary artery a main artery of the body.

4. Follow-Up

The patients were followed up and blood samples for the studied biomarkers levels were taken during the short term follow up period (period of time from the operation to the second post operative week) and during the long term follow up (after the second post operative week).

The mean follow up time was 377 days ±527, with a maximum follow up time of 1695 days and a minimum follow up time of 5 days.

Three patients died during the ventricular assistance time, one because of multi-organ failure, one because of hemorrhagic problem, and one because of pulmonary inflammation.

Three patients underwent heart transplantation 1695, 110 and 10 days after the ventricular assist device implantation.

5. Statistical analysis

Characteristics of the study population are described using means±Standar deviation. Data were analyzed for normal distribution by Kolmogorov-Smirnov test. Continuous variables between two groups were compared using t-test or Mann-Whitney test, as appropriate.

Comparison of continuous variables among groups was performed by Anova followed by Bonferroni post-test or by Kruskal-Wallis followed by Dunn's post-test, as appropriate.

Correlations between two variables were analyzed employing Pearson or Spearman test, as appropriate.

Probability values (p) less than 0.05 were considered significant.

Analyses were conducted with Prism, version 4.0c for Macintosh software.

IV. Results

1. Baseline clinical characteristics of the patients

The characteristics of the studied population, including cardiovascular risk factors, cardiac functionality parameters and therapy, are shown in Table 1.

The majority of the patients belonged to NYHA class IV (80 %).

According to INTERMACS classification, one patient was in class 3, four patients were in class 2 and four patients were between class 1 and 2.

All patients, when possible, were receiving maximal pharmacological therapy including angiotensin II receptors blockers, β-blockers, antialdosterone drugs, diuretics and digitalis based on the severity of their clinical status.

The 50 % of the cases required a continuous infusion of inotropes and in the 10% of patients an intra-aortic balloon pump (IABP) was inserted before the VAD insertion.

All patients received an ICD.

On cardiac catheterization pulmonary hypertension was found in the 50% of the patients.

Table 1: clinical features of the DCM group of patients.

	Average	St dev	Percentage
Total number of pts 10			
Age	58	12	
NYHA 4			80
Heart Failure duration	122 months	66 months	
Weight (kg)	73	16	
Height (cm)	173	9	
Body Max Index	22	4	
Diabetes			40
History of Hyper Tension			20
Hypercholesterolemia			40
Chronic renal failure			60
Pre-operative inotropes			50
Antialdosterone drugs			90
Pre-op IABP			10
Furosemide			100
Digoxin			50
ACE-Inhib			0
Amiodaron			60
β-blockers			50
bilirubina_b	1.85	1.02	
BNP (pg/mL)	673.33	413.43	
BUN	27.00	8.45	
DBP	66.38	6.96	
HT polm reversible			50
CVP mmHg	12.00	6.54	
PAPm mmHg	44.44	11.39	
SBP	102.50	11.41	
PAWP mmHg	31.86	6.69	
Death			30

New York Health Association scale (**NYHA**); pre-operative intraortic balloon pump (**pre-op IABP**), angiotensin converting enzyme-inhibitors (**ACE-Inhib**); systolic (**SBP**) and diastolic (**DBP**) blood pressure; pulmonary artery wedge pressure (**PAPW**); pulmonary artery mean pressure (**PAPm**); central venous pressure (**CVP**)

2. Base line circulatory biomarkers

Table 2. Pre-operative assessment of biomarker levels

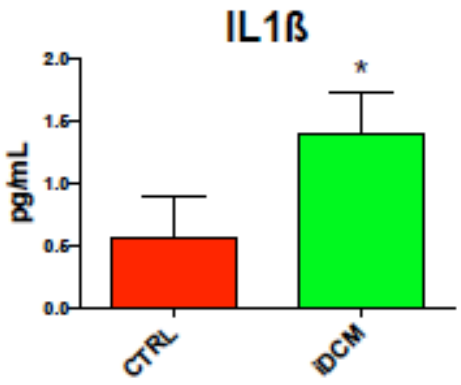
	DCM		Controls		
CONCENTRATION pg/ml	**Mean**	**st dev**	**Mean**	**st dev**	**p value**
IL1ß	1.40	1.01	0.76	1.67	0.0034
IL1RA	10.39	15.80	345.00	1055.00	0.3234
IL1ß/IL1RA	0.17	0.20	0.002	0.0050	0.0006
IL4	1.38	1.31	0.38	0.59	0.04
IL6	85.70	143.86	0.81	2.76	< 0.0001
IL10	7.23	14.84	5.07	7.23	1.00
GHRELIN	1298.45	824.75	1054	628.8	0.392
LEPTIN	2245.39	2400.52	4280.00	4174.00	0.4375

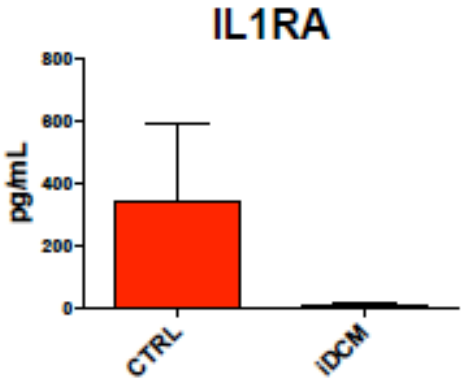
The circulatory biomarkers characteristics of the DCM patients and of the control group, summarized in the table 2, showed statistically significant differences in concentrations of IL1ß, IL4, IL6 and of IL1ß/ IL1RA ratio. All the mentioned biomarkers presented higher plasma concentration in the DCM group.

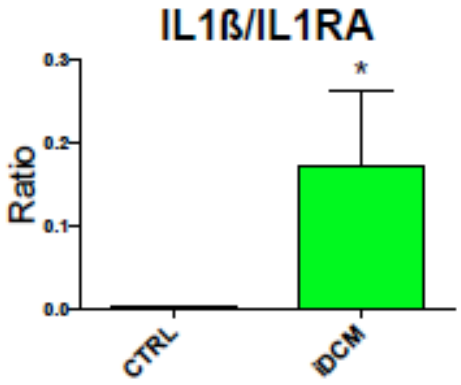
A tendency toward a higher concentration of the IL1ß receptor antagonist (IL1RA), even if not statistically significant, was observed in the control group.

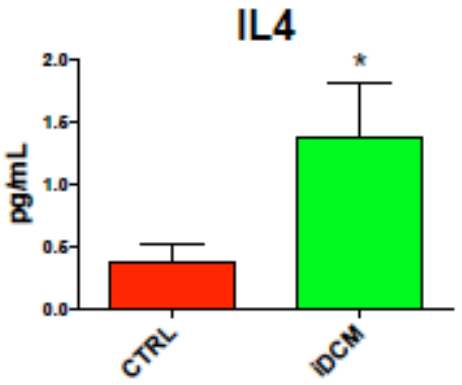
IL10 concentration was higher in the study group, although the difference was not significant.

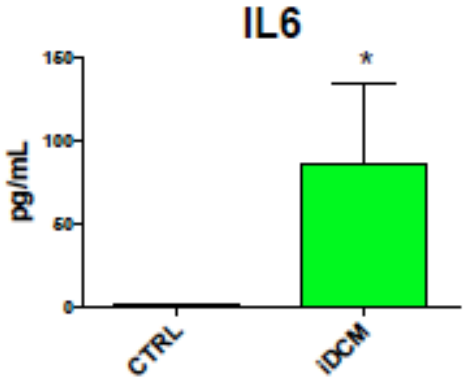
Regarding Grelin and Leptin, the concentrations were not significantly different but we observed a higher concentration of Ghrelin in the diseased patients and a higher concentration of Leptin in the healthy group.

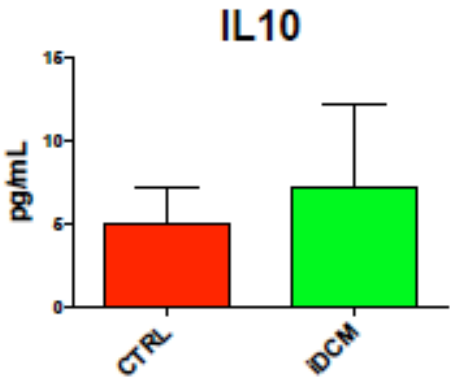

IL1ß
pg/mL
2.0
1.5
1.0
0.5
0.0
*
CTRL
iDCM

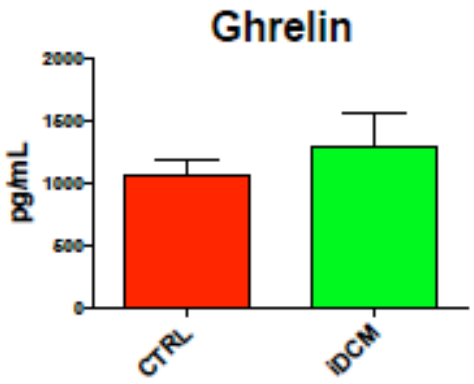

IL1RA
pg/mL
800
600
400
200
0
CTRL
iDCM

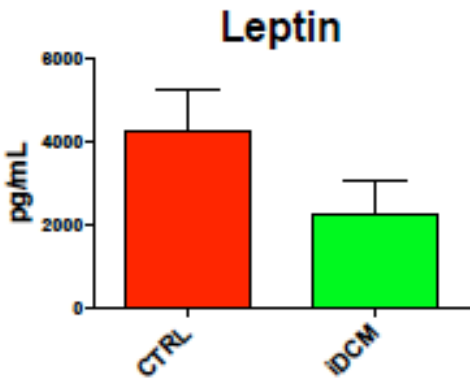

IL1ß/IL1RA
Ratio
0.3
0.2
0.1
0.0
*
CTRL
iDCM


IL4
pg/mL
2.0
1.5
1.0
0.5
0.0
*
CTRL
iDCM


IL6
pg/mL
150
100
50
0
*
CTRL
iDCM


IL10
pg/mL
15
10
5
0
CTRL
iDCM


Ghrelin
pg/mL
2000
1500
1000
500
0
CTRL
iDCM


Leptin
pg/mL
6000
4000
2000
0
CTRL
iDCM

3. Follow up analysis of the circulatory biomarkers

Table 3. Post-operative assessment of biomarker levels

	base		short term FU		Long term Fu		P values
(pg/mL)	Mean	St dev	Mean	St dev	Mean	St dev	
BNP	630.5	440.9	834.6	578.6	222.7	166.7	0.0827
IL1ß	1.4	1.01	1.216	0.8872	2.172	1.527	0.5083
IL4	1.38	1.31	1.35	1.086	3.068	1.722	0.6107
IL6	85.7	143.86	322.3	331.9	35.34	23.81	0.0064
IL10	7.23	14.84	49.34	83.06	34.07	19.67	0.1683
GHRELIN	1298.45	824.75	2379	1038	2069	1167	0.2068
LEPTIN	2245.39	2400.52	771.6	555.3	2690	1102	0.0280

The follow up analysis of the circulatory BNP levels showed that after the ventricular assist device insertion, there was an augmentation during the short term follow up and then a reduction during the long-term follow-up.

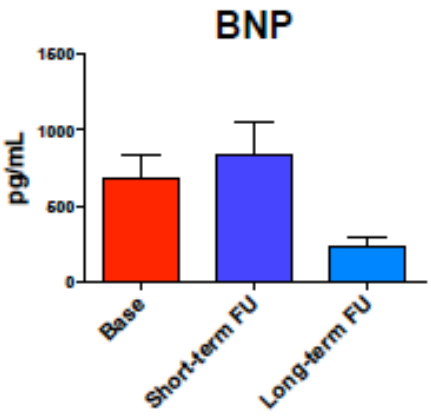
This result, even if not statistically significant, was probably due to the initial stress of the operation and to a reduction of the myocardial shear stress in the long-term period.

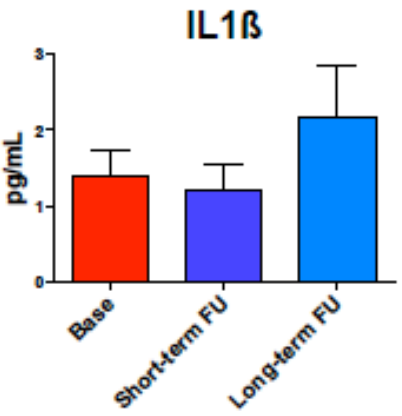
Regarding the IL1β and the IL4 levels we did not found a significant variation but we observed a tendency toward an augmentation of their concentration during the long term follow up.

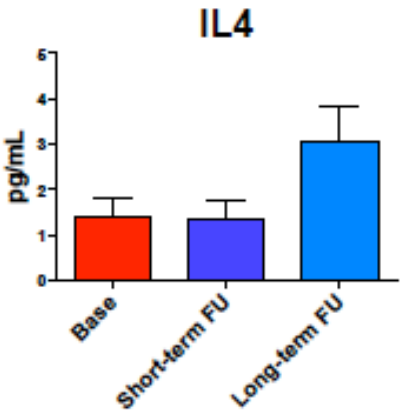
Regarding IL10 and IL6 we observed an augmentation during the first week and then a return to lower circulatory levels. This pattern of variation was significant only for the IL6.

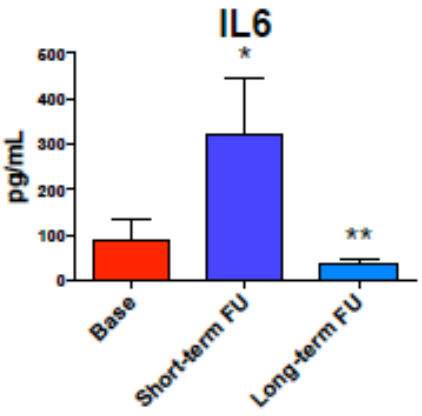
Regarding the Ghrelin, we observed an increase during the first week and then a low decrement, but the variations were not significant.

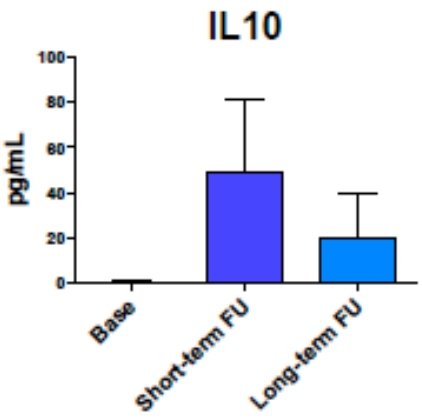
For the Leptin, after a first decrement we observed a significant increment of the levels in the long tem follow up.

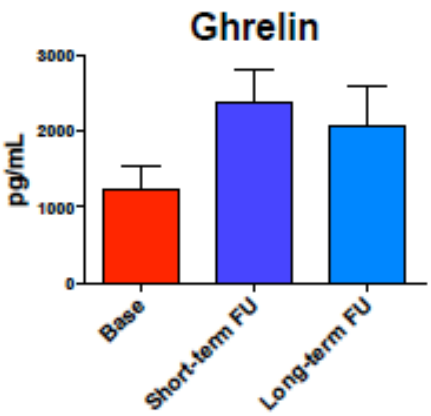

BNP
pg/mL
1500
1000
500
0
Base
Short-term FU
Long-term FU

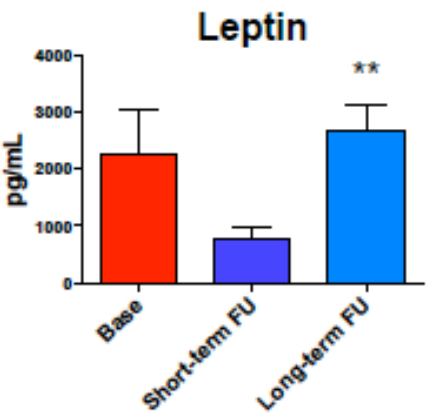

IL1ß
pg/mL
3
2
1
0
Base
Short-term FU
Long-term FU


IL4
pg/mL
5
4
3
2
1
0
Base
Short-term FU
Long-term FU


IL6
pg/mL
600
400
300
200
100
0
*
**
Base
Short-term FU
Long-term FU


IL10
pg/mL
100
80
60
40
20
0
Base
Short-term FU
Long-term FU


Ghrelin
pg/mL
3000
2000
1000
0
Base
Short-term FU
Long-term FU


Leptin
pg/mL
4000
3000
2000
1000
0
**
Base
Short-term FU
Long-term FU

4. Correlations

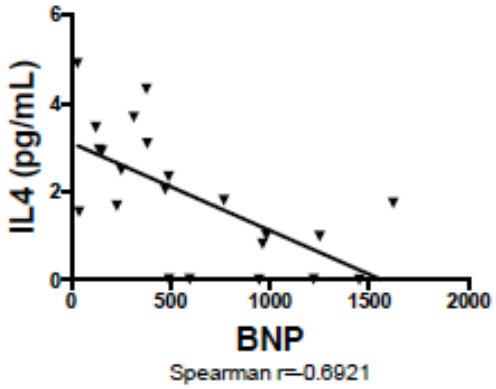
		BNP	IL1ß	IL4	IL6	IL10	GHRELIN	LEPTIN
BNP	Spearman r	1	-0.4292	-0,692**	0,475*	0.011	-0.055	-,462*
	P		0.0462	0,004	0.026	0.962	0.802	0.026
IL1ß	Spearman r	-0.4292	1	0,6667**	-0.171	-0.3	0,6278**	0.191
	P	0.0462		0.0005	0.447	0.187	0.0013	0.396
IL4	Spearman r	-0,692**	0,6667**	1	-0.18	-0.045	0,507*	0.404
	P	0,004	0.0005		0.412	0.842	0.0135	0.056
IL6	Spearman r	,475*	-0.171	-0.18	1	0.211	0.204	-0.2
	P	0.026	0.447	0.412		0.347	0.352	0.361
IL10	Spearman r	0.011	-0.3	-0.045	0.211	1	0.04	-0.255
	P	0.962	0.187	0.842	0.347		0.861	0.252
GHRELIN	Spearman r	-0.055	0,6278**	0,507*	0.204	0.04	1	-0.331
	P	0.802	0.0013	0.0135	0.352	0.861		0.114
LEPTIN	Spearman r	-,462*	0.191	0.404	-0.2	-0.255	-0.331	1
	P	0.026	0.396	0.056	0.361	0.252	0.114	

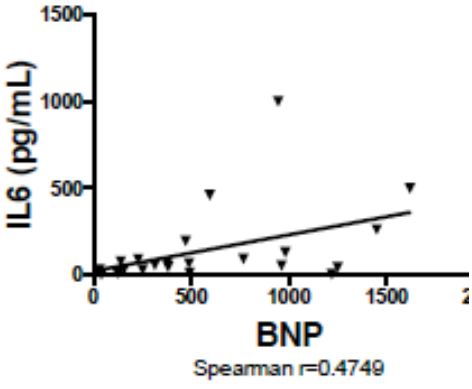
The table above shows the correlation between the different biomarkers.

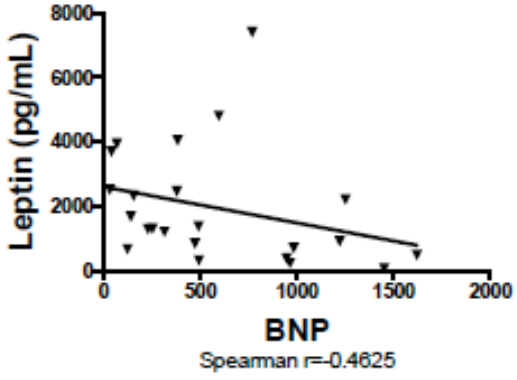
In our study population we found that BNP significantly correlates in a negative way to IL1ß (P=0.0462; Spearman r = -0.4292), IL4 (P=0.004; Speerman r = -0,692), and Leptin (P=0.026; Speerman r = -0,462) and in a positive way to IL 6 (P=0.026; Speerman r = 0,475).

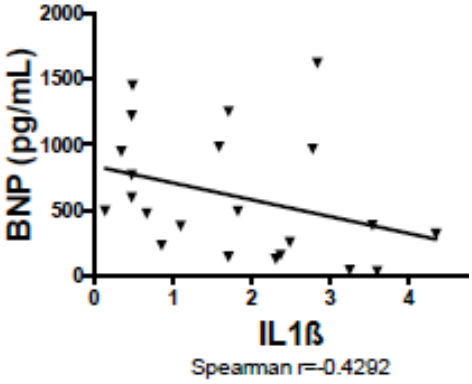
IL1ß was also correlated in a significant positive way to IL4 (P=0.0005; Speerman r = 0,6667), and Ghrelin (P=0.0013; Speerman r = 0,6278).

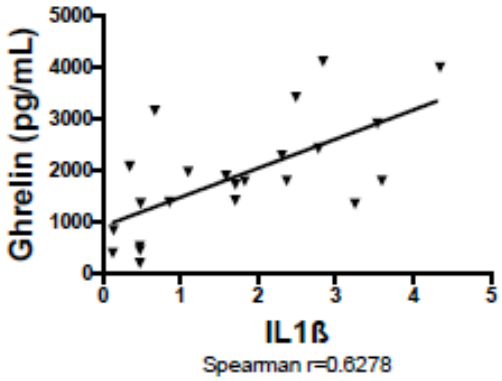
A significant positive correlation was also found between Ghrelin and IL 4 (P=0.0013; Speerman r = 0,6278).

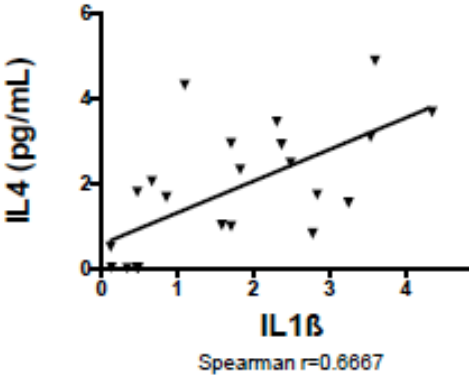

IL4 (pg/mL)
BNP
Spearman r=-0.6921

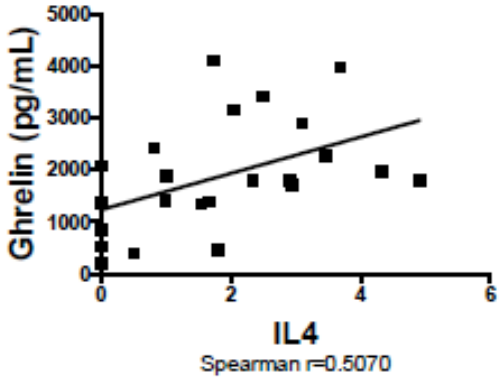

IL6 (pg/mL)
BNP
Spearman r=0.4749


Leptin (pg/mL)
BNP
Spearman r=-0.4625


BNP (pg/mL)
IL1ß
Spearman r=-0.4292


Ghrelin (pg/mL)
IL1ß
Spearman r=0.6278


IL4 (pg/mL)
IL1ß
Spearman r=0.6667


Ghrelin (pg/mL)
IL4
Spearman r=0.5070

V. Discussion

In our observational study we analyzed the cytokine profile of end stage heart failure patients affected by idiopathic dilated cardiomyopathy who underwent ventricular assist device implantation as destination therapy or as a bridge to transplant.

As shown by different authors, the activation of the inflammasome and the consequent establishment of sterile inflammation and pyroptosis play an important role in the clinical development of cardiomyopathy. In this pathogenetic process a pivotal task is covered by the pro-inflammatory cytokines that have been found in concentrations proportionally related to the worsening of the clinical status. (128 , 130).

Our results showed that in a selected population of patients affected by idiopatic dilated cardiomyopathy it is possible to find high concentration of pro-inflammatory cytokines such us IL1ß, IL4, IL6 and reduced levels of anti-inflammatory messengers like IL1RA and IL 10.
These results suggest that the pro-inflammatory environment rich in pro-inflammatory cytokines can be related to a chronic inflammasome activation, which can play an important role in the detrimental loop that leads to the end-stage heart failure in DCM patients.

The VAD insertion was a destination therapy in the 20 % of our patients and the device used in the 90 % of the patients was an LVAD generating a continuous flow. Despite the relatively low percentage of patients receiving the VAD as a destination therapy, we registered a relatively long follow-up (mean 377 days ±527), with a patient that underwent heart trasplantation after a ventricular assistance of 1695 days.

During the follow-up of the biochemical markers, we observed a tendency toward a decrement of the BNP levels in the long-term period, probably due to the hemodynamic effect of the VAD that can allow a reversal of stress-related compensatory responses of the overloaded myocardium leading to a subsequent structural and functional 'reverse remodeling'. [1 4]

Despite the reduced levels of BNP, we found steady high levels of pro-inflammatory cytokines IL 1β and IL 4, suggesting a continuation of the pro-inflammatory state and a continuation of the negative effect of the inflammasome on the heart function.
The above situation can be considered an obstacle to the recovery of the failing hearts or, especially in case of LVAD implantation as destination therapy, can have deleterious effects, leading to a progression of the dysfunction that in a mid-long term period can lead to right heart failure.

The BNP negative correlation to IL1ß, IL4 and Leptin was probably due to the reduced level of BNP following the VAD insertion.

Taken together, these results suggest that the reduction of the volume overload obtained by the VAD insertion does not reverse the mechanisms of chronic inflammation. Hence, targeting directly the inflammasome or its cytokines may be a valuable therapeutic strategy to improve the reversal cardiac remodeling and to reduce the effect of the inflammatory cytokines to the right ventricle in patients undergoing LVAD implantation.

While interleukin-1 blockade is an established therapeutic option for patients with rheumatoid arthritis and other autoinflammatory conditions (Abbate et al. 2012), two pilot studies with IL-1 blockade in patients with acute myocardial infarction and chronic heart failure have shown beneficial effects in terms of cardiac remodeling and aerobic exercise performance, and a large secondary prevention trial in patients with prior acute myocardial infarction is currently ongoing.

Targeting the components of the chronic inflammatory pathways could represent an adjuvant therapy option that could improve the mid and long term results in ventricular assisted patients.

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