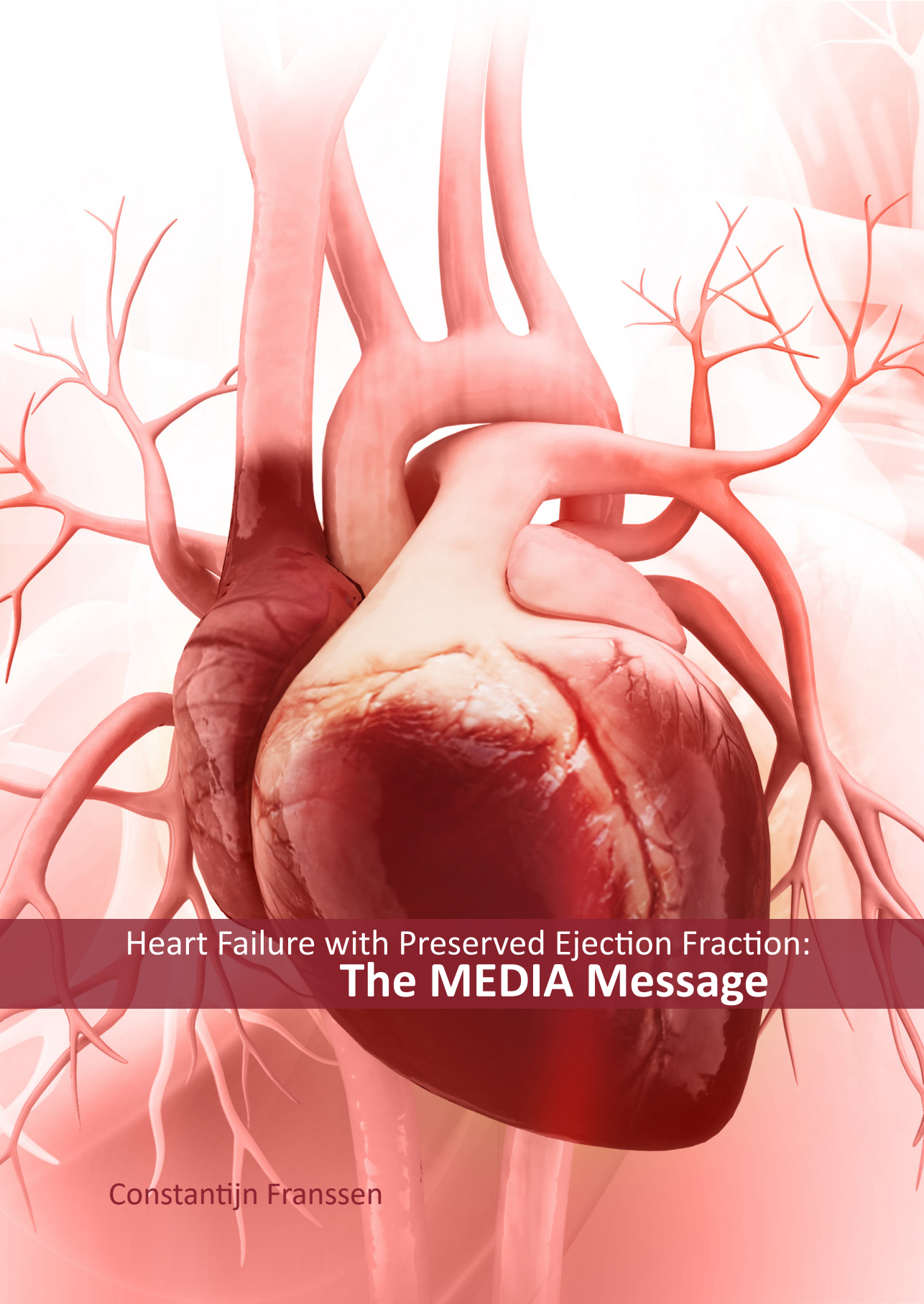

Heart Failure with Preserved Ejection Fraction: The MEDIA Message

Constantijn Franssen

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Heart Failure with Preserved Ejection Fraction: The MEDIA Message

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Ter verkrijging van de graad Doctor aan
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Door
Constantijn Paulus Matheus Franssen
geboren te Heerlen

Promotor: Prof. dr. W.J. Paulus

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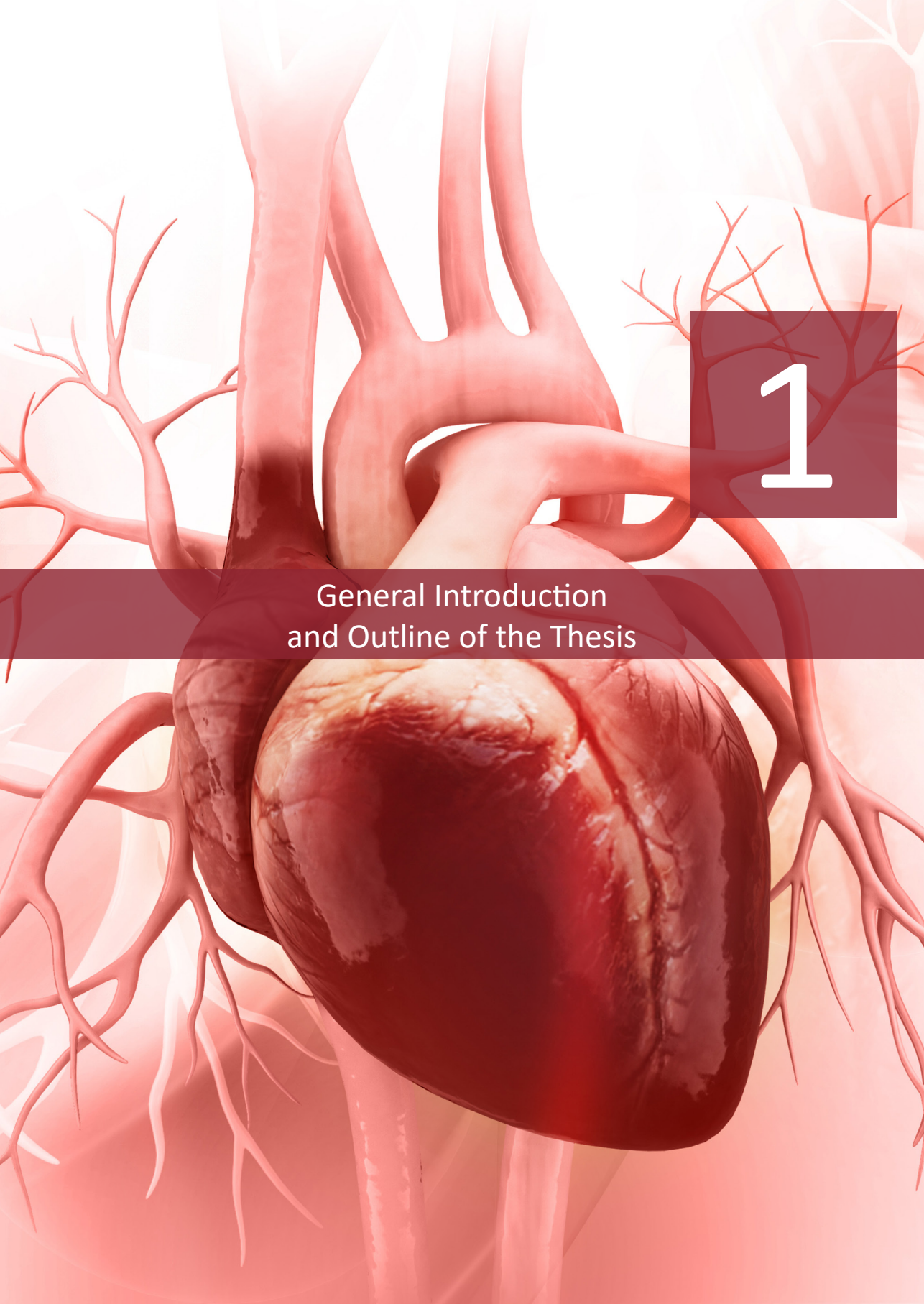

1

General Introduction and Outline of the Thesis

INTRODUCTION

Background

Heart Failure (HF) is a complex syndrome caused by an abnormality of cardiac structure or function leading to the inability of the heart to deliver oxygen at a rate commensurate with the requirements of the metabolizing tissues, or if the heart is only feasible to achieve this at the expense of increased filling pressures of the left ventricle (LV).[1] The diagnosis of HF requires typical symptoms (such as breathlessness, ankle swelling and fatigue) in combination with signs (such as elevated jugular venous pressure, displaced apex beat and pulmonary crackles).[2] Since these signs and symptoms can have many different causes or are difficult to detect in an elderly or obese population of HF patients, further evidence of cardiac dysfunction is warranted for the diagnosis of HF.

Heart Failure with Reduced and Preserved Ejection Fraction

In the beginning of the 1980s, the importance of LV ejection fraction (LVEF) originated from industry-driven clinical trials and the emphasis on statistics in the light of the novel principle of evidence-based medicine[3]. In these trials, only HF patients with a LVEF < 40- 45 % were included because they were expected to have a grim prognosis and this selection bias was aimed at increasing the statistical power with a reasonable number of patients.[3] This approach led to a tremendous improvement in therapeutic options in patients with HF and a reduced LVEF (HFrEF or systolic heart failure) as described in the current guidelines on the treatment of HF from the Heart Failure Association of the European Society of Cardiology and the American College of Cardiology Foundation/American Heart Association.[2,4] However, during the past decade it became more and more evident that the population of HF patients with a preserved LVEF (HFpEF) increases gradually.[5,6] Since HFpEF is characterized by diastolic LV dysfunction, this type of HF is also referred to as diastolic heart failure. Currently, HFpEF and HFrEF account for roughly equal proportions of HF patients,[7] but in the elderly HFpEF has already become the most common form of HF.[8] Moreover, since modern therapeutic strategies have proven to prevent deterioration of LVEF, it has been suggested that many patients have shifted from a HFrEF to a HFpEF population.[7] Also, mortality in HFpEF patients is only slightly lower than in HFrEF patients,[9] indicative of the burden of this type of HF with its

increasing prevalence. Unfortunately and in stark contrast to HFrEF, no treatment strategy studied to date in large HFpEF trials has proven to improve disease progression and survival, including betablockers,[10] angiotensin-converting enzyme inhibitors,[11] angiotensin 2 receptor blockers,[12,13] and digoxin.[14] Probably the most important reason for these disappointing trials and the lack of evidence-based treatment options is the absence of a profound knowledge about HFpEF pathophysiology. Indeed, just over a decade ago, knowledge about myocardial structure and function in HFpEF was very poor.[15] The following years many studies addressed epidemiological, clinical and fundamental aspects in HFpEF, gradually elucidating its pathophysiology.

Clinical characteristics of HFpEF patients

HFpEF is characterised by a high incidence of non-cardiac comorbidities. Although waist circumference and overweight patients are often not reported in studies, one third of patients has a body mass index ≥30kg/m^2.[6,16] Diabetes mellitus prevalence ranges from 37 to 45% in various registries, while arterial hypertension shows an even higher prevalence, ranging from 76 to 96%.[6,16–18] Furthermore, around 1/3 of HFpEF patients suffer from chronic obstructive pulmonary disease (COPD) and 26-52% of patients have chronic kidney disease.[6,18] Moreover, many patients have more than one comorbidity and the number of comorbidities correlates with prognosis.[19,20] These comorbidities share the capacity to induce a chronic, low-grade inflammatory state and oxidative stress, as will be discussed in detail later.

Cardiac characteristics of HFpEF

The LV in HFpEF is characterized by concentric remodeling, whereas HFrEF is characterized by eccentric LV remodeling as illustrated in Figure 1.[21]

Besides morphological differences between HFrEF and HFpEF, pressure-volume (PV) loop analyses reveal two distinct hemodynamical profiles (Figure 2).[22] The dominant functional abnormalities in HFrEF are decreased LV contractility, as evidenced by a decrease in the slope of the end-systolic PV relationship (systolic elastance) and a global down- and rightward shift of the PV-curve. In contrast, in HFpEF the PV-loop is shifted upward and to the left, indicative of increased LV diastolic stiffness and higher filling pressures in a smaller LV cavity volume.[2] Many studies thereafter focused on the myocardial mechanisms underlying the increased LV diastolic stiffness observed in HFpEF.

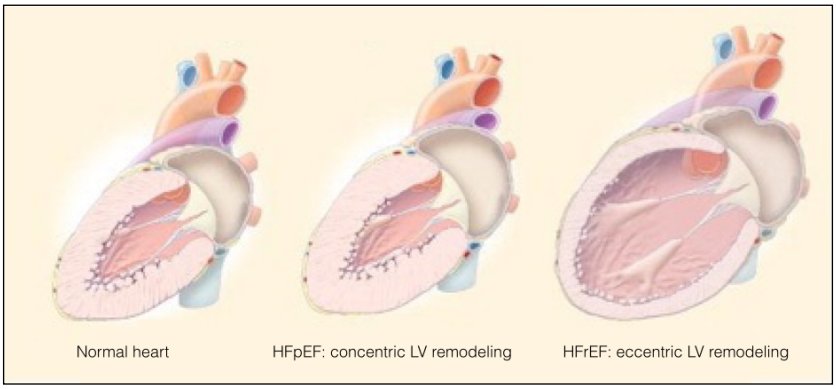

Figure 1: Compared to the normal heart in the left panel, the HFpEF heart is characterized by a normal-sized LV cavity with thickened ventricular walls (concentric LV hypertrophy) and preserved systolic function. In contrast, in HFrEF (right panel), the LV walls are thinner with eccentric remodeling and an overall decrease in systolic function. Reproduced with permission, Copyright Massachusetts Medical Society.[21]

Myocardial characteristics of HFpEF

The myocardium consists of 2 major compartments contributing to LV diastolic stiffness: the extracellular matrix (ECM) and the cardiomyocytes.[23] Studies on LV endomyocardial biopsy samples expanded our knowledge on HFpEF pathophysiology enormously. The first experimental studies showed myocardial fibrosis with an increased collagen volume fraction (CVF) in HFpEF patients compared to controls.[24] Associations of myocardial CVF with parameters related to diastolic dysfunction such as LV end-diastolic pressure[24] or the E:E' ratio (the ratio of transmitral E velocity to early diastolic mitral annular velocity)[25] have been found in HFpEF patients. However, quantification of total collagen content with CVF seems to have less functional implications than the relative amount of the stiffer collagen type I over the more compliant collagen type III, or the amount of cross-linked collagen by lysyl oxidase (LOX).[26,27] For example, human HFpEF myocardial biopsy samples contained increased levels of collagen type I, enhanced collagen cross-linking and LOX expression and these findings were associated with parameters of diastolic dysfunction on tissue Doppler echocardiography.[25] Myocardial fibrosis in HFpEF is mainly interstitial, whereas HFrEF is characterized by replacement fibrosis following cardiomyocyte cell death, suggesting different pathophysiological mechanisms in both HF entities.[28]

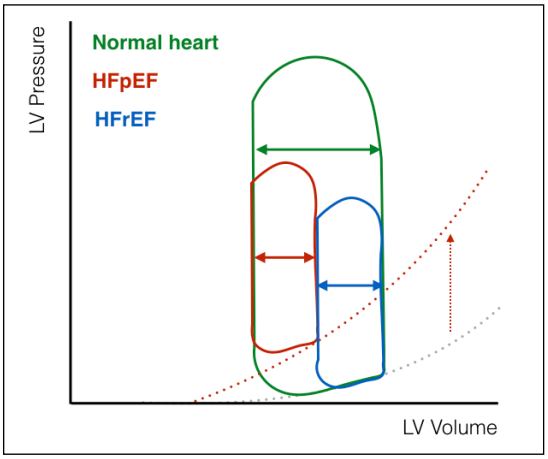

Figure 2: Schematic LV pressure-volume relationship through 1 cardiac cycle in normal hearts (green), HFpEF (red) and HFrEF (blue). HFrEF is characterized by increased LV volume, decreased stroke volume (SV, blue arrow) compared to a normal heart (green arrow), but the end-diastolic pressure-volume (PV) relationship is comparable (dotted grey line). In HFpEF, the SV is decreased (red arrow) and the end-diastolic PV-relationship is shifted upward and to the left (red dotted line), indicating increased diastolic stiffness.

However, besides alterations in the ECM, cardiomyocytes undergo typical changes in HFpEF. For example, irrespective of CVF, cardiomyocyte diameter was larger in biopsy samples from HFpEF patients compared to HFrEF patients.[29] Also, when mounted between a force transducer and piezzolectric motor (Figure 3), HFpEF cardiomyocytes appeared to be stiffer than control or HFrEF cardiomyocytes when stretched over a wide range of sarcomere lengths (SL).[24,29] Moreover, this increased passive stiffness ($F_{passive}$) upon stretch correlated with LV end-diastolic pressures and LV diastolic stiffness.[24]

Also, cardiomyocyte stiffness was higher in HFpEF than in HFrEF and highest in HFpEF patients with DM.[29,30] Upon administration of protein kinase A (PKA), cardiomyocyte stiffness declined more in HFpEF than in HFrEF cardiomyocytes, suggesting that a phosphorylation deficit contributes to myocardial and diastolic stiffness in HFpEF. Finally, in failing hearts, Ca^{2+} reuptake into the sarcoplasmic reticulum by the SERCA (sarcoplasmic/endoplasmic reticulum calcium- ATPase) pump is delayed.[31] This leads to increased cytoplasmic Ca^{2+} concentrations and subsequent increased diastolic stiffness.

It has been hypothesized that when the heart fills and the myocardium is stretched within a physiological range, $F_{passive}$ is primarily caused by titin in the cardiomyocytes.[32] In pathological settings where the myocardium is acutely

stretched or in the case of myocardial remodeling, it has been suggested that the ECM plays a more important role, probably to protect the cardiomyocytes from overstretching.[32]

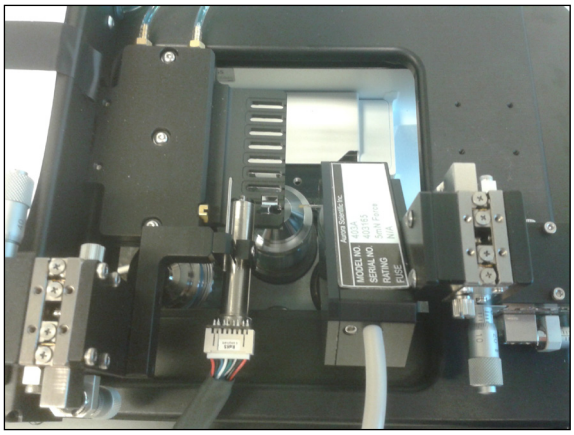

Figure 3: A myocardial muscle strip mounted between a force transducer and piezoelectric motor above an inverted microscope.

Molecular characteristics of HFpEF - Titin

Since administration of PKA could lower $F_{passive}$ in HFpEF cardiomyocytes and this to a larger extent than in HFrEF cardiomyocytes, molecular changes in HFpEF cardiomyocytes were to be expected.[29] The giant protein titin is the main determinant of myocardial stiffness in cardiomyocytes supporting diastolic distensibility. Titin modulates cardiomyocyte stiffness, via either phosphorylation or oxidation.[32] Titin can, for example, be phosphorylated by protein kinase A (PKA)[24], PKG[33], by PKC[34], calcium/calmodulin-dependent kinase II (CaMKII)[35] and extracellular signal-regulated kinase (ERK).[36] PKG is stimulated by cyclic guanosine monophosphate (cGMP), which on its turn is synthesized from guanylyl cyclase.[37] The latter has 2 isoforms and is either stimulated by nitric oxide (NO) via soluble GC (sGC) or natriuretic peptides (NP) via particulate GC (pGC).[38] Low PKG activity and cGMP concentration were observed in human HFpEF LV myocardium and $F_{passive}$ of isolated cardiomyocytes decreased upon *in vitro* PKG-administration.[39]

It was recently proposed that the low grade inflammatory state that is induced by metabolic comorbidities, leads to microvascular endothelial inflammation and oxidative stress.[28] These inflammatory and oxidative processes may decrease NO bioavailability and subsequently NO-sGC-cGMP-PKG signalling, eventually leading to titin hypophosphorylation and increased myocardial stiffness. However, besides titin hypophosphorylation, other mechanisms inside

the cardiomyocytes are expected to increase $F_{passive}$. Recent studies suggested a role for proteotoxicity in cardiac dysfunction, as previously demonstrated in neurological diseases such as Alzheimer's and Huntington's disease.[40] The basis for this proteotoxicity resides in failing quality-control and/or repair mechanisms to correct for aggregated or damaged proteins as a consequence of inflammation and oxidative stress, but it is also part of normal (cardiac) aging and it can affect mitochondrial dysfunction.[40,41] Knowledge about the role for proteotoxicity and mitochondrial dysfunction in relation to aging, inflammation and oxidative stress in HFpEF is still premature, but might explain the absence of benefit from different treatment strategies so far.[42]

The goal of the current thesis is to investigate the pathophysiological mechanisms leading to increased myocardial stiffness in HFpEF. Moreover, a more thorough knowledge about the pathophysiology might help to improve diagnostic strategies and identify potential therapeutic targets.

1.1 AIM, OBJECTIVES AND OUTLINE

The aim of this thesis was to gain more insight in the pathophysiology (chapter 2, 3, 4), diagnostic (chapter 5 ,6) and therapeutic options (chapter 7, 8) of HFpEF. For this, we used a translational approach, ranging from a rat model to human endomyocardial biopsy samples (Figure 4).

In **Chapter 2**, the focus is on myocardial stiffness in HFpEF and its contributors. In this study, a novel, metabolically induced ZSF1-HFpEF rat model was characterized with echocardiography, invasive hemodynamics, metabolic cage studies and on tissue and protein level after sacrifice. Force measurements were performed on small muscle strips and isolated cardiomyocytes to discern the different factors contributing to myocardial stiffness.

Chapter 3 investigates if systemic, low-grade inflammation of metabolic risk contributes to HFpEF through coronary microvascular endothelial activation. Inflammatory endothelial activation, myocardial oxidative stress, NO bioavailability and cGMP-PKG signalling were investigated in human HFpEF myocardial biopsies and validated in ZSF1-HFpEF rats.

In **Chapter 4**, the role of titin and ECM remodeling in HFpEF are reviewed. Different techniques to assess ECM quality, quantity and its relevance are discussed, followed by changes in cardiomyocytes observed in HFpEF. Also, cardiomyocyte and ECM cross-talk is discussed.

In **Chapter 5**, the focus changes to diagnostic aspects of HFpEF. Weaknesses of the current diagnostic algorithm are discussed, followed by the potential strengths of several biomarkers reflecting myocardial remodelling.

Chapter 6 highlights the difficulties clinicians are confronted with in patients with pulmonary hypertension (PH), when its aetiology is unclear. Pulmonary capillary wedge pressure (PCWP) aids in this diagnosis. If PCWP is high, PH is likely to be caused by left sided heart failure. However, many patients with HFpEF have a normal PCWP at rest and this chapter focuses on how to cope with this diagnostic trap.

Chapter 7 focuses on cardiomyocyte based stiffness and a potential therapeutic option (α-B crystallin, a heat shock protein) is examined in vitro. This study uses human endomyocardial biopsy specimens on which force measurements are performed on muscle strips and single cardiomyocytes.

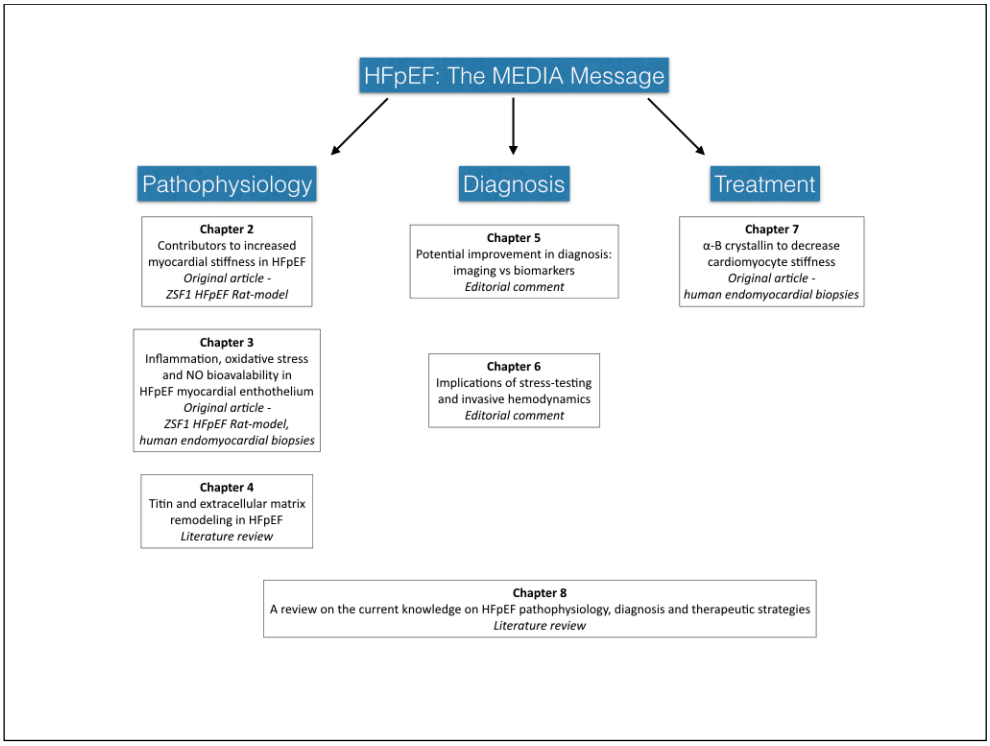

Figure 4. Outline of the thesis.

Chapter 8 concludes with a review on the current knowledge on HFpEF, including diagnosis, pathophysiology and current and potential future therapeutic strategies.

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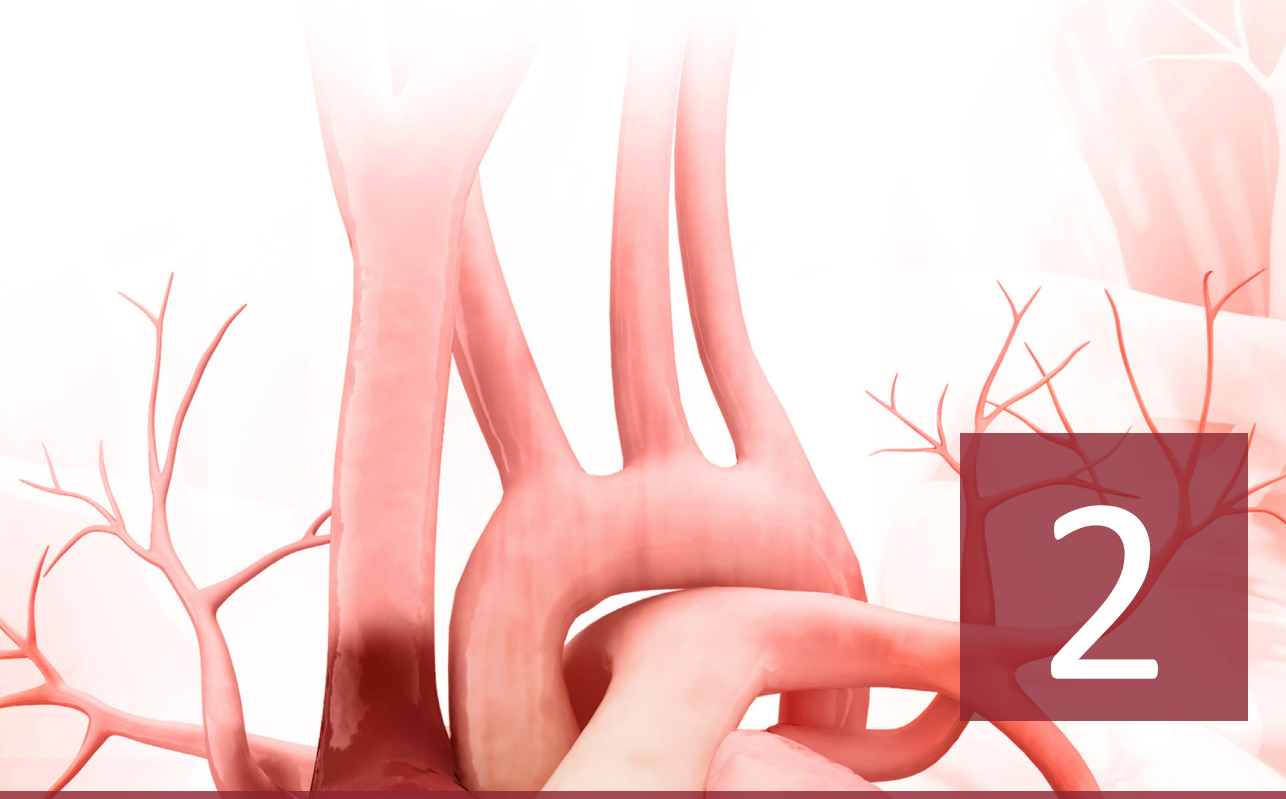

2

Myocardial Titin Hypophosphorylation Importantly Contributes to Heart Failure with Preserved Ejection Fraction in a Rat Metabolic Risk Model

Hamdani N*, Franssen C*, Lourenço A*, Falcão-Pires A, Fontoura D, Leite S, Plettig L, López B, Ottenheijm CA, Becher MP, González A, Tschöpe C, Díez J, Linke WA, Leite-Moreira AF, Paulus WJ.

*Authors contributed equally

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Myocardial Titin Hypophosphorylation Importantly Contributes to Heart Failure with Preserved Ejection Fraction in a Rat Metabolic Risk Model

by

Nazha Hamdani[1,2*], Constantijn Franssen[1*], André Lourenço[3*], Inês Falcão-Pires[3], Dulce Fontoura[3], Sara Leite[3], Luisa Plettig[2], Begoña López[4], Coen A. Ottenheijm[1], Moritz P. Becher[5], Arantxa González[4], Carsten Tschöpe[5], Javier Díez[4], Wolfgang A. Linke[2], Adelino F. Leite-Moreira[3], Walter J. Paulus[1]

[*] N.H., C.F. and A.L. contributed equally to this article.

From

[1] Department of Physiology, Institute for Cardiovascular Research, VU University Medical Center, Amsterdam, The Netherlands

[2] Department of Cardiovascular Physiology, Ruhr University Bochum, Germany

[3] Department of Physiology and Cardiothoracic Surgery, University of Porto, Portugal

[4] Division of Cardiovascular Sciences, Centre for Applied Medical Research, University of Navarra, Pamplona, Spain

[5] Department of Cardiology and Pneumology, Charité – Universitätsmedizin Berlin, Campus Benjamin Franklin, Berlin, Germany

ABSTRACT

Background—Obesity and diabetes are important metabolic risk factors and frequent comorbidities in heart failure with preserved ejection fraction (HFPEF). They contribute to myocardial diastolic dysfunction (DD) through collagen deposition or titin modification. The relative importance for myocardial DD of collagen deposition and titin modification was investigated in obese, diabetic ZSF1 rats after HFPEF development at 20 weeks of age.

Methods and Results—Four groups or rats (Wystar Kyoto, n=11; lean ZSF1, n=11; obese ZSF1, n=11 and obese ZSF1 with high fat diet, n=11) were followed over 20 weeks with repeat metabolic, renal and echocardiographic evaluations and hemodynamically assessed at sacrifice. Myocardial collagen, collagen crosslinking, titin isoforms and phosphorylation were also determined. Resting tension ($F_{passive}$)-sarcomere length relations were obtained in small muscle strips before and after KCl-KI treatment, which unanchors titin and allows contributions of titin and extracellular-matrix to $F_{passive}$ to be discerned. At 20 weeks of age, the lean ZSF1 group was hypertensive whereas both obese ZSF1 groups were hypertensive and diabetic. Only the obese ZSF1 groups had developed HFPEF, which was evident from increased lung weight, preserved LVEF and LV DD. The underlying myocardial DD was obvious from high muscle strip stiffness, which was largely (±80%) attributable to titin hypophosphorylation. The latter occurred specifically at the S3991 site of the elastic N2Bus segment and at the S12884 site of the PEVK segment.

Conclusions—Obese ZSF1 rats developed HFPEF over a 20 weeks time span. Titin hypophosphorylation importantly contributed to the underlying myocardial DD.

Key Words: diastole; heart failure; diabetes mellitus; obesity; myocardium

INTRODUCTION

Heart failure with preserved ejection fraction (HFPEF) is currently observed in 50% of all heart failure patients.[1] The incidence of HFPEF relative to heart failure with reduced ejection fraction (HFREF) continues to rise and its prognosis fails to improve partly because of lack of a specific HFPEF therapy.[2]

Prevalence of comorbidities is higher in HFPEF than in HFREF.[3] Comorbidities such as obesity and diabetes mellitus (DM) are key constituents of metabolic risk and known to be associated with the progressive left ventricular (LV) remodeling and dysfunction characteristically observed in HFPEF.[4,5] In HFPEF, body mass index has a U-shaped relation to mortality in contrast to HFREF where it displays an inverse relation with mortality.[6] DM has long time been recognized to be associated with LV diastolic dysfunction.[7] In HFPEF, HFREF and aortic stenosis (AS), DM worsens diastolic LV stiffness through a variety of mechanisms such as myocardial fibrosis, advanced glycation endproducts (AGEs) deposition and high cardiomyocyte stiffness.[8;9] High cardiomyocyte stiffness was especially evident in HFPEF and AS patients with DM, was associated with hypophosphorylation of the giant cytoskeletal protein titin[9,10] and corrected in-vitro by administration of protein kinase A (PKA) or G (PKG).[9,11] Furthermore, patients with high metabolic risk frequently suffer from salt-sensitive hypertension, which is like obesity associated with systemic oxidative stress.[12]

To elucidate the mechanisms underlying myocardial dysfunction in metabolic riskrelated HFPEF, the present study investigated: 1) LV hemodynamics; 2) myocardial histology; 3) in-vitro stiffness of small muscle strips; 4) cardiomyocyte stiffness and 5) myocardial titin phosphorylation in hypertensive ZSF1 rats, which became over a 20 weeks period morbidly obese and diabetic because of absence of satiation and unlimited access to a regular (ZSF1-obese) or high-fat diet (ZSF1-obese+HFD).

METHODS

An expanded Methods section is available in the Online Data Supplement.

Animal model

Nine-week old male ZSF1 lean (ZSF1-Lean, n=11), ZSF1 obese (ZSF1-Obese, n=22) and Wistar-Kyoto rats (WKY, n=11) were obtained from Charles River (Barcelona, Spain) and fed with Purina Diet (#5008). After a 1 week laboratory adaptation period, animals underwent phenotypic evaluation consisting of metabolic cage studies, blood sample collection and echocardiographic evaluation. To assess diastolic function, peak velocity of early (E) and late (A) mitral inflow signals and the ratio of E over E' (peak velocity of early diastolic lateral mitral annular motion) were measured as an indication of LV pressure.[13] From this point onward, a subgroup of ZSF1 obese rats (ZSF1-Obese+HFD, n=11) was randomly allocated to receive HFD (Research Diet Inc. #D12468). Weight gain and energy intake were recorded every third day. Phenotypic evaluation was repeated at the 14th and 18th week of life.

At 20 weeks of age, animals underwent hemodynamic evaluation under anesthesia and were subsequently sacrificed with procurement of myocardial tissue samples for histological, biochemical and biomechanical studies. Animals were kept in individually ventilated chambers in a controlled environment with a 12-h-light/-dark cycle at 20°C room temperature and had unlimited access to food. Investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the NIH (NIH Publication no. 85–23, revised 1996) and was approved by the ethics committee of the Faculty of Medicine of Porto.

Histology and Collagen Gene Expression

Collagen volume fraction (CVF) was determined by quantitative morphometry with an automated image analysis system in sections stained with collagen-specific picrosirius red.[8,9] A sircol-based assay was performed to obtain and quantify total, soluble and insoluble collagen, which was calculated by subtracting the amount of soluble collagen from the amount of total collagen. The degree of cross-linking was calculated as the ratio between soluble and insoluble collagen. Gene expression of Collagen1A1 and Collagen3A1 was performed using real-time PCR.

Force measurements on small muscle strips and cardiomyocytes

Cardiomyocytes and muscle strips were incubated respectively for 5 and 30 minutes in relaxing solution supplemented with 0.2 % TritonX-100 to remove all membrane structures and subsequently attached between a force transducer and

length motor. Resting tension ($F_{passive}$) was recorded between 1.9 and 2.3 m sarcomere length (SL). $F_{passive}$ of cardiomyocytes was measured before and after PKG incubation. In muscle strips, thick and thin filaments were extracted by immersing the preparation in relaxing containing 0.6M KCl (45 min at 20°C) followed by relaxing solution containing 1.0 min at 20°C). Following the extraction procedure, the muscle bundles were stretched again and the passive force remaining after KCl/KI treatment was assumed to be extracellular matrix-based. Titin-based passive force was determined as total passive force minus extracellular matrix-based passive force.[14]

Titin analysis

Titin isoform separation

Homogenized myocardial samples were analyzed by 1.8% sodium dodecyl-sulfate polyacrylamide-gel electrophoresis (SDS-PAGE). Protein bands were visualized by Coomassie blue or SYPRO Ruby, scanned, and analyzed densitometrically.[15,16]

Titin phosphorylation assays

Following 1.8% SDS-PAGE, gels were stained with Pro-Q Diamond for 1 hour and subsequently with Sypro Ruby overnight. Phosphorylation signals on Pro-Q Diamond-stained gels were indexed to Sypro Ruby-stained titin signals.[9,10,15,16]

Titin/Phosphotitin immunoblots

1.8% SDS-PAGE and Western blot were performed to measure site-specific phosphorylation and expression of titin using custom-made, affinity-purified, phosphosite-specific antibodies against phospho-S3991 (N2Bus), phospho-S12742 (PEVK) and phospho-S12884 (PEVK) (positions in mouse (Mus musculus) titin according to UniProtKB identifyer A2ASS6), and antibodies recognizing the corresponding nonphosphorylated sequence around these sites.[16]

Statistical analysis

Groups were compared by two-way repeated measures ANOVA whenever serial acquisitions were obtained for the same animal, and by one-way ANOVA for single acquisitions. Pressure-volume loop analysis was analyzed using LabChart 7 Pro v7.3.1. Values are given as mean ± SEM. A 2-tailed test with a probability of value <0.05 was considered significant. Single comparisons were assessed by an unpaired Student t test. Bonferroni-adjusted t tests were used subsequent for

multiple comparisons after repeated measure ANOVA. Statistical analysis was performed with SPSS (Version 15.0; SPSS Inc,Chicago,Ill).

RESULTS

Cardiometabolic risk in obese ZSF1 rats

ZSF1-Obese and ZSF1-Obese+HFD rats had higher weight gain at 20 weeks of age (Figure 1A). Energy intake was initially also higher in these animals, but leveled off at 20 weeks of age (Figure 1A). In both obese groups, glycemia levels, glucose tolerance and insulin resistance were higher (Figure 1B, Table 1). Hyperglycemia caused glycosuria, increased urine output and compensatory water intake. Proteinuria suggested presence of diabetic nephropathy despite preserved creatinine clearance and plasma protein levels (Table 1).

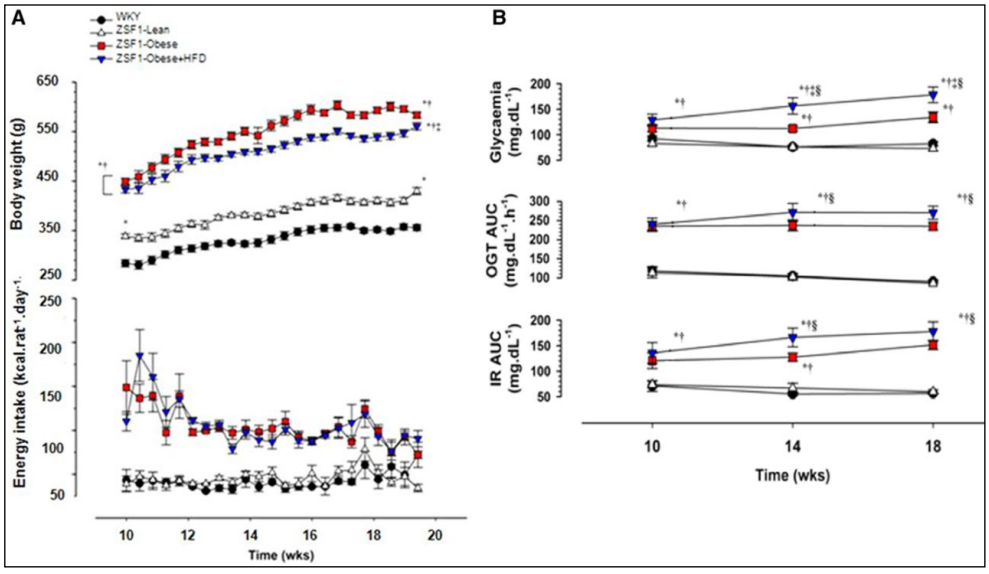

Figure 1. Body weight, energy intake and metabolism. **A**. Body weight and energy intake in all groups. **B**. Glycemia, oral glucose tolerance (OGT) and insulin resistance (IR) in all groups at 10, 14 and 18 weeks of age. *P<0.05 vs. WKY, †P<0.05 vs. ZSF1-Lean, ‡P<0.05 vs ZSF1-Obese.

Echocardiography, hemodynamics and morphometrics

Serial echocardiographic studies at 10, 14 and 18 weeks of age demonstrated normal systolic function in all groups (Figure 2A, 2B and Table 2). Concentric LV

remodeling was present throughout the entire study in both obese groups (Figure 2A, 2B and Table 2), which progressively developed diastolic LV dysfunction, evident from a restrictive LV-inflow signal, higher E/E' and increased left atrial area (LAA) at 14 and 18 weeks (Figure 2B and Table 2).

At 20 weeks of age, hemodynamic evaluation confirmed normal LV systolic performance, evident from LVEF, LVdP/dtmax and ESPVR EESI (Figure 2C and Table 3). Diastolic LV dysfunction was again evident from a prolonged , elevated LVEDP, an upward shift of the LV diastolic pressure-volume relationship and a higher LV diastolic chamber stiffness constant (β) (Figure 2C and Table 3). At sacrifice, lung and heart weights were increased in both obese groups (Figure 3A and Table 4). There was evidence of visceral adiposity with more perirenal and perigonadal fat (Table 4).

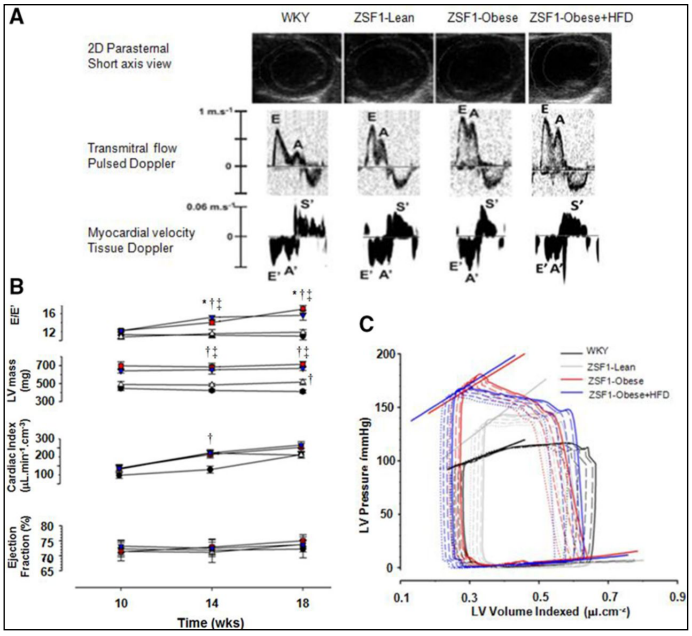

Figure 2. Echocardiography. **A**. Representative examples of sequential Dopplerechocardiographic imaging in all groups. **B**. LV Mass, Cardiac Index and LV EF in all groups at 10, 14 and 18 weeks of age. **C**. Representative LV pressure-volume from all groups at 20 weeks of age. *P<0.05 vs. WKY, †P<0.05 vs. ZSF1-Lean, ‡P<0.05 vs. ZSF1-Obese.

Histology

Cardiomyocyte hypertrophy was confirmed histologically in both ZSF1-Obese groups (Figure 3B). Collagen volume fraction, collagen cross-linking, procollagen carboxyl-terminal proteinase type I (PCP) and PCP enhancer (PCPE) were similar in all groups (Figure 4). In line with these findings, the relative mRNA expression of collagen 1A1 and collagen 3A3 were also similar among all groups (Figure 4). No

significant differences of Lysyl oxidase (LOX) expression were observed between all groups (WKY (1.75±0.44), ZSF1-Lean (1.76±0.19), ZSF1-Obese (1.50±0.35) and ZSF1-Obese+HFD (1.70±0.12)).

$F_{passive}$ in small muscle strips and cardiomyocytes

The relative contributions of collagen and titin were determined in small muscle strips (Figure 5). $F_{passive}$-SL relations were constructed for SL ranging from 1.9 to 2.3µm. $F_{passive}$ was higher in both obese groups from a SL of 2.075µm onwards (Figure 5A). To discern the contribution of extracellular matrix (E-matrix), $F_{passive}$-SL relations were also constructed following extraction with KCl/KI (Figure 5B). The contribution of titin was calculated by subtracting at each SL E-matrix based $F_{passive}$ from total $F_{passive}$ (Figure 5C). $F_{passive}$ attributable to E-matrix and titin were higher in both obese groups respectively from a SL of 2.175 and 2.025µm onwards. At the upper limit of the physiological SL-range (2.2µm) titin accounted for 82 and 78% of $F_{passive}$ in respectively ZSF1-Obese and ZSF1-Obese+HFD groups.

Table 1. Metabolism and renal function

	10^{th} wk	14^{th} wk	18^{th} wk	10^{th} wk	14^{th} wk	18^{th} wk
	WKY			**ZSF1-Lean**		
Plasma proteins ($g.L^{-1}$)	5.9 ± 0.1	5.7 ± 0.1	6.5 ± 0.1*	6.3 ± 0.1	6.3 ± 0.1	6.2 ± 0.1
Proteinuria ($mg.d^{-1}$)	23 ± 1	22 ± 1	20 ± 2	30 ± 2	29 ± 1	29 ± 1
Glycaemia ($mg.dL^{-1}$)	92 ± 5	76 ± 4	82 ± 4	83 ± 5	76 ± 3	73 ± 5
OGT AUC ($mg.dL^{-1}.h^{-1}$)	138 ± 11	124 ± 5	109 ± 5	132 ± 13	122 ± 9	105 ± 5
IR AUC ($mg.dL^{-1}.h^{-1}$)	59 ± 5	55 ± 3	56 ± 1	69 ± 7	58 ± 4	58 ± 3
Glycosuria ($mg.d^{-1}$)	18 ± 2	22 ± 1	20 ± 1	19 ± 2	18 ± 2	18 ± 1
Plasma creatinine ($mg.dL^{-1}$)	0.39 ± 0.02	0.41 ± 0.01	0.42 ± 0.01	0.40 ± 0.02	0.41 ± 0.02	0.40 ± 0.01
Urine output ($mL.Kg^{-1}.d^{-1}$)	77 ± 3	64 ± 2	56 ± 3	36 ± 2	32 ± 2	30 ± 2
C_{Cr} ($mL.min^{-1}.Kg^{-1}$)	8.6 ± 0.3	8.1 ± 0.2	7.5 ± 0.2	8.0 ± 0.3	7.7 ± 0.3	7.6 ± 0.2
Water intake ($mL.Kg^{-1}.d^{-1}$)	135 ± 6	100 ± 3*	85 ± 3*	76 ± 5†	52 ± 3†	41 ± 5*
	ZSF1-Obese			**ZSF1-Obese+HFD**		
Plasma proteins ($g.L^{-1}$)	7.9 ± 0.3†‡	8.7 ± 0.3*†‡	9.7 ± 0.4*†‡	7.2 ± 0.2†‡	6.9 ± 0.2†‡	8.3 ± 0.2*†‡§
Proteinuria ($mg.d^{-1}$)	92 ± 9†‡	115 ± 9*†‡	174 ± 10*†‡	105 ± 13†‡	88 ± 14*†‡§	126 ± 13*†‡§
Glycaemia ($mg.dL^{-1}$)	113 ± 7	112 ± 5†‡	134 ± 10†‡	121 ± 8	156 ± 16*†‡§	178 ± 16*†‡§
OGT AUC ($mg.dL^{-1}.h^{-1}$)	223 ± 13†‡	226 ± 14†‡	224 ± 10†‡	228 ± 16†‡	260 ± 24*†‡	259 ± 17*†‡
IR AUC ($mg.dL^{-1}.h^{-1}$)	111 ± 12†‡	123 ± 6*†‡	152 ± 9*†‡	117 ± 9†‡	154 ± 13*†‡§	185 ± 17*†‡§
Glycosuria ($mg.d^{-1}$)	739 ± 75†‡	730 ± 90*†‡	405 ± 45*†‡	759 ± 69†‡	1070 ± 6*†‡§	1070 ± 93*†‡§
Plasma creatinine ($mg.dL^{-1}$)	0.34 ± 0.02	0.31±0.02†‡	0.35 ± 0.03	0.37 ± 0.02	0.39 ± 0.01§	0.40 ± 0.03
Urine output ($mL.Kg^{-1}.d^{-1}$)	187 ± 23†‡	98 ± 17*‡	79 ± 18*‡	186 ± 24†‡	179 ± 19†‡§	177 ± 18†‡§
C_{Cr} ($mL.min^{-1}.Kg^{-1}$)	10.1 ± 0.4	9.2 ± 0.8	7.4 ± 0.8*	9.3 ± 0.5	5.6 ± 0.2*†‡§	4.4 ± 0.2*†‡§
Water intake ($mL.Kg^{-1}.d^{-1}$)	203 ± 23†‡	110 ± 16*‡	91 ± 16*‡	208 ± 24†‡	179 ± 20†‡§	182 ± 17†‡§

OGT, oral glucose tolerance; AUC, area under curve; IR, insulin resistance; C_{Cr}, creatinine clearance. Values are mean ± SEM, n=11, each group. $^{*}P<0.05$ *vs* 10^{th} wk; $^{\dagger}P<0.05$ *vs* WKY; $^{\ddagger}P<0.05$ *vs* ZSF1-Lean; §*vs* ZSF1-Obese.

$F_{passive}$-SL relations of isolated skinned cardiomyocytes were steeper in ZSF1-Obese and ZSF1-Obese+HFD (Figure 5D). Incubation with PKG returned the $F_{passive}$-SL relations to control levels (Figure 5D). No significant differences of active tension were observed between groups in single skinned small strips as well as skinned cardiomyocytes.

Table 2. Echocardiography

	10th wk	14th wk	18th wk	10th wk	14th wk	18th wk
	WKY			ZSF1-Lean		
HR (bpm)	281 ± 16	301 ± 10	290 ± 9	383 ± 11†	372 ± 11†	336 ± 9*†
dLVPW (mm)	1.28 ± 0.07	1.28 ± 0.05	1.26 ± 0.06	1.45 ± 0.03	1.39 ± 0.10	1.35 ± 0.09
LV mass (mg)	447 ± 24	423 ± 16	412 ± 18	490 ± 32	485 ± 16	517 ± 29†
CI ($\mu L.min^{-1}.cm^{-2}$)	99 ± 16	131 ± 20*	214 ± 18*	133 ± 20	223 ± 20*†	212 ± 16*
FS (%)	37 ± 2	36 ± 1	38 ± 2	36 ± 1	36 ± 3	38 ± 2
EF (%)	72 ± 3	72 ± 1	72 ± 4	71 ± 1	71 ± 3	74 ± 2
S ($mm.s^{-1}$)	66 ± 7	61 ± 3	54 ± 2	56 ± 4	58 ±1	64 ± 5
E/A	1.64 ± 0.09	1.75 ± 0.08	1.88 ± 0.09	1.38 ± 0.04	1.53 ± 0.11	1.61 ± 0.12
E/E'	11.5 ± 1.0	11.4 ± 0.4	11.2 ± 0.8	11.0 ± 0.4	11.7 ± 0.9	12.0 ± 0.6
MPI (Tei)	0.70 ± 0.03	0.78 ± 0.02	0.78 ± 0.03	0.77 ± 0.02	0.76 ± 0.04	0.76 ± 0.05
EDVI ($\mu L.cm^{-2}$)	1.51 ± 0.05	1.62 ± 0.10	1.90 ± 0.08*	1.60 ± 0.05	1.64 ± 0.11	1.87 ± 0.11*
LAA (mm^2)	2.0 ± 0.2	2.2 ± 0.2	2.2 ± 0.3	2.1 ± 0.2	2.6 ±0.2	2.6 ± 0.2
	ZSF1-Obese			ZSF1-Obese+HFD		
HR (bpm)	344 ± 11†‡	347 ± 11†	328 ± 16	335 ± 12†‡	329 ± 10	321 ± 11
dLVPW (mm)	1.56 ± 0.04†	1.50 ± 0.05†	1.49 ± 0.06†	1.53 ± 0.12	1.56 ± 0.11	1.44 ± 0.07
LV mass (mg)	698 ± 45	686 ± 42†‡	714 ± 33†‡	642 ± 24	654 ± 48†‡	671 ± 24†‡
CI ($\mu L.min^{-1}.cm^{-2}$)	136 ± 23	215 ± 20*†	255 ± 22*	138 ± 16	224 ± 12*†	268 ± 19*
FS (%)	36 ± 2	37 ± 2	39 ± 2	38 ± 2	40 ± 2	38 ± 1
EF (%)	71 ± 3	73 ± 3	75 ± 2	73 ± 2	72 ± 3	74 ± 1
S ($mm.s^{-1}$)	66 ± 4	67 ± 4	63 ± 6	69 ± 5	66 ± 4	59 ± 3
E/A	1.33 ± 0.03	1.34 ± 0.09†	1.32 ± 0.08†	1.44 ± 0.04	1.36 ± 0.15†	1.40 ± 0.10†
E/E'	12.4 ± 0.4	14.2 ± 0.5*†‡	17.2 ± 0.8*†‡	12.3 ± 0.2	15.4 ± 0.4*†‡	15.8 ± 1.1*†‡
MPI (Tei)	0.78 ±0.05	0.77 ± 0.06	0.78 ± 0.02	0.77 ± 0.03	0.76 ± 0.04	0.79 ± 0.04
EDVI ($\mu L.cm^{-2}$)	1.58 ± 0.13	1.59 ± 0.07	1.86 ± 0.07*	1.59 ± 0.17	1.60 ± 0.12	1.91 ± 0.03*
LAA (mm^2)	2.5 ± 0.2	3.0 ± 0.2†	3.6 ± 0.2*†‡	2.6 ± 0.1	2.7 ± 0.1	3.4 ± 0.1*†‡

HR, heart rate; dLVPW, left ventricular posterior wall measured in diastole; LV, lef ventricular; CI, cardiac index; FS, fractional shortening; EF, ejection fraction; S, peak systolic tissue Doppler velocity; E/A, ratio between peak E and A waves of pulsed-wave Doppler mitral flow velocity; E/E', ratio between peak E wave velocity of pulsed-wave Doppler mitral flow and peak E' wave velocity of tissue Doppler at the lateral mitral annulus; MPI, myocardial performance or Tei index; EDVI, end-diastolic volume indexed for body surface area; LAA, left atrial area; Values are mean ± SEM, n=6, each group. $^{*}P<0.05$ *vs* 10th wk; $^{\dagger}P<0.05$ *vs* WKY; $^{\ddagger}P<0.05$ *vs* ZSF1-Lean; §*vs* ZSF1-Obese.

Titin hypophosphorylation

N2B titin isoform expression relative to WKY was similar in all groups (WKY 100±9.4%; ZSF1-Lean 100.5±9.1%; ZSF1-Obese 95.03±9.6%; ZSF1-Obese+HFD 71.58± 11.4%), but titin phosphorylation decreased by 67 and 82% in ZSF1-Obese and ZSF1-Obese+HFD rats respectively (Figure 6A). Ex vivo phosphorylation by PKG significantly increased all-titin phosphorylation in ZSF1-Obese and ZSF1-

Obese+HFD, up to the level measured in WKY and ZSF1-Lean (Figure 6B). Using affinity-purified phosphospecific antibodies phosphorylation status was assessed by Western blot at a conserved serine within the N2Bus segment (S3991 of full-length mouse titin) and at two conserved serines within the PEVK segment (S12742 and S12884) (Figure 6C, D and E). In the obese groups, significant hypophosphorylation was observed at the phospho-N2Bus S3991 site and at the phospho-PEVK S12884 site but comparable phosphorylation at the phospho-PEVK S12742 site. Protein loading was checked by a sequence-specific antibody that corresponded with the phosphospecific antibody.

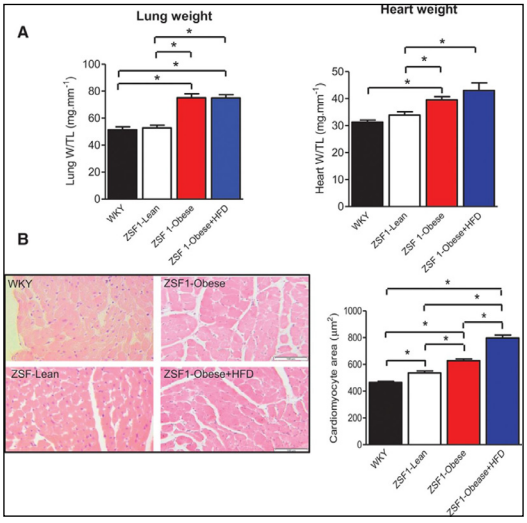

Figure 3. Lung weight, heart weight and cardiomyocyte hypertrophy. **A**. Lung and heart weights in all groups at sacrifice (20 weeks of age). **B**. Representative histological images of LV myocardium in all groups showing progressive cardiomyocyte hypertrophy. *P<0.05.

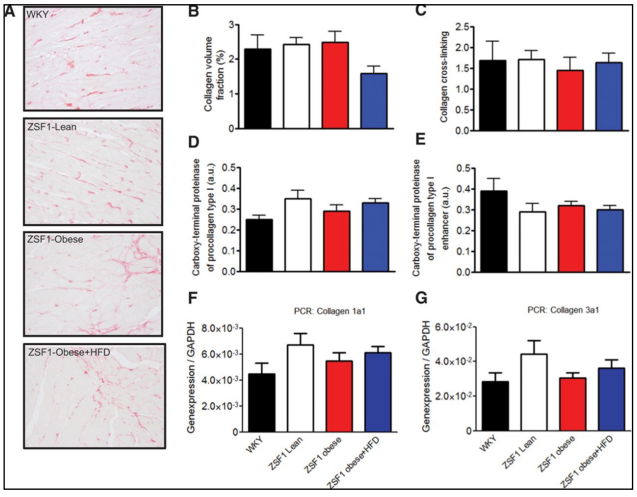

Figure 4. Collagen-volume fraction and cross-linking. **A**. Representative images of myocardial fibrosis (Picrosirius red; 200x magnification) in all groups. **B-G**. Collagen volume fraction, collagen cross-linking, procollagen carboxyl-terminal proteinase type I (PCP), PCP enhancer (PCPE), relative mRNA expressions of Collagen1A1 and Collagen3A1 in all groups.

Table 3. Hemodynamics

	WKY	ZSF1-Lean	ZSF1-Obese	ZSF1-Obese+HFD
BSA (cm^2)	468 ± 5	508 ± 7*	651 ± 5*†	616 ± 4*†‡
SAP (mmHg)	117 ± 4	146 ±6*	181 ± 6*†	170 ± 6*†
MAP (mmHg)	94 ± 6	127 ± 6*	149 ± 5*†	140 ± 5*
DAP (mmHg)	75 ± 7	106 ± 7*	125 ± 6*	113 ± 5*
HR (bpm)	346 ± 10	391 ± 9*	354 ± 12†	336 ± 8†
EDP (mmHg)	5 ± 0	4 ± 1	9 ± 1*†	7 ± 1*†
dP/dt_{max} ($mmHg.s^{-1}$)	9430 ± 770	11700 ± 964	13000 ± 680*	11000 ± 497
dP/dt_{min} ($mmHg.s^{-1}$)	-7880 ± 838	-12600 ± 686*	-11700 ± 541*	-11700 ± 429*
τ(ms)	8.3 ± 0.3	7.6 ± 0.4	10.5 ± 0.6*†	9.5 ± 0.4*†
EF (%)	60 ± 4	55 ± 3	59 ± 4	63 ± 3
ESPVR $E_{ES}I$ ($mmHg.\ \mu L^{-1}.cm^{-2}$)	0.64 ± 0.15	2.49 ± 0.71*	2.00 ± 0.39*	2.04 ± 0.39*
EDPVR β I ($\mu L^{-1}.cm^{-2}$)	0.016 ± 0.002	0.023 ± 0.002	0.028 ± 0.002*	0.029 ± 0.004*

BSA, body surface area; SAP, systolic arterial pressure; MAP, mean blood pressure; DAP, diastolic arterial pressure; HR, heart rate; EDP, end-diastolic pressure; dP/dt_{max}, maximum rate of pressure rise; dP/dt_{min}, maximum rate of pressure fall; τ, time constant of isovolumetric relaxation; EF, ejection fraction; $E_{ES}I$, slope of linear ESPVR for indexed volumes; EDPVR, end-diastolic pressure-volume relationship; β I, chamber stiffness constant for indexed volumes, derived from exponential EDPVR. For the purpose of volume indexation, BSA was estimated as 9.1*(body weight in g)$^{2/3}$. Values are mean ± SEM, n=11, each group. $^{*}P<0.05$ *vs* WKY; $^{\dagger}P<0.05$ *vs* ZSF1-Lean; $^{\ddagger}P<0.05$ *vs* ZSF1-Obese.

Table 4. Morphometrics

	WKY	ZSF1-Lean	ZSF1-Obese	ZSF1-Obese+HFD
TL (mm)	41.5 ± 0.1	42.1 ± 0.4	40.9 ± 0.4†	39.6 ± 0.3*†‡
LV+IVS weight/ TL ($mg.mm^{-1}$)	15.4 ± 0.6	16.6 ± 0.8	19.7 ± 1.1*	20.2 ± 1.3*†
RV weight/ TL ($mg.mm^{-1}$)	4.8 ± 0.4	4.5 ± 0.3	5.5 ± 0.3	5.6 ± 0.2†
Lung weight/ TL ($mg.mm^{-1}$)	51 ± 2	53 ± 2	75 ± 3*†	75 ± 2*†
Liver weight/ TL ($mg.mm^{-1}$)	252 ± 9	314 ± 12	906 ± 55*†	769 ± 29*†
Kidney weight/ TL ($mg.mm^{-1}$)	23.3 ± 0.3	26.0 ± 0.8	37.1 ± 0.9*†	33.9 ± 1.5*†
Perirenal fat weight/ TL ($mg.mm^{-1}$)	64 ± 4	58 ± 6	362 ± 12*†	415 ± 14*†
Perigonadal fat weight/ TL ($mg.mm^{-1}$)	62 ± 3	56 ± 5	161 ± 6*†	145 ± 5*†‡
Gastrocnemius weight/ TL ($mg.mm^{-1}$)	56 ± 1	63 ± 2	53 ± 1†	48 ± 1*†

TL, tibial length; LV, left ventricle; IVS, interventricular septum; RV, right ventricle. Values are mean ± SEM, n=11, each group. $^{*}P<0.05$ *vs* WKY; $^{\dagger}P<0.05$ *vs* ZSF1-Lean; $^{\ddagger}P<0.05$ *vs* ZSF1-Obese.

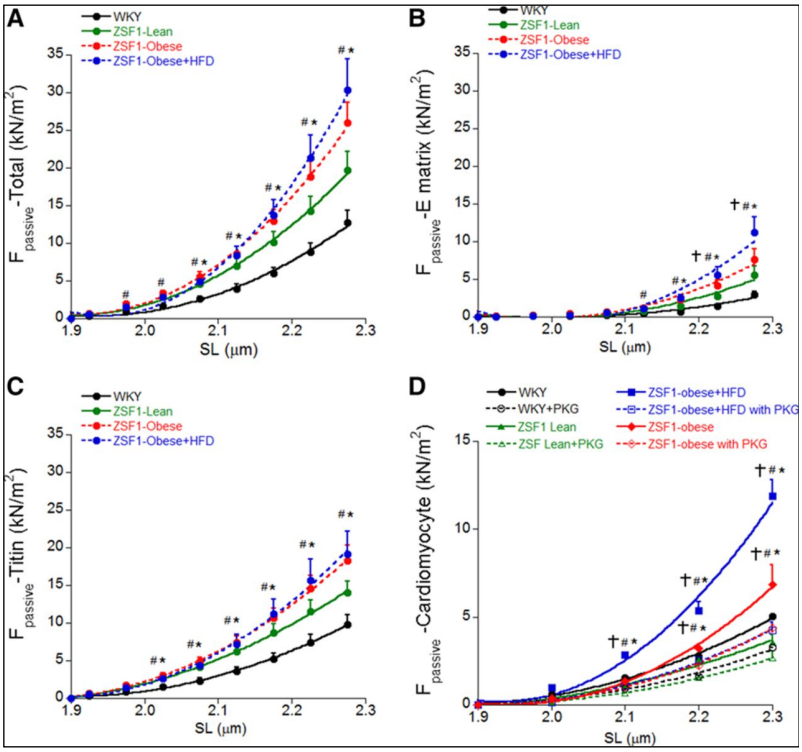

Figure 5. $F_{passive}$ of small muscle strips and cardiomyocytes. **A**, $F_{passive}$total vs sarcomere length (SL) curves for all groups. **B**, $F_{passive}$-extracellular matrix vs SL curves for all groups. **C**, $F_{passive}$-titin vs SL curves for all groups. **D**, $F_{passive}$-cardiomyocyte vs SL curves for all groups before (solid curves) and after protein kinase G (PKG) treatment (dashed curves). Curves are second-order polynomial fits to the mean values (n=16/4 muscle strips/group and n=15/4 cardiomyocytes/group). A to C, *P<0.05 ZSF1-Obese+high-fat diet (HFD) vs Wistar-Kyoto rats (WKY); #P<0.05 ZSF1-Obese vs WKY; and †P<0.05 ZSF1-Obese+HFD vs ZSF1-Lean. D, *P<0.05 ZSF1-Obese+HFD vs WKY and ZSF1-Lean; #P<0.05 ZSF1-Obese vs WKY and ZSF1-Lean; and †P<0.05 effect of PKG.

DISCUSSION

The present study identified cardiac titin hypophosphorylation to be associated with high myocardial stiffness and HFPEF in an obese ZSF1 rat model with high metabolic risk.

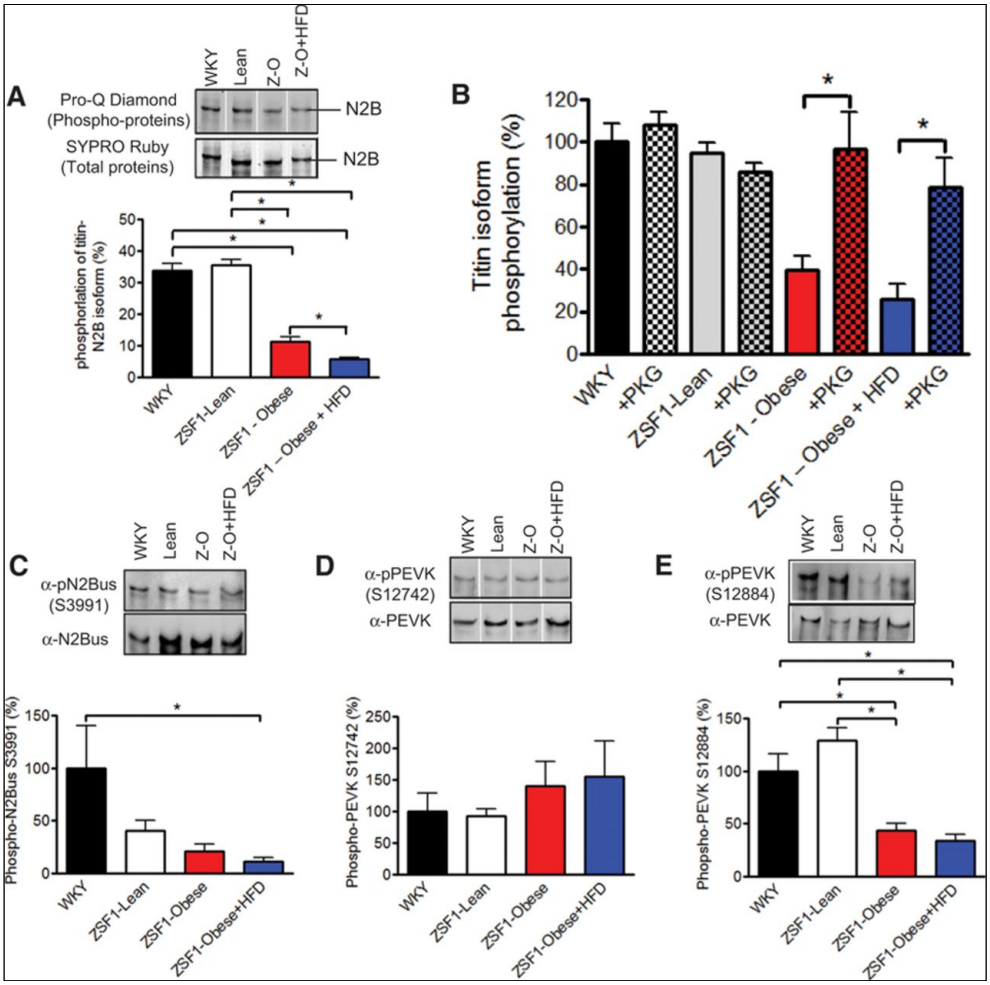

Figure 6. Total and site-specific phosphorylation of titin N2Bus/PEVK segments. **A**. Total titin phosphorylation in all groups. **B**. Titin phosphorylation at S3991 site in all groups. **C**. Titin phosphorylation at S12742 site in all groups. *P<0.05.

Metabolic risk-related HFPEF model

At the time of sacrifice at 20 weeks of age, high metabolic risk was clearly evident in the obese ZSF1 rats fed either regular diet or high fat diet. Compared to lean ZSF1 rats or WKY rats, obese ZSF1 rats showed many features of high metabolic risk such as visceral obesity evident from elevated perirenal and perigonadal fat, insulin resistance, hyperglycemia and physical inactivity evident from striated muscle wasting. Arterial blood pressure was elevated in both obese and lean ZSF1 rats. At the time of sacrifice, HFPEF was however only present in the obese ZSF1

rats and high metabolic risk therefore seemed to be a prerequisite in this model for HFPEF development. As such, the current model differs from previous experimental HFPEF models, which largely disregarded metabolic risk as they were carried out in old, hypertensive dogs[15,16] or in Dahl salt sensitive hypertensive rats.[17] The current model however closely resembles clinical HFPEF where metabolic risk is highly prevalent as evident from numerous HFPEF registries or large outcome trials.[3,18,19]

The HFPEF presentation observed in this metabolic risk model also shares characteristic features with clinical HFPEF presentation. After 18 weeks, during closed chest echocardiographic evaluation, the E/E' ratio was diagnostic of diastolic LV dysfunction[20] (ZSF1-Obese: 17.2±0.8; ZSF1-Obese+HFD: 15.8±1.1). At sacrifice, lung weight was 60% higher in obese animals. The latter probably resulted from episodes of pulmonary edema occurring during physical activity. A similar situation occurs in HFPEF patients who frequently have moderate abnormalities in diastolic LV function at rest but striking elevations of left ventricular filling pressure during exercise[21,22] because of a steep diastolic LV pressure-volume relation.[23] Steep diastolic LV pressure-volume and myocardial Fpassive-length relations were also present in the ZSF1 obese rats. The limited elevation of LVEDP in ZSF1 obese rats during open chest hemodynamic evaluation probably resulted from a reverse effect: thoracotomy and anaesthesia reduced venous return to the heart, which led to a prompt fall in LV filling pressures because of steep diastolic LV pressure-volume and myocardial $F_{passive}$-length relations. Apart from elevated E/E', high diastolic LV chamber stiffness and high myocardial stiffness, obese ZSF1 rats also had other evidence of diastolic LV dysfunction such as progressive LA enlargement and a significant increase in τ. The latter could however also be partially accounted for by the higher SAP in ZSF1-obese rats.

Systolic LV function in the ZSF1 obese rats closely resembled systolic LV function of HFPEF patients[24] as global indices of LV systolic performance (LV dP/dtmax, LVEF and Ees) were all preserved. In ZSF1 obese rats, Ees was even higher than in control WKY rats because of a steep end-systolic LV pressure-volume relation. The simultaneous presence of steep end-systolic and end-diastolic LV pressure-volume relations forces the left ventricle to function as a fixed stroke volume pump and explains the swings from pulmonary edema to low output frequently observed in HFPEF patients.[25]

Titin versus extracellular-matrix

ZSF1-Obese rats had a steeper myocardial $F_{passive}$-sarcomere length relation (Figure 5A). After extraction of the cardiac muscle strips with KCl/KI, which depolymerised thick and thin filaments thereby leaving titin unanchored, the contribution of the E-matrix to myocardial $F_{passive}$ became evident (Figure 5B). Subsequently, the contribution of titin could be calculated by subtracting at each SL the contribution of the E-matrix from the measured $F_{passive}$ (Figure 5C). For SLs ranging from 2.0 to 2.2µm, the contribution of titin greatly exceeded the contribution of the E-matrix. At 2.0µm, the contribution of titin was 6.8 and 9.3 times larger than of E-matrix for ZSF1-obese and ZSF1-obese+HFD rats. At 2.2µm, the contribution of titin was still 4.6 and 3.6 times larger. SLs ranging from 2.0 to 2.2µm covered the physiological range of LV filling pressures (from 5 to 40 mmHg). Using a thick wall ellipsoid model of the LV and the measured LVEDP of 5 mmHg (Table 3; WKY rats), the calculated LV end-diastolic wall stress (=1.25 kN/m^2) corresponded with measured $F_{passive}$ (1.26 kN/m^2) at a 2.0µm SL. Similarly, after adjusting the values of LVEDVI and dLVPW-thickness for a 2.2µm SL and substituting LV end-diastolic wall stress by the measured $F_{passive}$ at 2.2µm SL (13.70 kN/m^2 in ZSF1-obese+HFD), the same thick wall ellipsoid model yielded a LVEDP of 41 mmHg. Hence, up to filling pressures exceeding 40 mmHg, titin accounted for 82 and 78% of $F_{passive}$ in ZSF1-obese and ZSF1-obese+HFD rats respectively. High titin-based stiffness is therefore the main contributor to high myocardial stiffness and likely also to HFPEF development in this metabolic risk-related rat HFPEF model. The importance of intrinsic cardiomyocyte $F_{passive}$-SL relation of isolated skinned cardiomyocytes (Figure 5D), which was steeper and shifted upward in both ZSF1- obese and ZSF1-obese+HFD rats. Furthermore, in-vitro administration of PKG to the isolated cardiomyocytes corrected the $F_{passive}$-SL relations. This in-vitro reversibility suggests the high $F_{passive}$ of cardiomyocytes of obese ZSF1 rats to result more from altered phosphorylation status, than from structural changes of titin, such as isoform shifts or oxidative damage. In-vitro reversibility of high $F_{passive}$ was also observed in the ZSF1-obese+HFD rats, which were exposed to the highest systemic oxidative stress.

Limited involvement of the E-matrix in the high $F_{passive}$ of the obese ZSF1 rats was evident also from histological/biochemical analyses of myocardial tissue. Global myocardial collagen volume fraction, collagen cross linking, collagen 1A1 or collagen 3A3 gene expression and PCP or PCPE activity were unaltered in the obese ZSF1 rats (Figure 4). Despite these findings, there was a small increase in

myocardial $F_{passive}$ attributable to the Ematrix at SL > 2.175µm in ZSF1-obese and ZSF1-obese+HFD rats (Figure 5B). This increase could have resulted from subtle alterations in endomysial collagen which remained undetected by histological analysis or by components of the E-matrix other than collagen.

Titin hypophosphorylation

Titin stiffness can be modulated mainly through isoform shifts or alterations of the phosphorylation status. In patients presenting with eccentric LV remodeling after myocardial infarction or with dilated cardiomyopathy, a titin isoform shift from the stiff N2B to the compliant N2BA isoform has been reported.[26,27] In patients with concentric LV remodeling related to HFPEF or AS, most studies failed to observe a major shift in titin isoform expression.[9,28] Rat hearts predominantly express N2B titin isoform, the proportion of which remained unaffected in the present study by the concentric LV remodeling observed in lean and obese ZSF1 rats.

As previously observed in HFPEF patients, AS patients with type 2 diabetes and old hypertensive dogs with HFPEF[9,10,16], overall N2B titin isoform phosphorylation was greatly reduced in the obese ZSF1 rats, especially when exposed to HFD (Figure 6A). Using site-specific antibodies, the S3991 site and the S12884 of the N2Bus and PEVK segments of titin were identified as being hypophosphorylated (Figure 6C and E). The S3991 site can be phosphorylated by both PKA and ERK2 (extracellular regulated kinase 2)[29,30] and was recently also shown to be hypophosphorylated in old hypertensive HFPEF dogs.[16] Phosphorylation of the S12742 site of the PEVK segment was unaltered in contrast to old hypertensive HFPEF dogs where it was hyperphosphorylated.[16] Increased phosphorylation of the N2Bus segment is reported after PKA or PKG administration[31-35] and shown to lower $F_{passive}$ whereas increased phosphorylation of the S12742-PEVK occurs after PKC administration and raises $F_{passive}$.[36] Both PKC and CaMKII can phosphorylate the S12884- PEVK site. Phosphorylation of the S12884-PEVK site by CaMKII leads to a reduction of $F_{passive}$.[37] The elevated $F_{passive}$ observed in the present study in the obese ZSF1 rats is consistent with the observed hypophosphorylation of the S3991 site within the N2Bus segment and the observed hypopohosphorylation of the S12884 site within the PEVK segment.

CONCLUSIONS

Obese ZSF1 rats with a high metabolic risk profile developed HFPEF at 20 weeks of age. The diagnosis of HFPEF was based on lung congestion, preserved global LV systolic function and diastolic LV dysfunction. The latter was evident from elevated E/E', LA enlargement, high LV diastolic chamber stiffness and high myocardial stiffness. High myocardial stiffness was largely (±80%) attributable to high cardiomyocyte stiffness, which resulted from hypophosphorylation of titin.

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DISCLOSURES

None.

CLINICAL PERSPECTIVE

Heart failure with preserved ejection fraction (HFPEF) accounts for >50% of all heart failure cases. Both arterial hypertension and metabolic comorbidities, such as overweight/obesity and type 2 diabetes mellitus, are prevalent in HFPEF. Hitherto, experimental studies mainly tried to reproduce HFPEF in arterial hypertension models such as old dogs with bilateral renal wrapping or Dahl salt-sensitive rats and largely overlooked the prominent involvement of metabolic comorbidities. The present experimental study, therefore, investigated ZSF1 rats that are first generation hybrids between the Zucker diabetic fatty and spontaneously hypertensive heart failure rats. Lean and obese ZSF1 rats are hypertensive as they inherited the hypertension gene from male spontaneously hypertensive heart failure rats. Obese ZSF1 rats also inherited 2 different leptin receptor mutations from female Zucker diabetic fatty and male spontaneously hypertensive heart failure rats. At 20 weeks, the obese, but not the lean ZSF1, rats

had developed HFPEF, which was evident from increased lung weight, preserved left ventricular ejection fraction, normal left ventricular end-diastolic volume index, elevated left ventricular filling pressures, left atrial enlargement, and a high diastolic left ventricular stiffness modulus. High myocardial stiffness was also obvious in isolated cardiac muscle strips and could be attributed after myofilamentary extraction to stiffer titin and not to collagen deposition. High titin stiffness resulted from hypophosphorylation of its elastic segments. Titin hypophosphorylation was, therefore, identified as the main contributor to HFPEF in an experimental animal model that comes close to the clinical HFPEF presentation as it combines metabolic comorbidities with arterial hypertension.

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ONLINE DATA SUPPLEMENT: METHODS AND MATERIALS

Animal model

Nine-week old male ZSF1 lean (ZSF1-Lean, n=11), ZSF1 obese (ZSF1-Obese, n=22) and Wistar- Kyoto rats (WKY, n=11) were obtained from Charles River (Barcelona, Spain) and fed with Purina Diet (#5008). After a 1 week laboratory adaptation period, animals underwent phenotypic evaluation consisting of metabolic cage studies, blood sample collection and echocardiographic evaluation. From this point onward, a subgroup of ZSF1 obese rats (ZSF1-Obese+HFD, n=11) was randomly allocated to receive HFD (Research Diet Inc. #D12468). Weight gain and energy intake were recorded every third day. Phenotypic evaluation was repeated at the 14th and 18th week of life. At 20 weeks of age, animals underwent hemodynamic evaluation under anaesthesia and were subsequently sacrificed with procurement of myocardial tissue samples for histological, biochemical and biomechanical studies. Animals were kept in individually ventilated chambers in a controlled environment with a 12-h-light/- dark cycle at 22°C room temperature and had unlimited access to food. Investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the NIH (NIH Publication no. 85–23, revised 1996) and was approved by the ethics committee of the Faculty of Medicine of Porto.

ZSF1 rats (Charles River, Barcelona, Spain) are first generation hybrids between the ZDF (Zucker diabetic fatty) and SHHF (spontaneously hypertensive heart failure) rats, which carry two different leptin receptor mutations (*fa* and fa^{cp}). A female ZDF (+/*fa*) is crossbred with a male SHHF (+/fa^{cp}) rat, leading to either lean ZSF1 or obese ZSF1 rats (*fa*/fa^{cp}).[1] Both ZSF1 rats are hypertensive, since they inherit the hypertension gene from the SHHF rats but only obese ZSF1 rats develop diabetes and dyslipidemia.[2]

Metabolic studies and renal function

After a 24h acclimatization period, water and energy intake, weight gain and urine output were recorded, and a 24h urine sample was collected in metabolic cages (Techniplast, Buguggiate). After successive 24h rest intervals, all rats underwent oral glucose and insulin resistance testing, at the end of 12h feed-deprivation periods. Glycemia was recorded at baseline and 15, 30, 60, 90 and 120 min (Freestyle-Mini) after a 1 $g.Kg^{-1}$ glucose gavage or a 0.5 $U.Kg^{-1}$ intraperitoneal

insulin injection, respectively. Echocardiographic studies were conducted, after another 24h rest period, and a blood sample (1.5 mL) was collected from the subclavian vein under anaesthesia, at the end of the procedure.

Echocardiography

Rats (n=11 per group) were anaesthetized by inhalation of 8% sevoflurane in vented containers, orotracheally intubated and mechanically ventilated. Anaesthesia was maintained with sevoflurane (1- 2.5%) titrated to avoid the toe pinch reflex. Rats were placed in left-lateral decubitus on a heating pad, the ECG was monitored (lead II) and their temperature was kept at 38°C. The skin was shaved and depilated. After applying prewarmed echocardiography gel a linear 15MHz probe (Sequoia 15L8W) was gently positioned on the thorax. Systolic and diastolic wall thickness and cavity dimensions were recorded, in M-mode and 2D echocardiography, at the level just above the papillary muscles in the parasternal short axis view. The long axis diastolic dimensions of the left ventricle and transverse aortic root diameter were recorded by 2D and M-mode echocardiography, respectively, in the parasternal long axis view. Aortic flow velocity was recorded by pulsed-wave Doppler just above the aortic valve. Mitral flow velocity tracings were obtained with pulsed-wave Doppler just above the mitral leaflets, peak systolic tissue velocity and E' were measured with tissue Doppler at the medial mitral annulus and lateral mitral annulus, respectively, and left atrial dimensions were measured, at their maximum, by 2D echocardiography in the four chamber view. Acquisitions were done while transiently suspending mechanical ventilation and recordings were averaged from three consecutive heartbeats (Siemens Acuson Sequoia C512). Left ventricular (LV) mass and volumes were calculated by the 2D area-length method. Myocardial performance index was retrieved from the mitral flow pattern.

Haemodynamic evaluation

After sedation (100 $\mu g.kg^{-1}$ and 5 $mg.kg^{-1}$ intraperitoneal fentanyl and midazolam, respectively), anaesthesia (8 and 2.5–3% sevoflurane for induction and maintenance, respectively; Penlon Sigma Delta), endotracheal intubation, mechanical ventilation (TOPO, Kent scientific), 8 $mL.kg^{-1}.h^{-1}$ intravenous warm Ringer's solution infusion (NE-1000, New Era Pump Systems), temperature maintenance at 38°C on a heating pad, left thoracotomy, LV and right ventricular (RV) pressure- volume catheter insertion through the apex (SPR-838 and PVR-1045

Millar Instruments, respectively), and ascending aorta probe placement (Transonics) that allowed CO measurement (Active Redirection Transit Time Flowmeter, Triton Technology), signals were continuously acquired (MPVS 300,Millar Instruments), recorded at 1000 Hz (ML880 PowerLab 16/30, ADinstruments), and analyzed (PVAN 3.5, Millar Instruments). Recordings were obtained at suspended end-expiration. The LV catheter was advanced to record systemic arterial pressure. Parallel conductance was assessed with hypertonic saline. After euthanasia (100 $mg.kg^{-1}$ intravenous pentobarbital), blood (4mL) was collected for storage (-80°C) and for volume calibration (910–1048, Millar instruments). LV volumes were varied using transient inferior vena cava constrictions by adjusting a sling around the inferior vena cava. Organs were weighed, RV and LV + interventricular septum (IVS) were weighed after dissection, and tibia length (TL) was measured. Samples were either snap-frozen and stored at -80^{o}C or processed for histology. Weights were normalized to TL due to the large body weight differences between groups.

Histomorphological analysis was performed on elastica-von-Giesson and hematoxylin-eosin stained, 4 µm thick, sections of tissue placed in 4% buffered formaldehyde solution. As previously validated,3 MyD was determined perpendicularly to the outer contour of the cell membrane at nucleus level.

Collagen- volume fraction and cross-linking

The collagen volume fraction (CVF) was determined by quantitative morphometry with an automated image analysis system in sections stained with collagen-specific picro-sirius red, as previously reported in ZSF1 lean (ZSF1-Lean, n=11), ZSF1 obese (ZSF1-Obese, n=22) and Wistar-Kyoto rats (WKY, n=11).[3,4] All measurements were performed in duplicate by 2 independent observers. The inter- and intra-observer coefficients of variation were <4%. To distinguish between cross-linked (insoluble) and non-cross-linked (soluble) collagen a colorimetric procedure was employed. First, a fast green-sirius red assay was performed to identify and quantify total collagen. In a second step, a sircol-based assay was performed to obtain and quantify soluble collagen. The amount of insoluble collagen was calculated by subtracting the amount of soluble collagen from the amount of total collagen. The degree of cross-linking was calculated as the ratio between the insoluble and the soluble forms of collagen. All measurements were performed in duplicate. The inter- and intra-assay coefficients of variation were 5 and 3%, respectively.[3-5]

RNA isolation and gene expression analysis

Frozen tissue sections ZSF1 lean (ZSF1-Lean, n=5), ZSF1 obese (ZSF1-Obese, n=11) and Wistar- Kyoto rats (WKY, n=5) were minced in Trizol and further disrupted during 10 minutes of vigorous shaking. To extract the RNA, chlorophorm was added, mixed, and centrifuged. The aqueous phase containing the RNA was collected in a separate tube, and isopropanol was added. For precipitation, the RNA solution was centrifuged 15 minutes at 4°C at high speed. The RNA pellet was then further purified using the RNeasy Mini Kit (Qiagen) according to manufacturer's protocol. One µg of RNA was reverse transcribed into cDNA using the High Capacity Kit (Applied Biosystems) and then further diluted to a final concentration of 5 ng/µL cDNA.

The relative quantification of mRNA levels was carried out on a 7900 HT (Applied Biosystem). To assess the mRNA expression of the target genes, real-time PCR was performed using 5 µL of the gene expression master mix (Applied Biosystems) and 0.5 µL of the gene expression assay for Col1A1 (Rn01463848_m1) and Col3A1 (Rn01437683_m1) (each includes forward and reverse primers as well the fluorescently FAM-labelled probe) from Applied Biosystems, and 1 µL of cDNA in a final volume of 10 µL. Quantification of the house keeping gene GAPDH (Rn99999916_s1) as an internal control was performed for each sample. Data were normalized to 18S RNA level as an endogenous control and are expressed using the formula $2^{-\Delta Ct}$ in comparison to the corresponding untreated controls.

Force measurements on skinned cardiomyocytes and strips

Single skinned cardiomyocytes

Force measurements were performed on single skinned cardiomyocytes as described.[3,4,6] Cardiomyocytes were isolated from WKY, ZSF1-Lean, ZSF1-Obese and ZSF1-Obese+HFD rat hearts (n=15/4 cardiomyocytes/group). Briefly, samples were defrozen in relaxing solution (in mmol/L: free Mg, 1; KCl, 100; EGTA, 2; Mg-ATP, 4; imidazole, 10; pH 7.0), mechanically disrupted and incubated for 5 min in relaxing solution supplemented with 0.5% Triton X-100. The cell suspension was washed 5 times in relaxing solution. Single cardiomyocytes were selected under an inverted microscope (Zeiss Axiovert 135, 40x objective) and attached with silicone adhesive between a force transducer and a piezoelectric motor as part of a "Permeabilized Myocyte Test System" (1600A; with force transducer 403A; Aurora Scientific, Aurora, Ontario, Canada).

Cardiomyocyte $F_{passive}$ was measured in relaxing buffer at room temperature within a sarcomere-length range between 1.9 and 2.3 µm. Force values were normalized to myocyte cross- sectional area calculated from the diameter of the cells, assuming a circular shape. As a test of cell viability, each cardiomyocyte was also transferred from relaxing to maximally activating solution (pCa4.5), at which isometric force developed. Once a steady state force was reached, the cell was shortened within 1 ms to 80% of its original length to determine baseline force. Only cells developing active forces >20 kN/m^2 were included in the analysis. The passive tension was measured under steady state shear (viscous and elastic properties). Subsequently cardiomyocytes were incubated in relaxing solution supplemented with the PKG1α (0.1 U/mL; Sigma, batch 034K1336), guanosine cGMP (10 µmol/L, Sigma) and dithiothreitol (6 mmol/ L; Sigma). After 40-min-long incubation with PKG1α, $F_{passive}$ measurements were again performed in relaxing solution at SL 1.9-2.3 µm.

Skinned muscle strips

Left papillary muscles were dissected after sacrificing the animals (n=16/4 muscle strips/group). Small muscle strips were created and chemically permeabilized in a 1% Triton X-100 solution for 30 minutes. After clipping both ends, the strips were mounted between a length motor and a force transducer on top of an inverted microscope. Strips were activated at 20˚C with solutions containing a saturating Ca2+ concentration to determine maximal active tension (force/cross sectional area) at a sarcomere length (SL) of 2.0 µm. Subsequently, strips were stretched from SL 1.9 µm to 2.3 µm, with a velocity of 10% muscle length per second and while in a solution with low Ca2+ (pCa9), to determine passive tension generation. 7 Afterwards, the strips underwent an extraction protocol of 45 minutes in 0.6M KCl followed by 45 minutes in 1.0M KI to depolymerize thick and thin filaments respectively, leaving titin unanchored, and the strips were again passively stretched as described above. The remaining tension after this procedure is extraction-insensitive and caused by extracellular matrix, i.e. collagen. The passive tension was measured under steady state shear (viscous and elastic properties).

Titin-isoform separation and titin phosphorylation by Pro-Q Diamond/Sypro Ruby

Titin isoforms were separated as described.[6,8] Briefly, tissue samples from ZSF1 lean (ZSF1-Lean, n=5), ZSF1 obese (ZSF1-Obese, n=10) and Wistar-Kyoto rats (WKY,

n=5) were solubilized in 50 mM Tris-sodium dodecyl sulfate (SDS) buffer (pH 6.8) containing 8 µg/mL leupeptin (Peptin Institute, Japan) and phosphatase inhibitor cocktail (PIC [P2880], 10 µL/mL; Sigma). Samples were heated for 3 minutes at 96°C and centrifuged. Then, samples (20 µg; equal concentration checked by spectroscopic methods) were separated by agarose-strengthened 1.8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Gels were run at 5 mA constant current for 16 hours. The phosphorylation state of cardiac titin was determined using Pro-Q Diamond phosphoprotein. Titin gels were stained for one hour with Pro-Q Diamond, and then overnight with Sypro Ruby (Molecular Probes). Staining was visualized using the LAS-4000 Image Reader (Fuji Science Imaging Systems) and signals were analyzed using Multi Gauge V3.2 or AIDA software. Finally signals obtained from Pro-Q Diamond staining were normalized to signals obtained from Sypro Ruby staining.

Titin and phospho-titin analysis by Western blot

1.8% SDS-PAGE followed by Western blot was performed to measure expression and site-specific phosphorylation of titin. Samples from ZSF1 lean (ZSF1-Lean, n=5), ZSF1 obese (ZSF1-Obese, n=10) and Wistar-Kyoto rats (WKY, n=5) were applied at a concentration that was within the linear range of the detection system (30 µg dry weight; checked by spectroscopic methods). Sequence-specific or phosphosite-specific anti-titin antibodies were custom-made by Eurogentec, Belgium (positions in mouse (*Mus musculus*) titin according to UniProtKB identifyer A2ASS6)). The following affinity- purified antibodies were used[9,10]:

- Anti-titin- mouse N2Bus against EEGKSLSFPLA (rabbit polyclonal; 1:1000).
- Anti-phospho-N2Bus (S3991 in mouse titin and S4010 in human titin) against EEGKS(PO3H2)LSFPLA (rabbit polyclonal; 1:500).
- Anti-PEVK-domain against (cross-species conserved) sequence EVVLKSVLRK (1:1000) - Anti-phospho-PEVK-domain (S12742 in mouse titin and S11878 in human titin) against EVVLKS(PO3H2)VLRK (1:500)
- Anti-PEVK-domain against (cross-species conserved) sequence KLRPGSGGEKPP (1:100) - Anti-phospho-PEVK-domain (S12884 in mouse titin and S12022 in human titin) against KLRPGS(PO3H2)GGEKPP (1:500)

The amino acid sequences of rat are identical to the amino acid sequences of mouse.[9,10] Titin antibodies gave specific signals on Western blots with cardiac

tissue. Following SDS- PAGE, proteins were transferred to Hybond ECL nitrocellulose membranes. Blots were pre-incubated with 3% bovine serum albumin in Tween Tris-buffered saline (TTBS); 10 mmol/L Tris-HCl; pH 7.6; 75 mmol/L NaCl; 0.1% Tween) for 1 hour at room temperature. Then, blots were incubated overnight at 4°C with the primary antibodies against the respective (phospho) protein. After washing with TTBS, primary antibody binding was visualized using secondary horseradish peroxidase-labeled, goat-anti- rabbit/ mouse antibodies (dilution 1:1000; DakoCytomation) and enhanced chemiluminescence (ECL Western blotting detection; Amersham Biosciences). Staining was visualized using the LAS-4000 Image Reader and analyzed with Multi Gauge V3.2 or AIDA software. PVDF stains were saved for comparison of protein load. Loading was also controlled by comparing signals of the phospho-specific antibodies with those of the respective sequence-specific antibodies. Finally signals obtained from phospho-specific antibodies were normalized to signals obtained from sequence-specific antibodies.

Data analysis

Circumferential LV end-diastolic wall stress (σ) was computed using a thick wall ellipsoid model of the LV:

$$\sigma = PD/2h \times [1-(h/D)-(D^2/2L^2)]$$

where P is LV end-diastolic pressure, h is LV wall thickness, and D and L are LV short axis diameter and long axis length at the midwall.

Groups were compared by two-way repeated measures ANOVA whenever serial acquisitions were obtained for the same animal, and by one-way ANOVA for single acquisitions. Pressure-volume loop analysis was analyzed using LabChart 7 Pro v7.3.1. Values are given as mean±SEM. A 2-tailed test with a probability of value <0.05 was considered significant. Single comparisons were assessed by an unpaired Student t test. Bonferroni-adjusted t tests were used subsequent for multiple comparisons after repeated measure ANOVA. Statistical analysis was performed with SPSS (Version 15.0; SPSS Inc,Chicago,Ill).

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Mechanisms of Diastolic Dysfunction in Heart Failure With a Preserved Ejection Fraction: If It's Not One Thing It's Another

Martin M. LeWinter and Markus Meyer

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Editorial

Mechanisms of Diastolic Dysfunction in Heart Failure With a Preserved Ejection Fraction

If It's Not One Thing It's Another

Martin M. LeWinter, MD; Markus Meyer, MD

It has been nearly 30 years since the first series of patients with the syndrome of heart failure with a preserved ejection fraction (HFpEF) was reported.[1] It has proven to be a controversial topic. Because left ventricular (LV) EF is preserved, it was assumed that HFpEF results from altered diastolic properties. However, some argued that these patients did not truly have HF or had subtle forms of dilated HF. Symptomatic of this debate is reluctance to use the term diastolic HF (we prefer HFpEF because diastolic dysfunction is also present in HF with a reduced EF) as well as disagreement over the exact EF cutoff, that is, should a perfectly normal EF be required to diagnose HFpEF or does a modest reduction qualify?

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Although many questions remain, in the intervening years several features have emerged. HFpEF is a complex and extremely common syndrome, accounting for >50% of patients with HF.[2–6] It is more prevalent in women, and its prognosis is similar to HF with a reduced EF. The clinical presentation ranges from dyspnea with physical activity to a pattern of restrictive cardiomyopathy, with marked elevations of right and left filling pressure at rest, often with considerable pulmonary hypertension (HTN). Essentially all patients with HFpEF have diastolic dysfunction,[7] specifically, reduced LV passive compliance and/or slowed or incomplete relaxation. Various other cardiovascular abnormalities are common,[3–6] including subtle abnormalities of systolic function.

HFpEF Substrates

Although a small number of patients have HFpEF in association with specific cardiac diagnoses, for example, hypertrophic and infiltrative cardiomyopathy, constrictive pericarditis, all of which have profound effects on diastolic compliance, the vast majority have a history of HTN.[3,4,8] In many patients, especially elderly women, HTN is exclusively systolic,[9] resulting from reduced arterial compliance rather than changes in resistance vessels. Moreover, although there is considerable variation between patient cohorts, the great majority of patients with HTN-associated HFpEF have concentric LV remodeling, defined as either concentric hypertrophy (increased LV mass with normal or reduced chamber volume) or, in the absence of increased mass, increased mass:volume ratio or relative wall thickness.[8–12] In population studies, the progression from HTN to HFpEF is paralleled by declines in diastolic function.[9] These observations strongly support the concept that diastolic dysfunction is in fact a major underlying mechanism of this progression, resulting in the hemodynamic hallmark of HF, a depressed Frank–Starling relation.

HTN is not the only substrate in many if not most patients with HFpEF. Approximately one third have type 2 diabetes mellitus (DM2).[4,5,10,11,13] It is likely that a substantial additional number have insulin resistance in the absence of overt DM2. Insulin resistance/DM2 and associated hyperinsulinemia have pleiotropic effects on the myocardium,[14] including stimulation of hypertrophy, increased oxidative stress, and a proinflammatory/profibrotic state, which can modify cardiomyocyte function in multiple ways as well as extracellular matrix collagen, all of which can affect diastolic function. Obstructive sleep apnea and obesity are common in HFpEF[15] and also associated with a proinflammatory state and cardiac hypertrophy. HTN, DM2/insulin resistance, and obesity are components of the metabolic syndrome (MS). Recognition of the association between MS and HFpEF has led to the concept that in many patients HFpEF can be considered metabolic heart disease,[2,5,9,14] although the detailed mechanisms whereby metabolic derangements and associated oxidative stress and proinflammatory/profibrotic states cause diastolic dysfunction remain to be elucidated. Abnormal myocardial triglyceride accumulation associated with echocardiographic evidence of diastolic dysfunction in patient with elements of the MS provides direct evidence of this link.[16]

Mechanisms of Diastolic Dysfunction in HFpEF

The exact mechanisms leading to diastolic dysfunction in concentric remodeling and HFpEF have begun to be elucidated during the past 5 to 10 years. In discussing the article by Hamdani et al[17] in this issue of *Circulation: Heart Failure*, we will focus on its relationship to what is known about these mechanisms from studies on myocardial tissue from patients.

One well-documented mechanism studied in biopsy tissue from patients with HFpEF is hypophosphorylation of protein kinase (PK) A and PKG sites on cardiac titin,[18,19] the giant myofilament protein responsible for cardiomyocyte passive tension.[20,21] Titin's N terminus is anchored in the z-disc of the sarcomere, and its C terminus is anchored in the M-band. When the cardiomyocyte is stretched, titin lengthens and

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From the Cardiology Unit, University of Vermont, Burlington.

Correspondence to Martin M. LeWinter, MD, Cardiology Unit, University of Vermont, Fletcher Allen Health Care, 111 Colchester Ave, Burlington, VT 05401. E-mail martin.lewinter@vtmednet.org

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functions as a complex molecular spring, developing passive tension with a curvilinear length–tension relationship. Chemical disruption of titin's anchors in the M-band eliminates virtually all cardiomyocyte passive stiffness over the physiological sarcomere length range.[20] Using these chemical methods, the proportion of myocardial passive tension ascribable to titin versus extracellular matrix collagen has been dissected.[20] Although there are differences in absolute levels of passive tension, in all species studied, including humans, titin accounts for the majority of myocardial passive tension at short sarcomere lengths. With further lengthening, the relative contribution of collagen increases such that it accounts for ≈50% or more of passive tension at sarcomere lengths at the upper end of the physiological range.[20,21] Titin is also a key biomechanical sensing and signaling molecule and the most commonly mutated gene in human dilated cardiomyopathy. These and other functional and disease-specific aspects of titin have been discussed in recent reviews.[20,21]

Titin stiffness is modulated by isoform variation accomplished by alternative splicing and changes in phosphorylation state.[20,21] Two isoforms (N2B and N2BA) are present in the postnatal heart; N2B is smaller and markedly stiffer than N2BA. The N2BA:N2B ratio is ≈40:60 in normal adult human LV myocardium. In both ischemic and nonischemic dilated cardiomyopathy as well as HFpEF, a shift toward the more compliant N2BA isoform occurs, which reduces cardiomyocyte resting tension.[18–21] PKA/PKG phosphorylate multiple, identical sites on titin, which reduces cardiomyocyte resting tension. Changes in phosphorylation can rapidly alter myocardial passive stiffness, for example, during exercise. In HFpEF, the net effect of increased N2BA titin and hypophosphorylation of PKA/PKG sites is increased cardiomyocyte resting tension.[18,19] In addition to PKA/PKG, PKC-α has been shown to phosphorylate other titin sites.[21,22] CaM kinase targets these same sites. In contrast to PKA/PKG sites, PKC-α phosphorylation increases resting tension.

In their elegant study, Hamdani et al[17] used Zucker rats to demonstrate that the combination of obesity, DM, and HTN (with or without a high-fat diet) leads to HFpEF in association with increased passive myocardial stiffness and markedly reduced phosphorylation of titin's PKA/PKG sites compared with controls. There were no changes in isoforms or phosphorylation of one of the PKC-α sites. Importantly, phosphorylation of PKA/PKG sites was unchanged in lean, nondiabetic, but hypertensive Zucker rats. Thus, components of the MS besides HTN seem sufficient to cause changes in passive stiffness attributable to reduced titin phosphorylation in this experimental model. Although the underlying mechanism(s) of reduced phosphorylation was not elucidated, this article provides important insights into the pathophysiology of HFpEF that could play a role in patients.

Hamdani et al[17] have not shown that reduced titin phosphorylation is sufficient in and of itself to cause HFpEF in obese–diabetic–hypertensive rats. Lean hypertensive rats developed significant but modest increases in LV mass at the last, 18th week, measuring point, which were not associated with changes in diastolic function indexes. In contrast, increases in mass were much larger and occurred much earlier in obese–diabetic–hypertensive rats and were associated with abnormal diastolic function. Thus, it is important to consider determinants of diastolic function other than titin that could contribute to the development of diastolic dysfunction and HFpEF.

A modest amount of such information obtained in human tissue is now available, although it has not been specifically focused on metabolic heart disease. One determinant is changes in extracellular matrix collagen, but Hamdani et al[17] report that collagen volume fraction and cross-linking were unchanged. However, this differs from HFpEF in patients,[23] in whom collagen volume fraction and cross-linking are increased and underscores the potential for animal models to provide information that does not apply to patients. Using the chemical methods noted above, Hamdani et al[17] also report that collagen-dependent passive tension was unchanged. However, an unexplained finding is that collagen-dependent tension accounted for only ≈10% to 20% of total passive tension in all groups. As noted above, this is much smaller than what has been reported previously in several species.[20]

In addition to passive diastolic properties, LV relaxation is abnormal in patients with LV hypertrophy and HFpEF,[7,9,12] but the mechanisms have received less attention. At the level of the LV, increased arterial load, when present, slows relaxation rate. At the myocardial level, the speed and completeness of relaxation are dependent on deactivation of cross-bridges formed during contraction, which in turn depends on both the mechanisms that restore systolic $[Ca^{2+}]_{in}$ to diastolic levels and the kinetics of cross-bridge dissociation.

We recently reported the first evidence of abnormal calcium handling in patients with pressure overload–induced concentric remodeling.[24] In excitable tissue from LV epicardial biopsies obtained from patients with normal EF undergoing coronary bypass grafting, we found that isometrically contracting strips from patients with concentric remodeling (some of whom had HFpEF) displayed a progressive increase in diastolic tension beginning at stimulation frequencies in the 100 to 110 per minute range, that is, incomplete relaxation occurred at rates present during low-level physical activity. Additional experiments revealed a defect in sarcolemmal calcium extrusion. In more recent, unpublished work, we found that cytoplasmic $[Ca^{2+}]_{in}$ is indeed increased at these same rates. These results may provide a mechanism whereby patients with HFpEF increase filling pressures and become dyspneic with physical activity.[25] Correspondingly, patients with HFpEF display a reduced ability to maintain end-diastolic volume and cardiac output during increases in heart rate, which could reflect the same mechanism.[26]

In another recent report using demembranated (skinned) myocardial strips,[27] we showed that the kinetics of cross-bridge dissociation are slowed in patients with concentric remodeling compared with controls. Using the method of sinusoidal length perturbation, the apparent rate constant of cross-bridge dissociation was reduced at submaximal $[Ca^{2+}]$ and its mathematical inverse, cross-bridge on-time (the time the cross-bridge is attached and generating force) was prolonged. These changes in dissociation kinetics serve to slow relaxation. We also found that total phosphorylation of both cardiac troponin I and myosin binding protein C is reduced in concentrically remodeled LV myocardium. Recent, unpublished studies using site-specific phosphoantibodies reveal

that PKA/PKG sites on both proteins are hypophosphorylated. Because phosphorylation of these sites speeds actomyosin kinetics, hypophosphorylation may contribute to slowed relaxation.

In summary, although studies are limited, in patients with HFpEF or pressure overload–induced concentric remodeling abnormalities of every component of LV diastolic function, arterial load, mass:volume ratio, passive stiffness (titin and collagen), and cross-bridge deactivation (calcium handling and actomyosin kinetics) have been demonstrated or implicated. In future, it will be important to understand the relative importance and time course of these abnormalities in relation to the progression to HFpEF as well as the influence of substrates other than HTN.

Therapeutic Considerations

There are currently no therapies for HFpEF that have been shown to improve long-term outcomes. Perhaps the diverse abnormalities of diastolic function identified, which could have great interpatient variability, make it difficult for treatments to yield significant effects in clinical trials. Guidelines for treatment[28] are, therefore, largely empirical, emphasizing Na restriction, diuretics as needed, and blood pressure control. In patients with HFpEF with MS, common sense suggests that weight loss and perhaps exercise should be therapeutic goals. Small trials show that weight loss can improve diastolic function,[29] but the effects of exercise have been variable.[30] The study by Hamdani et al[17] links a specific component of diastolic dysfunction in HFpEF, that is, titin hypophosphorylation, to DM2 and obesity and suggests 1 potential mechanism whereby lifestyle changes can improve diastolic function.

Our knowledge of the mechanisms of diastolic dysfunction in concentric remodeling and HFpEF, although admittedly rudimentary, has other therapeutic implications. It is intriguing that a specific alteration at the level of the myofilaments, hypophosphorylation of PKA/PKG sites, may contribute to increased passive stiffness (titin) and slowed relaxation (cardiac troponin I/myosin binding protein C). Accordingly, pharmacological approaches that target this molecular abnormality offer promise. Unfortunately, the RELAX (Phosphodiesterase-5 Inhibition to Improve Clinical Status and Exercise Capacity in Heart Failure with Preserved Ejection Fraction) trial of sildenafil in HFpEF[31] did not demonstrate efficacy despite the fact that phosphodiesterase-5 inhibition has several effects that, in addition to potential normalization of titin and cardiac troponin I/myosin binding protein C phosphorylation, should be beneficial.[31] In the RELAX trial, sildenafil did not significantly increase plasma cGMP activity, suggesting that PKG activity may not have been effectively augmented. This in turn suggests that other approaches to increasing NO availability and PKG activity should be considered. Nitrates are an obvious choice.

Other considerations arise concerning exercise and the common use of β-blockers in HFpEF. Hypophosphorylation of PKA/PKG sites should be ameliorated during exercise in conjunction with increased adrenergic stimulation, that is, their importance may decrease with physical activity. β-Blockers could potentiate these same abnormalities at rest and during exercise. In contrast, rate-dependent incomplete relaxation and inadequate maintenance of end-diastolic volume are obviously more pronounced during exercise and could therefore be more important as a mechanism of exercise limitation. In that case, β-blockers may help by blunting increases in heart rate during exercise. These divergent heart rate effects might make it difficult to detect beneficial effects of β-blockers.

Targeting the extracellular matrix is obviously also a promising therapeutic approach. Aldosterone inhibition is potently antifibrotic and has other potentially beneficial effects.[32] The recent Aldo-DHF (Aldosterone Receptor Blockade in Diastolic Heart Failure) phase 2 trial of spironolactone[33] revealed improvements in resting diastolic function in HFpEF. The results of the larger, ongoing TOPCAT (Treatment of Preserved Cardiac Function with an Aldosterone Antagonist) trial of spironolactone in HFpEF[34] are therefore eagerly awaited.

Summary

We are beginning to gain a better understanding of the mechanisms of diastolic dysfunction in HFpEF. By demonstrating reduced titin phosphorylation in obese–diabetic–hypertensive rats, Hamdani et al[17] have provided important insights into the substantial number of patients with HFpEF with components of the MS, that is, metabolic heart disease. As progress is made in other mechanistic aspects of HFpEF, we will hopefully gain a more integrated understanding and a more rational basis for developing new treatments. In view of the multiple abnormalities of diastolic function identified, it may be particularly important to individualize and target treatment.

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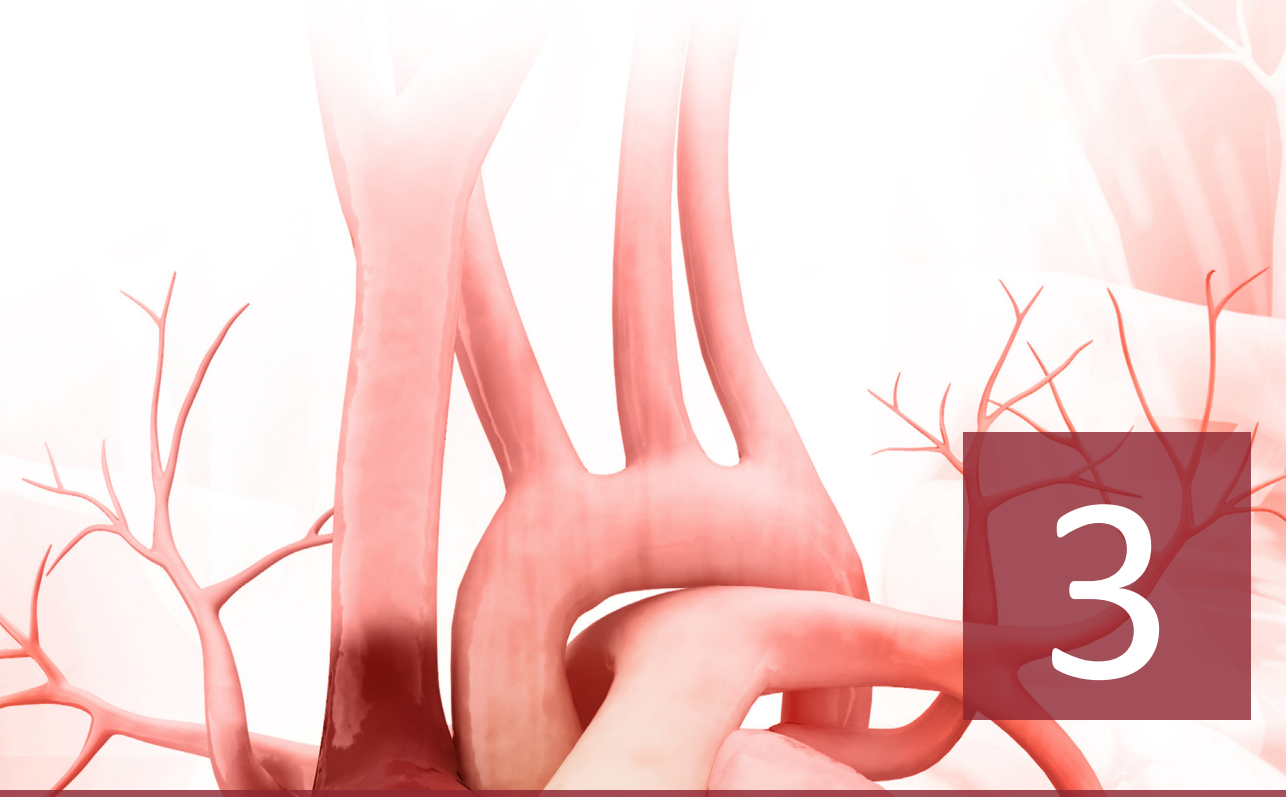
KEY WORDS: Editorials ■ diastole ■ diastolic heart failure ■ heart failure ■ myocardium ■ titin



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Myocardial Microvascular Inflammatory Endothelial Activation in Heart Failure with Preserved Ejection Fraction

Franssen C*, Chen S*, Unger A, Korkmaz HI, De Keulenaer GW, Tschöpe C, Leite-Moreira AF, Musters R, Niessen HW, Linke WA, Paulus WJ, Hamdani N.

*Authors contributed equally

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Myocardial Microvascular Inflammatory endothelial activation in Heart Failure with Preserved Ejection Fraction

By

Constantijn Franssen*[1], M.D., Sophia Chen*[1], B.Sc., Andreas Unger[2],Ph.D., H. Ibrahim Korkmaz[1,3], M.Sc., Gilles W. De Keulenaer[4], M.D., Ph.D., Carsten Tschöpe[5], M.D., Ph.D., Adelino F. Leite-Moreira[6], M.D., Ph.D., René Musters[1], Ph.D., Hans W.M. Niessen[3], M.D., Ph.D., Wolfgang A. Linke[2], Ph.D., Walter J. Paulus[1], M.D., Ph.D. and Nazha Hamdani[1,2],Ph.D.

From

[1]Department of Physiology, Institute for Cardiovascular Research, VU University Medical Center, Amsterdam, The Netherlands,
[2]Department of Cardiovascular Physiology, Ruhr University Bochum, Germany,
[3]Department of Pathology and Cardiac Surgery VU University Medical Center, Amsterdam, The Netherlands
[4]Laboratory of Physiology, University of Antwerp, Belgium,
[5]Department of Cardiology and Pneumology, Charité – Universitätsmedizin Berlin, Berlin, Germany,
[6]Department of Physiology and Cardiothoracic Surgery, University of Porto, Portugal,

* C.F. and S.C. equally contributed to this manuscript

Short title: Microvascular endothelial inflammation in HFpEF

ABSTRACT

Background - Metabolic risk is associated with diastolic LV dysfunction and heart failure with preserved ejection fraction (HFpEF).
Objectives - The present study investigates if systemic, low-grade inflammation of metabolic risk contributes to diastolic LV dysfunction and HFpEF through coronary microvascular endothelial activation, which alters paracrine signalling to cardiomyocytes and predisposes them to hypertrophy and high diastolic stiffness.
Methods - We explored inflammatory endothelial activation and its effects on oxidative stress, NO bioavailability and cGMP-PKG signalling in myocardial biopsies of HFpEF patients and validated our findings by comparing obese ZSF1-HFpEF rats to ZSF1-Control (Ctrl) rats.
Results - In myocardium of HFpEF patients and ZSF1-HFpEF rats we observed: 1) E-selectin and ICAM-1 to be upregulated, 2) NOX2 expression to be raised in macrophages and endothelial cells but not in cardiomyocytes and 3) uncoupling of eNOS which was associated with reduced myocardial nitrite/nitrate concentration, cGMP-content and PKG-activity.
Conclusions - HFPEF is associated with coronary microvascular endothelial activation and oxidative stress. This leads to a reduction of NO-dependent signalling from endothelial cells to cardiomyocytes, which can contribute to the high cardiomyocyte stiffness and hypertrophy observed in HFPEF.

CONDENSED ABSTRACT

The present study investigates if systemic, low-grade inflammation of metabolic risk contributes to HFpEF through coronary microvascular endothelial activation. Inflammatory endothelial activation, myocardial oxidative stress, NO bioavailability and cGMP-PKG signalling were investigated in human HFpEF myocardial biopsies and in obese ZSF1-HFpEF rats. Endothelial E-selectin and ICAM-1 were upregulated. NOX2 expression was raised in macrophages, but not in cardiomyocytes and 3-Nitrotyrosine expression was limited to endothelial cells. Myocardial eNOS was uncoupled and associated with reduced myocardial nitrite/nitrate concentration, cGMP-content and PKG-activity. Metabolic risk therefore contributes to HFpEF development through myocardial microvascular inflammation, which alters NO signalling from endothelial cells to cardiomyocytes.

KEYWORDS

Heart failure, inflammation, nitric oxide, endothelium, oxidative stress

ABBREVIATIONS

AS: aortic stenosis
cGMP: cyclic guanosine monophosphate
DM: diabetes mellitus
eNOS: endothelial nitric oxide synthase
HFpEF: heart failure with preserved ejection fraction
HFrEF: heart failure with reduced ejection fraction
ICAM-1: Intercellular Adhesion Molecule 1
NO: nitric oxide
NOX2: NADPH oxidase 2
PKG: protein kinase G

INTRODUCTION

Metabolic risk is increasingly recognized as an important contributor to diastolic left ventricular (LV) dysfunction and to heart failure with preserved ejection fraction (HFpEF). Recent longitudinal non-invasive studies over a 4 year time interval revealed close correlations between diastolic LV stiffness and body mass index (BMI) (1,2). These studies concluded that weight loss and reduction of central adiposity could prevent diastolic LV dysfunction and eventual HFpEF development. Similar evidence was already provided by the All-HAT trial, which enrolled patients with arterial hypertension and one additional cardiovascular risk factor, and observed a high BMI at enrolment to be the strongest predictor for HFpEF development (3). The latter finding was consistent with the high prevalence of overweight/obesity in large HFpEF outcome trials or registries, which almost uniformly reported a median BMI value of HFpEF patients in excess of 30 kg/m^2. In primates developing diet-induced obesity, endothelial inflammatory activation evident from adhesion molecule expression appears to be the earliest manifestation of vascular damage (4). Endothelial inflammatory activation is associated with microalbuminuria, which was recently shown to be associated with diastolic LV dysfunction (5) and to predict HFpEF development (6). When endothelial inflammatory activation evolves to endothelial dysfunction, vasomotor responses become blunted as evident from a lower reactive hyperemic response (7), which provides both diagnostic and prognostic information in HFpEF (8,9). In HFpEF, endothelial dysfunction also closely relates to worsening of symptoms (10), functional capacity (10) and precapillary pulmonary hypertension (11).

This prominent involvement of metabolic risk and endothelial inflammatory activation recently led to a new paradigm for HFpEF development (12). In accordance to this paradigm, metabolic comorbidities drive LV remodeling and dysfunction in HFpEF through coronary microvascular endothelial inflammation, which alters paracrine signalling from endothelial cells to surrounding cardiomyocytes. Especially the fall in nitric oxide-cyclic guanosine monophosphate–protein kinase G (NO-cGMP-PKG) signalling predisposes cardiomyocytes to hypertrophy development and to high diastolic resting tension. Microvascular endothelial dysfunction as a mechanism of LV remodeling in HFpEF differs from heart failure with reduced ejection fraction (HFrEF), where eccentric LV remodeling results from cardiomyocyte cell death pathways such as accelerated autophagy, apoptosis or necrosis (13). It also differs from aortic stenosis (AS),

where concentric LV remodeling is induced by excessive systolic wall stress (14). To establish the validity of endothelial dysfunction controlling LV remodeling in HFpEF, the current study compared microvascular endothelial inflammatory activation and its effects on myocardial oxidative stress, NO bioavailability and cGMP content in myocardial biopsies of HFpEF, HFrEF and AS patients.

Furthermore, we studied the ability of metabolic risk to induce HFpEF through myocardial microvascular endothelial inflammatory activation in leptin-resistant, obese, hypertensive ZSF1 rats. These rats develop a HFpEF phenotype after 20 weeks, which was evident from elevated LV filling pressures with preserved LV systolic function, increased lung weight because of pulmonary congestion and increased stiffness of isolated myocardial strips (15). At that time, a similar assessment of myocardial microvascular endothelial inflammatory activation and its effects on oxidative stress, NO bioavailability, cGMP content and PKG signalling was performed and compared to age matched lean, hypertensive ZSF1 rats with normal diastolic LV function and no lung congestion.

METHODS

A detailed method section can be found in an Online Supplement.

Human samples

Human HFrEF and HFpEF samples were procured from LV biopsies [HFrEF (N=43) and HFpEF (N=36)]. HFrEF and HFpEF patients were hospitalized for HF and underwent transvascular LV endomyocardial biopsy procurement because of suspicion of an infiltrative or inflammatory cardiomyopathy. Patients were included if significant coronary artery disease (stenosis >50%) was ruled out by angiography and if histological analysis of the biopsy showed no evidence of infiltrative or inflammatory myocardial disease. Patients were classified as HFpEF if LVEF was >50%, LV end diastolic volume index <97 ml/m^2, and LV end-diastolic pressure was >16 mmHg (16). If LVEF was <45%, a patient was classified as HFrEF. AS patients (N=67) had severe AS (mean aortic valve area 0.53±0.04cm^2) without concomitant coronary artery disease. Biopsy specimens from this group were procured from endomyocardial tissue resected from the septum (Morrow procedure) during aortic valve replacement. The local ethics committee approved the study protocol. Written informed consent was obtained from all patients. Control human samples (N=4) were procured from patients with life-threatening

arrhythmias, suspected infiltrative heart disease or myocarditis and a preserved LVEF without coronary artery disease in whom histology ruled out myocarditis or infiltrative pathology. Because of limited availability of human myocardial tissue, histological and biochemical determinations could only be performed in subgroups of patients, randomly selected by blinded investigators.

TABLE 1 Baseline Characteristics and Hemodynamics of Patients

	HFpEF (n = 36)	HFrEF (n = 43)	AS (n = 67)	Control (n = 4)	p Value (HFpEF vs. HFrEF)	p Value (HFpEF vs. AS)
Age, yrs	63.8 ± 2.0	60.0 ± 2.1	65.3 ± 1.6	51 ± 4	0.21	0.57
% of males	56	70	47	25	0.24	0.53
% of hypertension	78	16	58	–	<0.0001	0.07
% of DM	47	30	26	–	0.16	0.047
BMI, kg/m^2	30.4 ± 1.0	27.5 ± 0.8	28.1 ± 0.6	–	0.023	0.031
GFR, ml/min/1.73 m^2	72.9 ± 2.3	73.3 ± 2.7	68.9 ± 18.5	–	0.49	0.2
% of atrial fibrillation	14	33	2	–	0.067	0.028
% taking medications						
ACEI/ARB	81	81	43	–	1.00	<0.0001
Beta-blocker	53	63	61	–	0.49	0.52
Diuretic agent	78	72	54	–	0.61	0.028
Aldosterone receptor antagonist	47	74	7	–	0.020	<0.0001
Digoxin	14	33	2	–	0.067	0.028
Statin	42	21	46	–	0.054	0.83
Metformin	17	14	3	–	0.76	0.049
Bronchodilators	17	19	9	–	1.00	0.34
Hemodynamics						
HR, beats/min	75 ± 2	82 ± 4	74 ± 2	80 ± 16	0.073	0.71
LVPSP, mm Hg	166 ± 6	120 ± 3	223 ± 4	135 ± 15	<0.0001	<0.0001
LVEDP, mm Hg	25.1 ± 1.1	22.3 ± 1.4	22.8 ± 1.4	13 ± 4	0.12	0.21
LVEDVI, ml/m^2	80 ± 3	127 ± 5	55 ± 2	78 ± 23	<0.0001	<0.0001
% of LVEF	58.4 ± 2.1	29.4 ± 1.5	64.0 ± 1.2	73 ± 3	<0.0001	0.016

Values are mean ± SD or%.

ACEI = angiotensin-converting enzyme inhibitor; ARB = angiotensin II receptor blocker; AS = aortic stenosis; BMI = body mass index; DM = diabetes mellitus; HFpEF = heart failure with preserved ejection fraction; HFrEF indicates heart failure with reduced ejection fraction; HR = heart rate; LVEDP = left ventricular end-diastolic pressure; LVEDVI = left ventricular end-diastolic volume index; LVEF = left ventricular ejection fraction; LVPSP = left ventricular peak-systolic pressure.

Rat samples

Obese ZSF1 rats were previously shown to develop a HFpEF phenotype over a 20 weeks lifespan (15) and are referred to as ZSF1-HFpEF (N=8) in the present study. These rats are hypertensive and develop obesity and diabetes mellitus (DM) because of leptin resistance. ZSF1-lean rats are hypertensive, but do not develop obesity or DM and have no HFpEF phenotype (15). The ZSF1-lean rats are referred to as ZSF1-Ctrls (N=8) in the present study. All rats were sacrificed at 20 weeks of age.

Western blotting

Expression of total and phospho-proteins was measured in homogenized samples. The number of samples analysed was: ICAM-1: ZSF1-Ctrls:n=16, ZSF1-HFpEF:n=16, AS:n=4, HFrEF:n=7, HFpEF:n=4; E-selectin: AS:n=6, HFrEF:n=8, HFpEF:n=8; eNOS: ZSF1-Ctrls:n=8, ZSF1-HFpEF:n=8, AS:n=5, HFrEF:n=7, HFpEF:n=7; Kinases: ZSF1-Ctrls:n=8-10, ZSF1-HFpEF:n=8-10.

Immunofluorescence

Myocardial ICAM-1 expression was measured by immunofluorescence in frozen sections of 10µm thick rat myocardium: ZSF1-HFpEF:n=12, ZSF1-Ctrl:n=12.

Immunohistochemistry

For immunohistochemical staining of myeloperoxidase (MPO), CD68 and NADPH oxidase-2 (NOX2), paraffin embedded myocardial samples were used: MPO and CD68: ZSF1-Ctrl:n=8, ZSF1-HFpEF:n=8; NOX2: ZSF1-Ctrl:n=8, ZSF1-HFpEF:n=8, HFpEF:n=4, Ctrl patients:n=4.

Myocardial hydrogen peroxide quantification

Hydrogen peroxide (H_2O_2) was assessed in human and rat myocardial tissue homogenates (ZSF1-Ctrl:n=10; ZSF1-HFpEF:n=10, AS:n=16, HFrEF:n=16, HFpEF:n=16.

Nitrate/nitrite concentration

The concentrations of nitrite/nitrate were measured in tissue homogenates (ZSF1-Ctrl:n=10, ZSF1-HFPEF:n=10, AS:n=16, HFpEF:n=16, HFrEF:n=16) by means of a colorimetric assay kit (BioVision).

Immunoelectron microscopic quantification of 3-nitrotyrosine

A standard pre-embedding immunogold electron microscopy protocol was used to quantify myocardial 3-nitrotyrosine in rats (ZSF1-HFpEF:n=6, ZSF1-Ctrl:n=6).

Myocardial PKA, PKC, PKG, cGMP and CaMKII activity

Kinase activities were assessed in myocardial homogenates. Activities (ZSF1-HFpEF:n=10, ZSF1-Ctrl:n=10) were analyzed as described previously for PKA and PKC (17) and for PKG and cGMP (18).

CaMKII activity was determined using a CycLex® CaMKII assay kit (ZSF1-HFpEF:n=10, ZSF1-Ctrl:n=10).

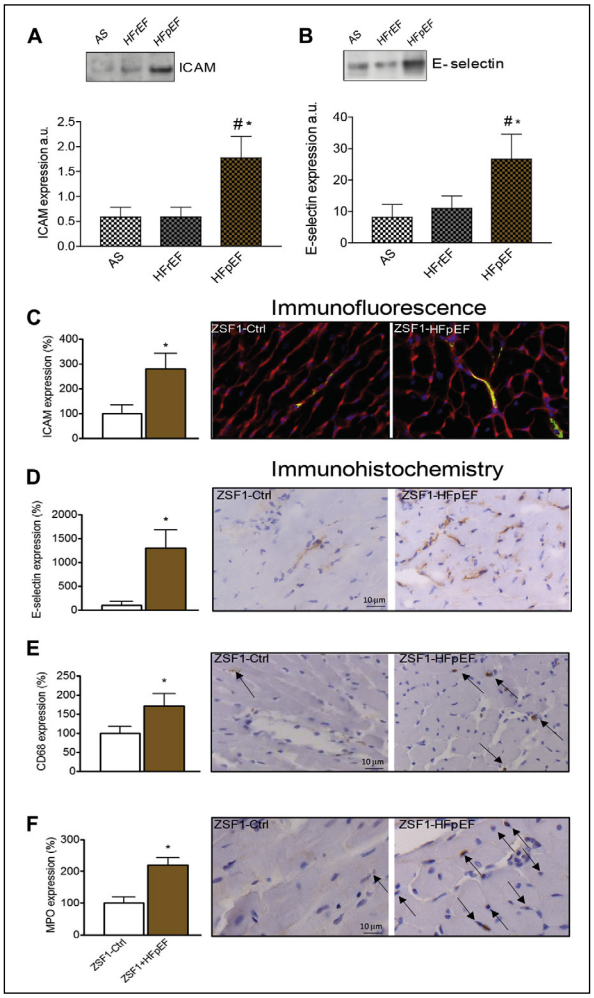

Figure 1. (**A**) ICAM-1 expression was higher in HFpEF than in AS (#p < 0.05 vs. AS;*p < 0.05 vs. HFrEF). (**B**) E-selectin levels were higher in HFpEF patients than in those with HFrEF and AS (#p < 0.05 vs. AS; *p < 0.05 vs. HFrEF). Expression levels of ICAM-1 (**C**) and E-selectin (**D**) were higher in ZSF1-HFpEF myocardium than in that of ZSF1-Ctrls (*p < 0.05). (E and F) ZSF1-HFpEF rats had higher levels of myocardial CD68 and MPO than ZSF1-Ctrls (*p < 0.05). AS = aortic stenosis; Ctrls = controls; HFpEF = heart failure with preserved ejection fraction; HFrEF = heart failure with reduced ejection fraction; ICAM = intercellular adhesion molecule; MPO = myeloperoxidase.

Statistical analysis

Differences between groups were analyzed by one-way ANOVA followed by Bonferroni-adjusted t tests. Single comparisons were assessed by an unpaired Student t test. All analyses were performed using Prism software (GraphPad Software Inc, version 6.0).

RESULTS

Patient characteristics

More HFpEF than HFrEF patients were hypertensive and DM was more prevalent in HFpEF compared to AS (see Table). Body mass index (BMI) was higher in HFpEF

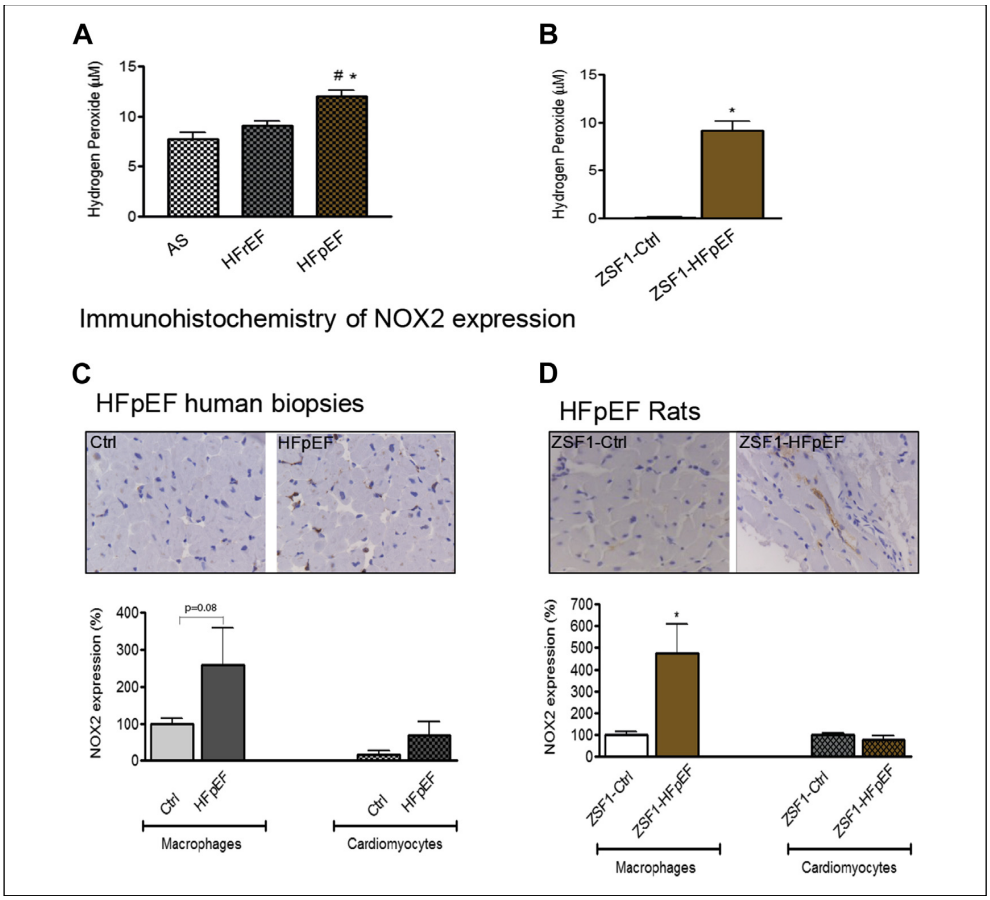

Figure 2. (**A**) HFpEF patients had higher myocardial concentrations of H_2O_2 than AS and HFrEF (#p < 0.05 vs. AS; *p < 0.05 vs. HFrEF). (**B**) Myocardial H_2O_2 concentrations in ZSF1-HFpEF were higher than in ZSF1-Ctrl rats (*p < 0.05). (**C**) Expression of NOX2 was comparable in cardiomyocytes but tended to be higher in macrophages of HFpEF patients. (**D**) Expression of NOX2 was comparable in cardiomyocytes of ZSF1-Ctrl and ZSF1- HFpEF rats. More NOX2-expressing macrophages were observed in ZSF1-HFpEF rats (*p < 0.05 vs. controls). NOX2 = NADPH oxidase 2; other abbreviations as in Figure 1.

versus AS patients. Angiotensin converting enzyme inhibitors or angiotensin II receptor blockers, diuretics and digoxin were more frequently used in HFpEF and HFrEF compared to AS patients. Aldosterone receptor antagonists were used more in HFpEF than in AS, but even more in HFrEF patients. LV peak systolic pressure and LVEF were higher in HFpEF than in HFrEF but highest in AS patients. LV end diastolic volume index was lower in HFpEF than in HFrEF but the lowest in AS patients. LV end diastolic pressures were equally elevated in all patient groups.

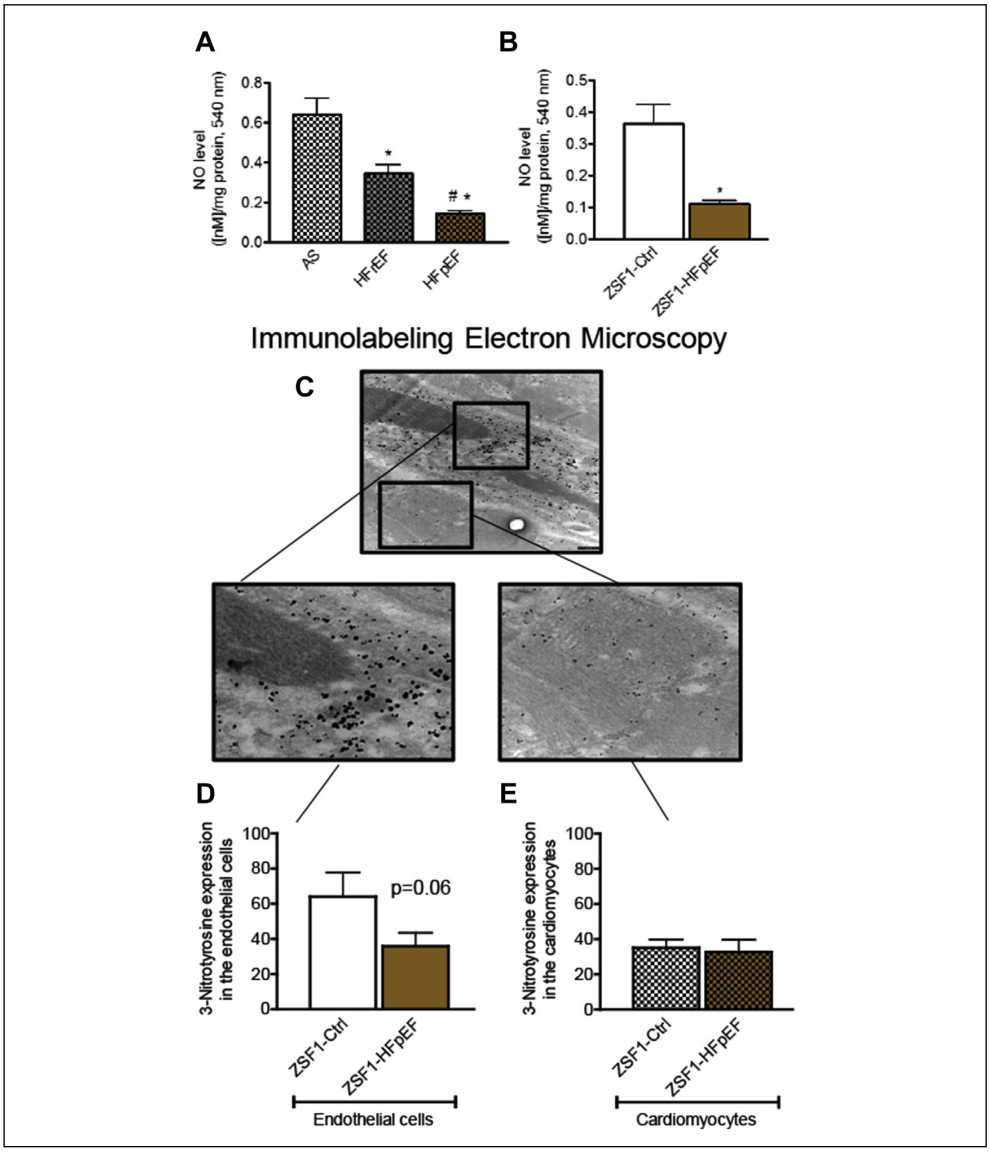

Figure 3. (**A**) Myocardial nitrite/nitrate concentrations were lower in HFpEF than in AS and HFrEF patients and lower in HFrEF than in AS patients (*p < 0.05 HFpEF vs. AS; #p < 0.05, HFpEF vs. HFrEF; *p < 0.05, HFrEF vs. AS). (**B**) Myocardial nitrite/nitrate concentrations were lower in ZSF1-HFpEF than in ZSF1-Ctrl rats (*p < 0.05 vs. controls). (**C**) Immunogold-labeled electron microscopy showed myocardial localization of 3-nitrotyrosine. (**D**) 3-Nitrotyrosine expression tended to decrease more in endothelial cells of ZSF1-HFpEF than those of ZSF1-Ctrl rats. (**E**) 3-Nitrotyrosine expression was similar in cardiomyocytes of ZSF1-HFpEF and ZSF1-Ctrl rats. NO = nitric oxide; other abbreviations as in Figure 1.

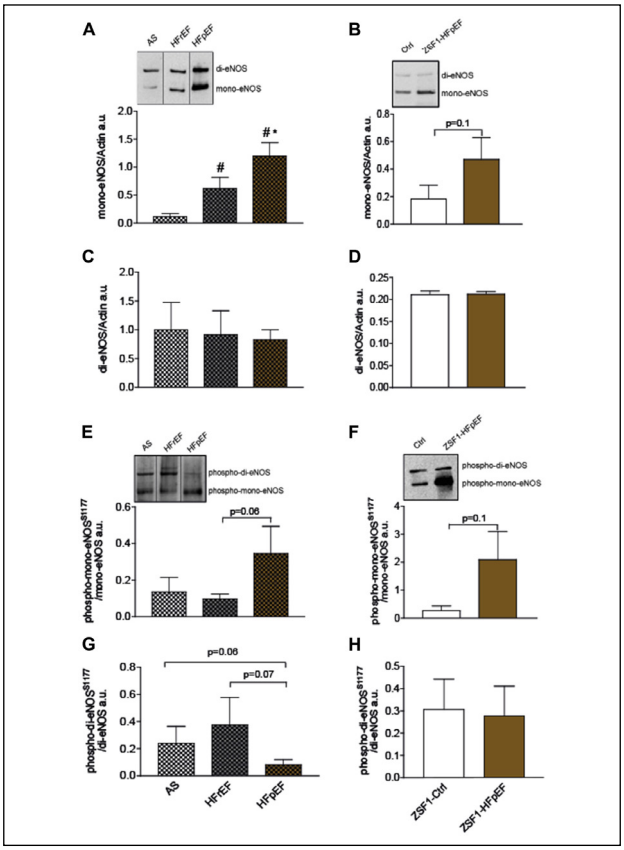

Figure 4. (**A**) Expression of eNOS monomer was higher in HFpEF than in AS and HFrEF and higher in HFrEF than in AS (#p < 0.05 vs. AS; *p < 0.05 vs. HFrEF). (**B**) A similar trend was observed in a comparison between ZSF1-HFpEF and ZSF1-Ctrl rats. (**C** and **D**) eNOS dimer concentrations were comparable in AS, HFrEF, and HFpEF patients as well as in ZSF1-HFpEF and ZSF1-Ctrl rats. (**E** and **F**) eNOS momomer phosphorylation tended to be higher in HFpEF patients and in ZSF1-HFpEF rats. (**G** and **H**) eNOS dimer phosphorylation tended to be lower in HFpEF patients but was comparable in rats. eNOS = endothelial nitric oxide synthase; other abbreviations as in Figure 1.

Microvascular inflammation and macrophage activation in HFpEF

Microvascular inflammation and macrophage activation were assessed by expression of the vascular adhesion molecules ICAM-1 and E-selectin. Both markers were increased in HFpEF compared to AS or HFrEF (Figure 1A-B), consistent with the comorbidities-induced pro-inflammatory status of HFpEF patients.

Similarly, immunofluorescence showed increased endothelial ICAM-1 and E-selectin expression in ZSF1-HFpEF rats compared to ZSF1-Ctrls (Figure 1C-D). ZSF1-HFpEF rats also had higher myocardial CD68 and MPO expression than ZSF1-Ctrl, indicating monocyte/macrophage recruitment and neutrophil activation (Figures 1E-F).

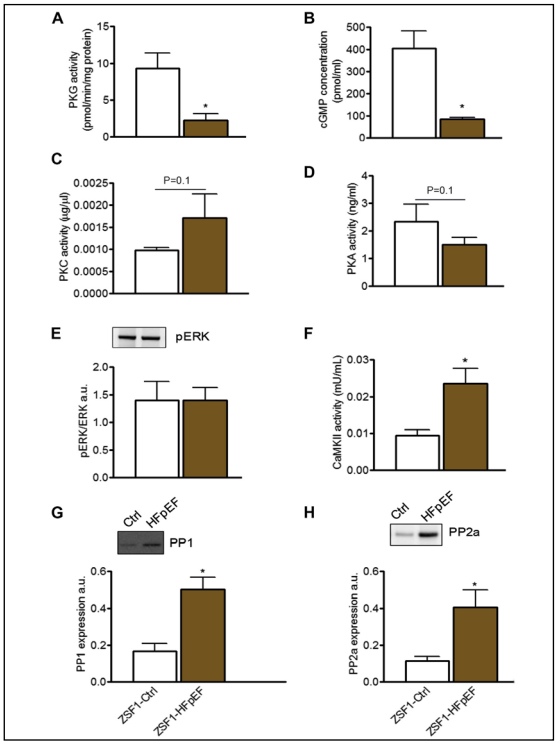

Figure 5. (**A** and **B**) Myocardial PKG activity and cGMP concentration were lower in ZSF1-HFpEF than in ZSF1-Ctrl rats. (**C** and **D**) Comparable activity levels of PKC and PKA were seen in ZSF1-HFpEF and ZSF1-Ctrl rats. (**E**) Comparable expression levels of phosphorylated ERK were seen in ZSF1-HFpEF and ZSF1-Ctrl rats. (**F**) Expression of CaMKII was higher in ZSF1-HFpEF than in ZSF1-Ctrl rats. (**G** and **H**) Higher expression of PP1 and PP2a was seen in ZSF1-HFpEF than in ZSF1-Ctrl rats (*p < 0.05). CaMKII = Ca^{2+}/calmodulin-dependent protein kinase II; cGMP = cyclic guanosine monophosphate; ERK = extracellular signal-regulated kinase; PKA = protein kinase A; PKC = protein kinase C; other abbreviations as in Figure 1.

Increased oxidative stress in HFpE

To quantify myocardial oxidative stress, H_2O_2 concentrations were shown to be higher in HFpEF than in HFrEF and AS patients (Figure 2A), again consistent with the comorbidities-induced pro-inflammatory status of the HFpEF patients. Findings were reproduced in ZSF1-HFpEF rats which also had increased myocardial H_2O_2 levels compared to ZSF1-Ctrl (Figure 2B).

To account for the myocardial oxidative stress, expression of NOX2 was compared between HFpEF patients and controls. Human HFpEF myocardium contained more NOX2 expressing macrophages than control (Figure 2C). Expression in cardiomyocytes was however comparable in both HFpEF patients and controls (Figure 2C). Findings were confirmed in ZSF1-HFpEF myocardium by the presence of more NOX2 expressing macrophages than in ZSF1-Ctrl. Similar to the human findings, NOX2 expression was equal in cardiomyocytes of ZSF1-HFpEF and ZSF1-Ctrl rats (Figure 2D). Furthermore, NOX2 expression was also manifest in microvascular endothelial cells of ZSF1-HFpEF rats (Figure 2D), indicative of a systemic pro-inflammatory status.

Decreased NO bioavailability in HFpEF

Because of the high oxidative stress, bioavailability of NO gets jeopardized. NO bioavailability was therefore assessed by measurement of myocardial nitrite/ nitrate concentrations in human biopsies. Nitrite/nitrate concentration was indeed lower in HFpEF than in AS and HFrEF (Figure 3A). These findings were also confirmed in the rat model (Figure 3B).

Furthermore, immunogold labeled electron microscopy allowed for quantification of 3-nitrotyrosine in different myocardial cellular compartments (Figure 3C). 3-Nitrotyrosine formation reflects concentration of peroxynitrite, which is generated from superoxide anion and NO. 3-Nitrotyrosine expression was higher in endothelial cells compared to cardiomyocytes (Figure 3D-E). Its endothelial expression tended to decrease in ZSF1-HFpEF (Figure 3D) probably as a result of reduced NO bioavailability, whereas cardiomyocyte expression remained unaltered (Figure 3E).

Uncoupling of nitric oxide synthase in HFpEF

Endothelial nitric oxide synthase (eNOS) produces NO as a dimer, but "uncouples" into monomers in the presence of inflammation/oxidative stress, producing superoxide anion. HFpEF patients had significantly higher expression of the eNOS monomer than HFrEF or AS patients (Figure 4A). In ZSF1-HFpEF rats, there was a similar trend for higher expression of the eNOS monomer compared to ZSF1-Ctrl (Figure 4B). Levels of eNOS dimers were equal among human groups and between the two groups of rats (Figures 4C-D). Phosphorylated and hence activated eNOS monomers tended to be higher in HFpEF compared to HFrEF and AS patients (P=0.06) and in ZSF1-HFpEF compared to ZSF1-Ctrl rats (Figures 4E-F). The concentration of active, phosphorylated NO-producing dimer was lower in HFpEF compared to AS (P=0.06) and HFrEF (P=0.07) patients (Figure 4G). In rats, phosphorylation of the eNOS dimer was similar (Figure 4H).

Decreased cGMP concentration and PKG activity in HFpEF

Because of decreased NO bioavailability, soluble guanylate cyclase (sGC) activity falls. This leads to reduced production of cGMP, which regulates PKG activity. PKG acitivity and cGMP concentration were indeed lower in myocardium of ZSF1-HFpEF rats compared to ZSF1-Ctrl (Figure 5A-B).. PKG lowers cardiomyocyte stiffness through phosphorylation of titin, the giant intracellular protein responsible for cardiomyocyte based stiffness (15, 17–19). Activity and expression

of other protein kinases and phosphatases, reported to modulate titin phosphorylation (19) were measured as well. Activities of protein kinase C (PKC) and A (PKA) were not significantly different between ZSF1-HFpEF and ZSF1- Ctrl rats (Figures 5C-D). There was no significant difference in expression of the active, phosphorylated state of extracellular-signal-regulated kinase (ERK) between ZSF1-HFpEF rats and Ctrl rats (Figure 5E). Ca^{2+}/calmodulin-dependent protein kinase-II (CaMKII) also lowers cardiomyocyte stiffness through titin phosphorylation (20). CaMKII activity was increased in ZSF1-HFpEF rats compared to ZSF1-Ctrl rats (Figure 5F) and altered CaMKII activity therefore fails to explain the increased cardiomyocyte stiffness observed in previous experiments in ZSF1-HFpEF rats (15). Finally, titin can also be affected by dephosphorylating protein phosphatases (PP) such as PP1 and PP2a (19). Both were increased in ZSF1-HFpEF compared to ZSF1-Ctrl rats (Figures 5G-H).

DISCUSSION

The present study provides comprehensive evidence for microvascular endothelial activation, high oxidative stress, eNOS-uncoupling and low NO level in LV myocardium of HFpEF patients. These findings were reproduced in leptin-resistant, obese hypertensive ZSF1 rats, which develop HFpEF after 20 weeks in contrast to lean hypertensive ZSF1 rats, which maintain normal LV function after a similar time period. The present study also demonstrated that the low myocardial NO level was associated with reduced myocardial cGMP/PKG signalling in ZSF1-HFPEF rats. A similar reduction was previously demonstrated in LV myocardium of HFpEF patients and shown to be associated with titin hypophosphorylation which contributes to high myocardial diastolic stiffness (18).

Microvascular inflammation

In the present study, myocardial expression of E-selectin and ICAM-1 was upregulated in HFpEF patients and ZSF1-HFpEF rats (Figure 6). Upregulated myocardial expression of Vascular Cell Adhesion Molecule (VCAM) and myocardial microvascular rarefaction compatible with microvascular inflammation had previously already been reported in HFpEF (22,23). Because of the similarity of findings in HFpEF patients and ZSF1-HFpEF rats, we attribute the endothelial inflammatory activation in HFpEF patients to their metabolic risk profile. HFpEF

patients had significantly higher BMI than AS and HFrEF patients and the prevalence of DM was also higher in the HFpEF than in AS patients. The high prevalence of a metabolic risk profile in HFpEF patients of the present study was consistent with the findings of the recent MEDIA European HFpEF registry (23). This registry was the first to report on the prevalence of metabolic syndrome in HFpEF and observed 85% of HFpEF patients to satisfy the National Cholesterol Education Program III criteria of metabolic syndrome.

Expression of adhesion molecules favours myocardial infiltration of inflammatory cells, which was evident in the current study in HFpEF patients and in ZSF1-HFpEF rats respectively by the presence of NOX2 producing macrophages and by the high expression of CD68 and MPO. In contrast to viral myocarditis, the myocardial presence in HFpEF of macrophages is not accompanied by evidence of cardiomyocyte cell death (25,26). A recent study provides an explanation for this intriguing finding as it observed macrophages activated by obesity to have a distinct proinflammatory phenotype (26). Hitherto macrophage activation was conceived to proceed either to a M1 phenotype with potent proinflammatory properties or to a M2 phenotype with anti-inflammatory properties. However, when macrophages are activated by obesity, a distinct phenotype is induced with low production of proinflammatory cytokines because of peroxisome proliferator-activated receptor ɣ (PPAR ɣ) partially inhibiting induction of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB). This last effect results from the abundance in obesity of free fatty acids such as palmitate, which stimulate PPAR ɣ activity. A recent study also demonstrated HFpEF development to be associated with monocytosis and monocyte differentiation into M2-macrophages (27).

Oxidative stress

Myocardial H_2O_2 concentration was significantly higher in HFpEF than in both HFrEF and AS. Similarly, ZSF1-HFpEF rats had higher myocardial H_2O_2 concentrations than ZSF1-Ctrl animals. H_2O_2 results from conversion of superoxide anion by superoxide dismutase and the high H_2O_2 concentrations therefore suggest increased superoxide anion production in HFpEF. Possible sources of superoxide anion production are NADPH oxidases (NOX2, NOX4), uncoupled NO synthases (eNOS, iNOS), xanthine oxidase and mitochondria (Figure 6). The cellular localization of NOX2 expression was immunohistochemically visualized in myocardial tissue of HFpEF patients and ZSF1-HFpEF rats. Upregulation of NOX2 expression was observed in macrophages and microvascular endothelium but not

in cardiomyocytes. The current findings of upregulated endothelial NOX2 expression and unaltered NOX2 expression in cardiomyocytes in HFpEF myocardium support myocardial remodeling in HFpEF to be driven by endothelial activation in contrast to HFrEF, where myocardial remodeling is driven by cardiomyocyte cell death triggered by upregulated NOX2 activity within cardiomyocytes (28).

NO-cGMP-PKG signalling

Similar to NOX2 expression, immunoelectron microscopy revealed myocardial 3-nitrotyrosine expression in ZSF1-Ctrl and ZSF1-HFpEF rats to be mainly localized in endothelial cells with less expression in cardiomyocytes. HFpEF development failed to affect 3-nitrotyrosine expression in cardiomyocytes but reduced 3-nitrotyrosine expression in endothelial cells. 3-Nitrotyrosine formation reflects peroxynitrite concentration, which is generated from superoxide anion and NO. Because of increased H_2O_2 concentration, the trend for reduced 3-nitrotyrosine in endothelial cells of HFpEF myocardium probably resulted from low NO production. The latter was consistent with the low nitrite/nitrate concentrations observed in myocardium of both HFpEF patients and ZSF1-HFpEF rats. Reduced NO production can be explained by eNOS uncoupling, which was confirmed in both HFpEF patients and ZSF1-HFpEF rats. eNOS uncoupling switches eNOS from the NO producing dimer to the superoxide anion generating monomer (29). Apart from eNOS uncoupling, the present study also observed modified eNOS phosphorylation in HFpEF patients with a higher phosphorylation of monomeric eNOS increasing superoxide production and a lower phosphorylation of dimeric eNOS decreasing NO production. Low NO production affects sGC activity and results in decreased levels of cGMP and low PKG activity (Figure 6). This was previously observed in human HFpEF myocardium (18) and currently confirmed in ZSF1-HFpEF myocardium. Low PKG activity increases diastolic stiffness through reduced phosphorylation of the giant cytoskeletal protein titin (Figure 6) (31,32).

The phosphorylation and distensibility of titin are also affected by phosphatases and other kinases, such as PKC, PKA, ERK and CaMKII (20,31,32). In the present study, ZSF1-HFpEF rats showed higher expression of PP1 and PP2a. PKC, PKA and ERK activities were comparable but CaMKII activity was increased. Phosphorylation of titin by CaMKII augments titin distensibility (32) and the higher CaMKII activity therefore cannot explain the high cardiomyocyte resting tension

previously observed in ZSF1-HFpEF rats (15). The latter more likely results from imbalanced activities of PKG and phosphatases.

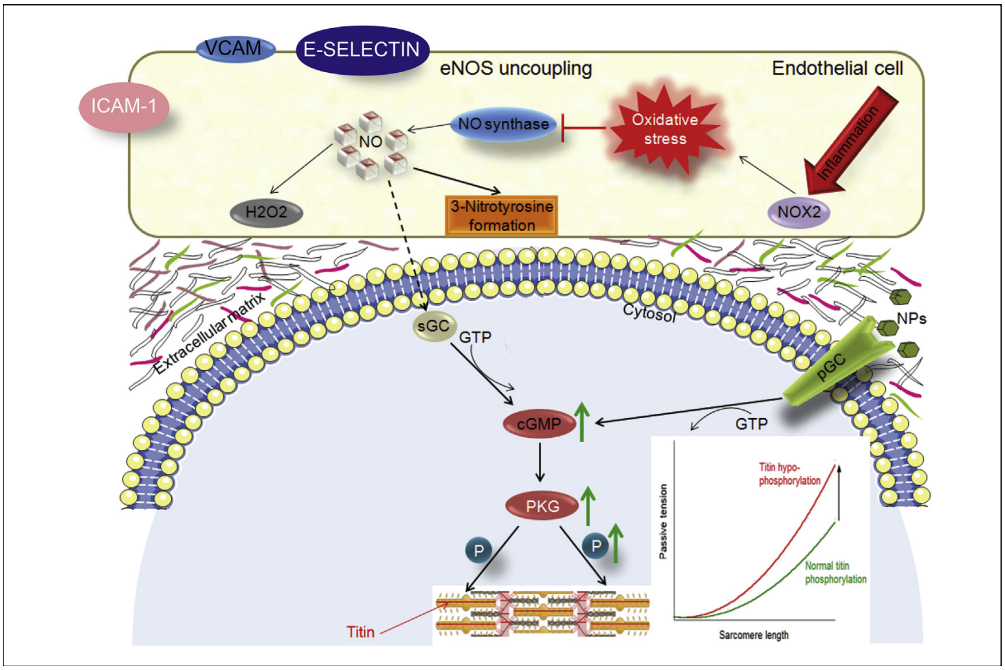

Figure 6. Systemic inflammation induces inflammatory activation of the endothelium of myocardial microcirculation. This leads to enhanced endothelial expression of adhesion molecules such as ICAM-1, VCAM, and E-selectin. Because of inflammatory activation, NOX2 is upregulated in endo- thelial cells. This results in oxidative stress, increased levels of H_2O_2, uncoupling of eNOS, decreased NO bioavailability, and formation of 3-nitrotyrosine. In cardiomyocytes, decreased NO bioavailability leads to less stimulation of sGC, reduced formation of cGMP, and diminished PKG activity. Lack of PKG activity is associated with decreased titin phosphorylation and increased passive stiffness of cardiomyocytes. cGMP can also be generated by NPs which activate pGC. Because of the low cGMP concentration, the latter pathway failed to compensate for the decreased NO-bioavailability. cGMP = cyclic guanosine monophosphate; eNOS = endothelial nitric oxide synthase; H_2O_2 = hydrogen peroxide; ICAM-1 = intercellular adhesion molecule-1; NO = nitric oxide; NOX2 = NADPH nicotinamide adenine dinucleotide phosphate oxidase 2; NPs = natriuretic peptides; pGC = particulate guanylate cyclase; PKG = protein kinase G; sGC = soluble guanylate cyclase; VCAM = vascular cell adhesion molecule.

LIMITATIONS

Except for immunohistochemical assessment of NOX2 expression, the present study compares myocardial tissue of HFpEF patients to tissue of HFrEF and AS patients because of limited availability of myocardial tissue from control subjects. This limitation was partially accounted for by inclusion of an animal model consisting of obese and lean ZSF1 rats (15). Both HFpEF and HFrEF patients were investigated following an acute heart failure admission. An inflammatory component related to the acute heart failure episode could have contributed to the microvascular inflammation.

CONCLUSIONS

Microvascular inflammatory endothelial activation, high oxidative stress, eNOS uncoupling and impaired cGMP-PKG signalling were observed in LV myocardium of HFpEF patients. Similar changes were reproduced in obese, leptin-resistant, hypertensive ZSF1 rats, which developed a HFpEF phenotype after 20 weeks, but not in lean, hypertensive ZSF1 rats. Because of these findings, myocardial microvascular inflammation induced by metabolic comorbidities could be an important contributor to HFpEF development.

PERSPECTIVES

Clinical competencies - Metabolic comorbidities such as obesity and DM induce chronic endothelial inflammation of the coronary microvasculature, which reduces myocardial NO bioavailability, PKG activity and cGMP concentration. The latter predisposes to cardiomyocyte stiffness and hypertrophy, both of which are characteristic for HFpEF.

Translational outlook – Future therapeutic strategies in HFPEF should target the metabolic risk-induced chronic inflammation of the myocardial microvasculature.

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ONLINE DATA SUPPLEMENT: METHOD SECTION

METHODS

Human samples

Human HFrEF and HFpEF samples were procured from LV biopsies [HFrEF (N=43) and HFpEF (N=36)]. HFrEF and HFpEF patients were hospitalized for HF and underwent transvascular LV endomyocardial biopsy procurement because of suspicion of an infiltrative or inflammatory cardiomyopathy. Patients were included if significant coronary artery disease (stenosis >50%) was ruled out by angiography and if histological analysis of the biopsy showed no evidence of infiltrative or inflammatory myocardial disease. Patients were classified as HFpEF if LVEF was >50%, LV end diastolic volume index <97 ml/m^2, and LV end-diastolic pressure was >16 mmHg (16). If LVEF was <45%, a patient was classified as HFrEF. AS patients (N=67) had severe AS (mean aortic valve area 0.53±0.04cm^2) without concomitant coronary artery disease. Biopsy specimens from this group were procured from endomyocardial tissue resected from the septum (Morrow procedure) during aortic valve replacement. The local ethics committee approved the study protocol. Written informed consent was obtained from all patients. Control human samples (N=4) were procured from patients with life-threatening arrhythmias, suspected infiltrative heart disease or myocarditis and a preserved LVEF without coronary artery disease in whom histology ruled out myocarditis or infiltrative pathology. Because of limited availability of human myocardial tissue, histological and biochemical determinations could only be performed in subgroups of patients, randomly selected by blinded investigators.

Rat samples

Obese ZSF1 rats were previously shown to develop a HFpEF phenotype over a 20 weeks lifespan (15) and are referred to as ZSF1-HFpEF (N=8) in the present study. These rats are hypertensive and develop obesity and diabetes mellitus (DM) because of leptin resistance. ZSF1-lean rats are hypertensive, but do not develop obesity or DM and have no HFpEF phenotype (15). The ZSF1-lean rats are referred to as ZSF1-Ctrls (N=8) in the present study. All rats were sacrificed at 20 weeks of age.

Western blotting

Expression of total and phospho-proteins was measured by 15% SDS-PAGE and Western blot in homogenized samples. Hybond ECL nitrocellulose membranes (GE Healthcare) were used in combination with the following primary rabbit or mouse polyclonal antibodies: against eNOS and phospho-eNOS (1:1000, Abcam), Intercellular Adhesion Molecule 1 (ICAM-1, 1:200, Abcam), E-selectin (1:250, Abcam), ERK (1:1000, Cell Signalling), phospho-ERK (1:1000, Cell Signalling), PP1 (1:200, Santa Cruz Biotechnology) and PP2a (1:500, Upstate). All signals were normalized to β-actin peroxidase antibody (dilution 1:1000; clone KJ43A; Sigma). Staining was visualized using the LAS-3000 Image Reader (460nm/605nm Ex/Em; 2s illumination) and signals were analyzed with AIDA software. The number of samples analysed was: ICAM-1: ZSF1-Ctrls:n=16, ZSF1-HFpEF: n=16, AS:n=4, HFrEF:n=7, HFpEF:n=4; E-selectin: AS:n=6, HFrEF:n=8, HFpEF:n=8; eNOS: ZSF1-Ctrls:n=8, ZSF1-HFpEF:n=8, AS:n=5, HFrEF:n=7, HFpEF:n=7; Kinases: ZSF1-HFpEF:n=8-10, ZSF1-Ctrls:n=8-10.

Immunofluorescence

Myocardial ICAM-1 expression was measured by immunofluorescence in rats. Frozen sections of 10µm thick rat myocardium (ZSF1-HFpEF:n=12, ZSF1-Ctrl:n=12;) were fixated in 4% paraformaldehyde and washed in 0.05% PBS-Tween. Tissue sections were incubated with primary antibody against ICAM-1 at 4°C (1:100, Abcam). After rinsing with PBS-Tween, sections were incubated with secondary antibody donkey anti-mouse Alexafluor-488 (1:1000, Invitrogen). After washing in PBS-Tween samples were incubated with Wheat Germ Agglutination Conjugate Alexafluor-555 (WGA, 1:1000, Invitrogen). After rinsing again in PBS-Tween and PBS, coverslips were mounted onto the glass slides using mounting medium with DAPI (Vectashield). Images were acquired with a fluorescence microscope and quantified in Slidebook (3i). ICAM-1 staining was displayed with FITC and sum intensities were acquired. The results were expressed as a percentage of the control (set at 100%).

Immunohistochemistry

For immunohistochemical staining of myeloperoxidase (MPO), CD68 and NADPH oxidase-2 (NOX2), paraffin embedded myocardial samples (MPO and CD68: ZSF1-HFpEF:n=8, ZSF1-Ctrl:n=8; NOX2: ZSF1-HFpEF: n=8, ZSF1-Ctrl:n=8; HFpEF: n=4, Ctrl patients:n=4) were dewaxed and rehydrated in xylene and alcohol (100%)

followed by incubation in 0,3% methanol/H_2O_2 to block endogenous peroxidases. Antigen retrieval was performed by either boiling slides in a citrate pH 6.0 (MPO) or a Tris-EDTA pH 9.0 (NOX2) solution or by incubation with 0,1% pepsin/HCl (CD68). Next, sections were incubated with rabbit anti-rat MPO (1:50, Abcam), mouse anti-rat CD68 (1:400, Serotec), or mouse anti-NOX2 (1:10, CLB). Sections were then incubated with undiluted goat anti-mouse/rabbit envision (Dako).

For immunohistochemical staining of E-selectin (CD62e), frozen myocardial tissues (ZSF1-HFpEF:n=8, ZSF1-Ctrl:n=8;) were fixated in followed by incubation in 0,3% methanol/H_2O_2 to block endogenous peroxidases. Next, sections were pre-incubated with 1:500 normal rabbit serum (Dako), followed by incubation with goat anti-rat CD62e (1:100, LifeSpan BioSciences) primary antibody. Sections were then incubated with rabbit anti-goat-HRP (1:100, Dako). Staining for all markers was visualized using 3,3'-diaminobenzidine (0.1mg/mL, 0.02% H2O2), and sections were counterstained with haematoxylin, dehydrated, and covered. Negative and positive controls were performed in parallel to all staining series.

Light microscopy (Olympus BX50) was used for the quantitative analysis. Macrophages and cardiomyocytes were manually counted. Analysis of tissue areas was performed using Slidebook (3i). The staining density was calculated as the number of stained macrophages or cardiomyocyte nuclei per unit area of myocardium. These results are expressed as a percentage of the control (set at 100%).

Myocardial hydrogen peroxide quantification

Hydrogen peroxide (H_2O_2) was assessed in human and rat myocardial tissue homogenates (ZSF1-HFpEF:n=10, ZSF1-Ctrl:n=10; AS:n=16, HFrEF:n=16, HFpEF: n=16). Samples containing equal amounts of total protein were analyzed for H_2O_2 formation and measured calorimetrically at 540nm. Results were converted using the standard curve for a known concentration of H_2O_2 and expressed in micromolar.

Nitrate/nitrite concentration

The concentrations of nitrite/nitrate were measured in tissue homogenates (ZSF1-HFPEF:n=10, ZSF1-Ctrl:n=10, AS:n=16; HFpEF:n=16; HFrEF:n=16) by means of a colorimetric assay kit (BioVision). This kit uses a two-step protocol. First, nitrate is

converted to nitrite using nitrate reductase. Subsequenlty Griess Reagents convert nitrite to an azochromophore reflecting NO concentration in the tissue and plasma samples. Nitrite levels can be measured independently from nitrate by omitting the first step.

Immunoelectron microscopic quantification of 3-nitrotyrosine

A standard pre-embedding immunogold electron microscopy protocol was used in this study. Briefly, cardiac samples (ZSF1-HFpEF:n=6, ZSF1-Ctrl:n=6;) were fixed in 4% paraformaldehyde, 15% saturated picric acid in 0.1M phosphate buffer pH 7.4. Sections were cut on a vibratome (Leica VT 1000S) for a thickness of 50µm, blocked in 20% NGS (Vector Laboratories) in PBS and were incubated with Nitrotyrosin (Millipore 06-284) primary antibody in phosphate-buffered saline containing 5% NGS. After washing, sections were incubated with 1.4nm gold-coupled secondary antibodies (Nanoprobes). After several washes sections were postfixed in 1% glutaraldehyde in PBS and then incubated with HQ Silver kit (Nanoprobes). After treatment with OsO4, sections were stained with uranyl acetate, dehydrated and at embedded in Durcupan resin (Fluka). Ultrathin sections were prepared (Ultracut S) and examined with a ZEISS LEO 910 electron microscope. Images were subsequently analyzed with Slidebook (3i). 3-Nitrotyrosine expression was calculated as the sum of 3-nitrotyrosine gold-labelling area related to the total area of cardiomyocytes or endothelium.

Myocardial PKA, PKC, PKG, cGMP and CaMKII activity

All kinase activities were assessed in myocardial homogenates. Activities (ZSF1-HFpEF:n=10, ZSF1-Ctrl: n=10) were analyzed as described previously for PKA and PKC (17) and for PKG and cGMP (18).

For CaMKII, activity was determined using a CycLex® CaMKII assay kit (CY-1173; MBL Corporation, MA) according to the manufacturer's guidelines (ZSF1-HFpEF:n=10, ZSF1-Ctrl:n=10;).

Statistical analysis

Differences between groups were analyzed by one-way ANOVA followed by Bonferroni-adjusted t tests. Single comparisons were assessed by an unpaired Student t test. All analyses were performed using Prism software (GraphPad Software Inc, version 6.0).

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EDITORIAL COMMENT

Inflammation in Heart Failure With Preserved Ejection Fraction

Time to Put Out the Fire*

Mardi Gomberg-Maitland, MD, MSc,[a] Sanijv J. Shah, MD,[b] Marco Guazzi, MD, PhD[c]

Heart failure with preserved ejection fraction (HFpEF) is a common and costly condition associated with a high frequency of comorbid conditions. HFpEF prevalence is rising compared to heart failure with reduced ejection fraction (HFrEF), without any change in clinical outcomes over the past 20 years (1).

In contrast to HFpEF, HFrEF outcomes have improved with the advent of multiple drug therapies. Most of the therapeutic approaches proven to be effective in HFrEF have been tested and developed in controlled experimental studies in animal models (2) by dissecting the role of abnormal signaling in intracellular molecular pathways (3). The most impressive examples come from studies on angiotensin-converting enzyme inhibitors and beta-blockers. Mechanistic research enabled appropriate therapeutic targets and favored the development of genetically manipulated small animal strains (3).

Thus far, therapeutic remedies have been ineffective in HFpEF likely because a multitude of phenotypes are grouped under the same definition (4). Another central reason for the lack of therapies for HFpEF may be related to the erroneous assumption that the therapeutics approved for treating HFrEF can be successfully transposed to HFpEF without a full appreciation of differences in cardiac adaptation and extent of diastolic impairment (5) or, more simply, without taking full advantage of translational science and appropriate animal models. Indeed, animal models of HFpEF are not as well established as many capture components of specific hemodynamic aspects, such as left ventricular (LV) vascular uncoupling (6), left atrial dysfunction, and pulmonary hypertension (7), but poorly reproduce the integrative complexity of human disease and related comorbidities such as obesity, diabetes, chronic kidney disease, and atrial fibrillation.

A systemic inflammatory state is central to these common comorbidities associated with HFpEF and oxidative stress and endothelial dysfunction are prominent features that characterize the so-called metabolic risk, especially in the presence of obesity (8). It is noteworthy that occurrence of an inflammatory activation is predictive of incident HFpEF but not HFrEF (9). Once exposed to inflammatory activation, the cardiac myocyte suffers from an impaired cellular signaling at a variety of levels. Specifically, endothelial dysfunction in the coronary microcirculation may alter the paracrine cross-talk signaling between coronary circulation and cardiomyocyte through decreased bioavailability of nitric oxide (NO) and its downstream cyclic guanosine monophosphate (cGMP) protein kinase G (PKG) pathway (10). Collagen turnover and titin homeostasis critically depend on cGMP and PKG signaling, which is absolutely relevant for passive properties (stiffness) of the LV, as demonstrated in animals, isolated human cardiomyocyte preparations (11,12), and the intact human myocardium (13). Recent research suggests that coronary microvascular endothelial inflammation is

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From the [a]Cardiology Section, Department of Medicine, University of Chicago, Chicago, Illinois; [b]Division of Cardiology, Northwestern University Feinberg School of Medicine, Chicago, Illinois; and the [c]Cardiology Department, University of Milan, I.R.C.C.S. Policlinico San Donato, San Donato Milanese, Milan, Italy. Dr. Shah has received research grant support from the National Institutes of Health (R01 HL107557 and R01 HL127028), Actelion, and Novartis; and has served as a consultant for AstraZeneca, Bayer, and Novartis. All other authors have reported that they have no relationships relevant to the contents of this paper to disclose.

also associated with coronary microvasculature rarefaction, with a resultant reduction in capillary density that may impair coronary flow reserve, thereby causing LV systolic and diastolic dysfunction (14). According to this emerging evidence, inflammatory activation and its multisignaling cascade of events on the coronary microvasculature and myocardium is increasingly recognized as a primary contributor in the pathogenesis of HFpEF, and thus has become an intriguing target for intervention (15).

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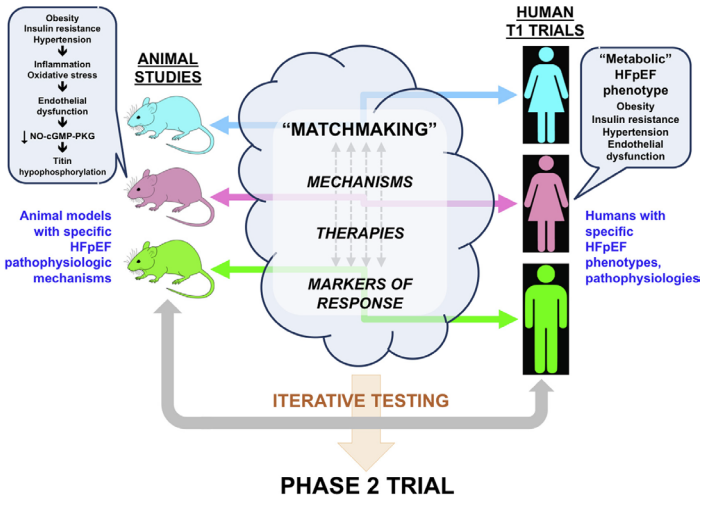
In this issue of *JACC: Heart Failure*, Franssen et al. (16) provide further demonstration of the putative role of myocardial microvascular inflammation in HFpEF by studying its effects on cardiac myocyte oxidative stress and NO pathway signaling in a parallel human and animal study. The authors designed an elegant study that included analysis of myocardial biopsies from a group of patients with increased body mass index and left ventricular hypertrophy (LVH) associated with HFpEF and 3 comparison groups: nonobese HFrEF, aortic stenosis, and control groups. The results of the human study were compared with findings obtained in 2 strains of ZSF1 rats—one leptin resistant, obese, hypertensive, and diabetic and the other nonobese, nondiabetic, and hypertensive. The ZSF1 leptin-resistant model develops LVH and signs of HF, increased LV filling pressure, myocyte stiffness, and congestion, with preservation of LV ejection fraction.

The authors found a similar inflammatory phenotype in HFpEF patients and ZSF1 obese rats, with the same expression of adhesion molecule (ICAM-I, E-selectin), oxidative stress (increased H_2O_2 and

FIGURE 1 Proposed Iterative Testing With Animal Models and Human T1 Mechanistic Phase Trials for the Development of Novel Therapies for Heart Failure With Preserved Ejection Fraction



Iterative testing of novel therapies on the basis of matching of animal models, molecular mechanisms, therapies, markers of treatment response, and humans with specific heart failure with preserved ejection fraction (HFpEF) phenotypes. Matching of animal models, molecular mechanisms, targeted therapies, markers of treatment response, and specific HFpEF phenotypes may lead to improved results in HFpEF clinical trials. For example, iterative testing with the ZSF1-HFpEF rat and patients with the metabolic HFpEF phenotype may provide a platform for the evaluation of therapies that specifically target deficiencies in the nitric oxide-cyclic guanosine monophosphate-protein kinase G (NO-cGMP-PKG) pathway.

reduced nitrite/nitrate concentration), and evidence of uncoupling of endothelial NO synthase. Findings typical of the HFpEF phenotype were not present in the HFrEF and aortic stenosis biopsy samples.

The HFpEF human "metabolic phenotype" adopted in this study was highly selected (body mass index >30 kg/m^2, hypertension, and diabetes) reproducing the most frequent constellation of comorbid disorders in HFpEF (17). Compared to previous observations by the same group (18) the findings of the present study have been extended to a wider characterization of molecular arrays, particularly the multilevel examination of microvascular inflammation, oxidative stress, NO-cGMP-PKG signaling, and titin analysis. The more extensive characterization of the molecular cascade allows us to draw solid conclusions on the associative and, in part, mechanistic role of these pathways in the context of the human HFpEF syndrome.

Because HF is by definition a syndrome with sign and symptoms of congestion, a full characterization of congestive state in both human and animals would have been useful especially considering that the patients included in the present study were hospitalized for an acute episode of decompensated HF whereas the rats that were studied had a more chronic, progressive congestive state. On the basis of the results of the study, these differences in the human subjects versus animal models do not appear relevant in terms of inflammatory activation; however, on the basis of the association of inflammatory markers such as C-reactive protein with the severity of LV end-diastolic pressure increases in HF (19), documentation of the severity of congestion in the patients and the animals in the study could have assisted with understanding whether the authors' results were more applicable to a more advanced stage of HFpEF.

The study by Franssen et al. (16) also could have benefited from a more detailed analysis of cardiac structure and function; indices such as degree of LVH, type of remodeling, and cardiac mechanics were lacking both in the humans and animal models included in the study. How much passive stiffness is affected by inflammatory activation per se rather than pressure overload and ventricular-vascular uncoupling in the in vivo setting remains an unanswered question.

Why is this study important and how do these parallel human and basic science analyses enhance knowledge in the field? The study not only elucidates potential causative metabolic processes but also helps us as clinical investigators better design clinical trials. Limiting our early-phase HFpEF therapeutic trials to patients with only certain comorbidities, similar to these animal models, may result in a more targeted clinical trial approach with early mechanistic studies in limited numbers of patients (i.e., T1 phase trials). An iterative, nimble exchange between animal studies and T1 human studies could result in more fruitful phase 2 and 3 HFpEF clinical trials (**Figure 1**). If successful this type of strategy would also help validate the use of specific animal models for HFpEF. Despite a few drawbacks, the findings by Franssen et al. (16) remain meritorious because they add evidence to the emerging prominent role of inflammatory activation in HFpEF and recall the necessity to optimize the bench to bedside methodological gaps at a level equivalent to what has been successful for HFrEF (i.e., establish animal models that may accurately reproduce the cellular pathways closest to the specific human failing heart phenotype and associated comorbidities). These emerging perspectives and an extended profiling of the cGMP-PKG signaling cascade seem preparatory to appropriately match anti-inflammatory therapeutic interventions to the "inflamed" HFpEF phenotype in large-scale clinical trials.

REPRINT REQUESTS AND CORRESPONDENCE TO: Dr. Mardi Gomberg-Maitland, Section of Cardiology, University of Chicago, 5841 South Maryland Avenue, MC 5403, Chicago, Illinois 60611. E-mail: mgomberg@bsd.uchicago.edu.

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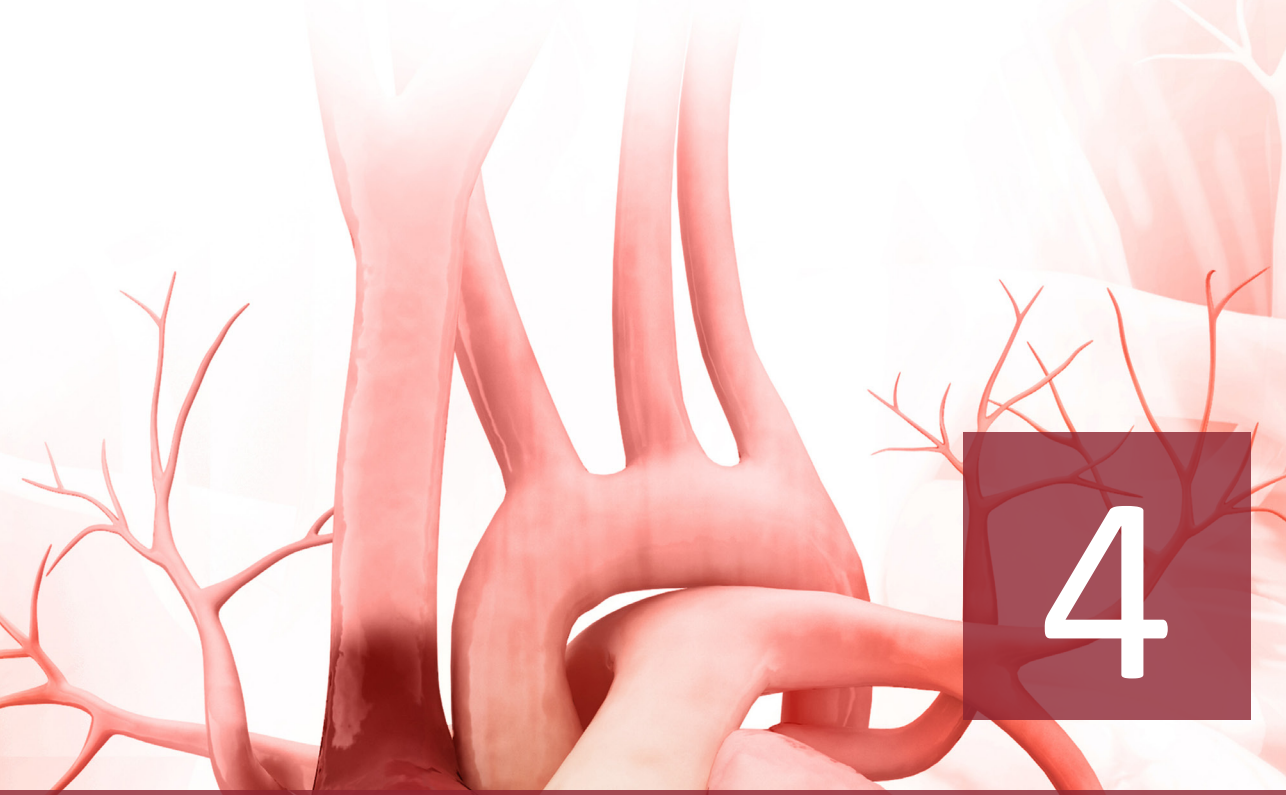
KEY WORDS heart failure preserved ejection fraction, Inflammation



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The role of titin and extracellular matrix remodelling in HFpEF

Franssen C, González Miqueo A

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The role of titin and extracellular matrix remodelling in HFpEF

by

Constantijn Franssen[1], Arantxa González Miqueo[2].

From

[1]Department of Physiology, Institute for Cardiovascular Research,
VU University Medical Center, Amsterdam, The Netherlands.
[2] Program of Cardiovascular Diseases, Centre for Applied Medical Research,
University of Navarra, Pamplona, Spain

Running Title: Titin and extracellular matrix in HFpEF

ABSTRACT

Heart failure with preserved ejection fraction (HFpEF) is characterized by a high incidence of metabolic comorbities that share the potential to induce both systemic and coronary microvascular inflammation and oxidative stress. These pathophysiological alterations contribute to increased myocardial passive stiffness and to diastolic dysfunction, both hallmarks of HFpEF. Myocardial passive stiffness depends mainly on two components: the extracellular matrix (ECM) and the cardiomyocytes. Quantitative and qualitative changes in collagen metabolism leading to myocardial fibrosis determine the ECM-based myocardial passive stiffness. Different non-invasive diagnostic tools to assess myocardial fibrosis are being developed, some of which have demonstrated to correlate with clinical status and prognosis. Cardiomyocytes mainly alter the passive stiffness through alterations in the giant myofilament titin, which serves as a spring. By modifying its phosphorylation state or by direct oxidative effects, titin determines cardiomyocyte-based passive stiffness. Probably the relative importance of cardiomyocyte-based changes is more important in the beginning of the disease, whereas ECM-based changes become more prominent in the more advanced stages. The present review focuses on these changes in ECM and cardiomyocytes in HFpEF and their potential prognostic and therapeutic implications.

INTRODUCTION

In contrast to heart failure with a reduced ejection fraction (HFrEF), patients with heart failure with a preserved ejection fraction (HFpEF) still do not benefit from evidence-based treatment options in the absence of a profound knowledge about its pathophysiology. Therefore, HFpEF therapy is aimed at comorbidities and at reducing signs and symptoms of congestion (1). Indeed, just over a decade ago, knowledge about myocardial structure and function in HFpEF was very poor (2). The following years many studies addressed epidemiological, clinical and fundamental aspects in HFpEF. This eventually led to a novel paradigm in HFpEF pathophysiology with a central role for metabolic comorbidities on top with downstream effects such as inflammation and oxidative stress, eventually interfering with normal myocardial function (3). Indeed, non-cardiac comorbidities such as obesity, arterial hypertension (AHT) and diabetes mellitus (DM) are highly

prevalent in HFpEF (4). These comorbidities generate a chronic, systemic inflammatory state and diverse markers of inflammation have been found to be associated with HFpEF diagnosis and prognosis (5,6). According to this HFpEF paradigm, the observed systemic inflammation also leads to myocardial microvascular endothelial activation and oxidative stress (3). At the cardiac level, this endothelial inflammation and oxidative stress induce myocardial stiffening.

Although there are many other pathophysiological findings in HFpEF, as recently discussed comprehensively elsewhere (7), this review will focus on myocardial abnormalities and the interplay between changes in the extracellular matrix (ECM) and the cardiomyocytes (and specifically titin) that cause myocardial stiffening.

INCREASED MYOCARDIAL STIFFNESS IN HFPEF

The signs and symptoms of HFpEF are based on increased myocardial stiffness, leading to diastolic left ventricular (LV) dysfunction, which is defined as the inability of the heart to fill to an adequate end-diastolic volume at acceptably low pressures in the absence of endocardial or pericardial disease (8). Although practically all patients with HF, regardless of LV ejection fraction (LVEF), have diastolic dysfunction to a higher or lesser degree, HFpEF patients have a non-dilated LV with a globally preserved systolic function (LVEF >50%). Diastolic dysfunction is either diagnosed invasively (by measuring elevated pulmonary capillary wedge pressure, LV end-diastolic pressure or prolonged LV isovolumic relaxation) or non-invasively with tissue Doppler echocardiography (9,10).
LV diastole can be subdivided into two components: myocardial inactivation and myocardial stiffness. This myocardial inactivation is the consequence of dissociating contractile myofilaments and calcium reuptake into the sarcoplasmic reticulum. Myocardial stiffness can be attributed to the viscoelastic properties of the myocardium (Figure 1) (11). The two myocardial compartments that regulate the viscoelastic properties and hence myocardial stiffness will be discussed next: the ECM, namely the collagen network, and the cardiomyocytes, in which the giant protein titin plays a key regulatory role.

Quantification of collagen content

The first indications about HFpEF pathophysiology were based on human myocardial biopsy samples, which showed myocardial fibrosis with an increased collagen volume fraction (CVF) in HFpEF patients compared to controls (12). These findings were recently confirmed in patients with an ante-mortem diagnosis of HFpEF expressing more myocardial fibrosis on autopsies than age-matched

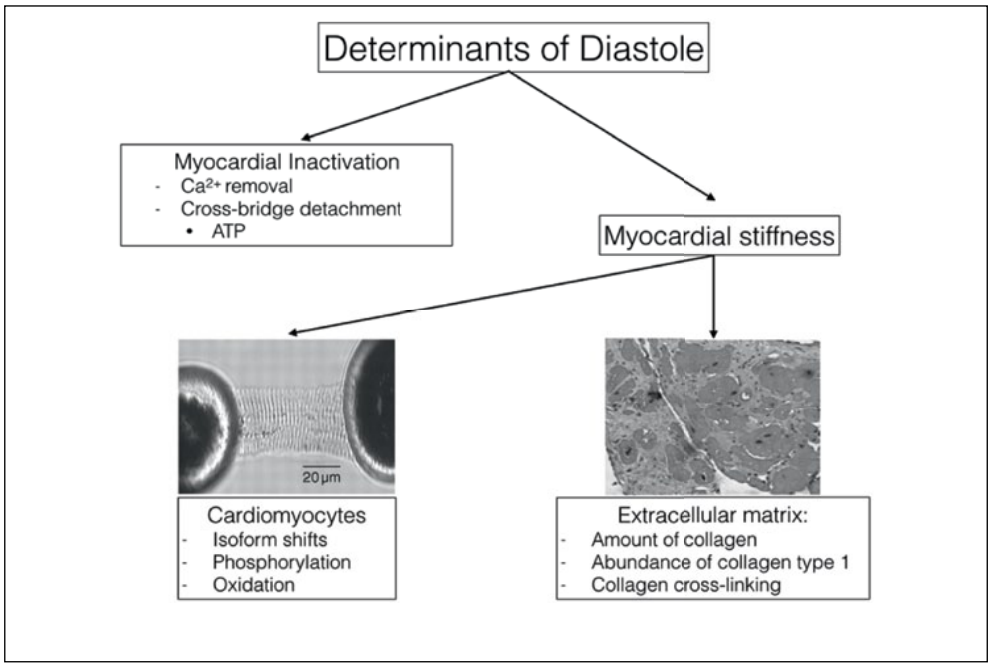

Figure 1. Determinants of diastole. LV diastole can be subdivided into two components: myocar- dial inactivation and myocardial stiffness. This myocardial inactivation is the consequence of dissociating contractile myofilaments and calcium reuptake into the sarcoplasmic reticulum. Myocardial stiffness can be attributed to the viscoelastic properties of the myocardium [11]. The two myocardial compartments that regulate the viscoelastic properties and hence myocardial stiffness are the ECM and the cardiomyocytes, in which the giant protein titin plays a key regulatory role. (Modified with permission from [59])

controls (13). Besides, it was demonstrated that an inflammatory trigger, such as it is present in HFpEF, can induce the differentiation of myocardial fibroblasts into

collagen producing myofibroblasts after stimulation with transforming growth factor-β (14).

However, quantification of total collagen content with CVF seems to have less functional implications than the relative amount of the stiffer collagen type I over the more compliant collagen type III, or the amount of cross-linked collagen by lysyl oxidase (LOX) (Figure 2) (13,14). For example, human HFpEF myocardial biospy samples contained increased levels of collagen type I, enhanced collagen cross-linking and LOX expression and these findings were associated with parameters of diastolic dysfunction on tissue Doppler echocardiography (17). Also, it was demonstrated that HFpEF patients with DM have increased deposition of advanced glycation end-products (AGEs) in the ECM, which are able to cross-link collagen and increase myocardial stiffness (Figure 2)(18). Moreover, AGEs are known to induce myocardial inflammation (19,20) and oxidative stress (21). The relevance of myocardial inflammation and oxidative stress will be discussed in more detail later.

Research has focused on non-invasive techniques to measure LV myocardial fibrosis to avoid the (low) risk of complications and sampling error, both inherent to the procurement of an endomyocardial biopsy. Cardiac magnetic resonance (CMR) imaging, for example, allows for quantification of diffuse myocardial fibrosis by measurement of longitudinal relaxation time (T1-mapping). Several T1 mapping methods have been validated with endomyocardial biopsies to assess diffuse myocardial fibrosis. These techniques include postcontrast T1 mapping, calculation of extracellular volume fraction (EVF) using MOLLI (Modified Look-Locker inversion recovery) sequences, and equilibrium contrast CMR (22). Indeed, EVF as a marker of diffuse myocardial fibrosis correlated with impaired diastolic function in HFpEF (23). On the other hand, numerous biomarkers related to collagen metabolism or its turnover, or molecules integrating cardiac stress injury, inflammation and fibrosis have been studied. However, blood levels of a valid biomarker of myocardial fibrosis should directly correlate with quantitative parameters used to define fibrosis in endomyocardial biopsies (24). Of all possible candidates, only PICP (the carboxy-terminal propeptide of procollagen type I) and PIIINP (the amino-terminal propeptide of procollagen type III) have been shown to be associated with myocardial fibrosis (Figure 2)(24). Possibly different biomarkers of collagen and ECM turnover vary during the transition from being at risk for HFpEF development to more advanced stages of the disease, although

further studies are needed to validate these biomarkers and their potential role in HFpEF diagnosis, treatment and prognosis.

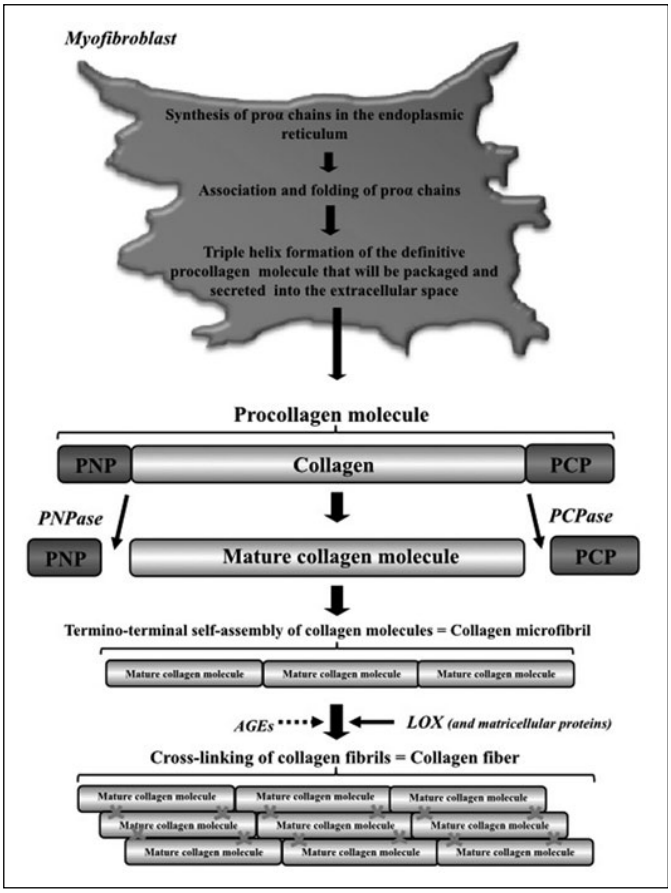

Figure 2. Schematic representation of the process of collagen bres formation. (Adapted from [60] with permission). *PCP* procollagen car- boxy-terminal propeptide, *PNP* procollagen amino-terminal propep- tide, *PCPase* PCP prcollagen carboxi-pepdtidase, *PNP* procollagen amino-peptidase, *AGEs* advanced end-glycation products

Prognostic relevance of myocardial fibrosis

After fibrosis is detected, its clinical or prognostic consequences need to be established. As mentioned above, EVF can be determined with CMR in HFpEF patients as a measure of diffuse myocardial fibrosis (23). EVF correlated with LV end-diastolic and systolic volumes, LV mass, LVEF, peak filling rate and peak ejection rate in HFpEF patients (23). A significant association has been reported between CMR T1 time (validated in LV endomyocardial biopsies) and cardiac outcomes (hospitalization for HF or death from cardiovascular causes) in HFpEF patients (25). However, further large-scale studies need to establish the prognostic relevance of these findings and their value in clinical decision making.

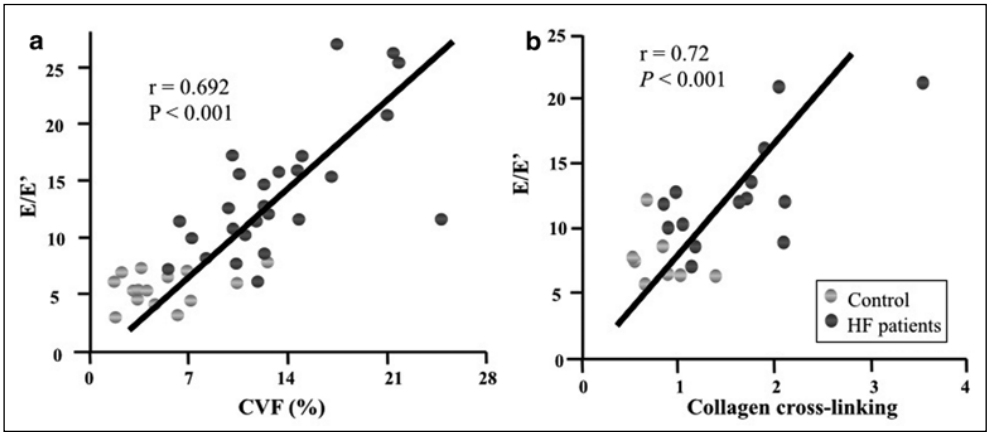

Figure 3. Association of (**a**) col- lagen volume fraction (CVF) and (**b**) collagen cross-linking with left-sided lling pressures echocardiographically esti- mated in heart failure patients with preserved ejection fraction (HFpEF). (Adapted from [61] with permission). *E* maximum early transmitral ow velocity in diastole, *E'*, maximum early diastolic velocity of the mitral annulus displacement.

The number of biomarkers that are currently being studied or developed reflecting myocardial fibrosis in HFpEF is growing exponentially and a detailed discussion of this topic is beyond the scope of this review. The most important and promising biomarkers were recently discussed elsewhere (26). Especially ST2 and galectin-3 have the potential to predict prognosis in HFpEF and, next to this, galectin-3 is related to aldosterone signalling and might identify patients for treatment with aldosterone antagonists (27). However, in a recent substudy of the RELAX-trial, galectin-3 correlated with renal dysfunction and, taking this into account, was not independently associated with the severity of HFpEF (28). Further research is needed to elucidate the role of biomarkers of fibrosis in HFpEF.

Functional relevance of myocardial fibrosis in diastolic dysfunction

Associations of myocardial CVF with parameters related to diastolic dysfunction such as LV end-diastolic pressure (12) or the E:E' ratio (the ratio of transmitral E velocity to early diastolic mitral annular velocity) (17) have been found in HFpEF patients (Figure 3). Moreover, associations between collagen-dependent stiffness and pulmonary capillary wedge pressure or left atrial diameter have been reported (29). However, it has to be considered that LV end-diastolic pressure, LV end-diastolic wall stress and myocardial stiffness modulus, were increased in

HFpEF patients versus controls, even in those patients with low CVF, suggesting that myocardial fibrosis is not the sole contributor to LV diastolic dysfunction (12).

In this regard, in HFpEF patients, passive stiffness ($F_{passive}$) of single isolated, membrane-permeabilized cardiomyocytes was shown to be significantly higher during muscle lengthening than in HFrEF, despite increased CVF in HFrEF versus HFpEF (30). Of course these single cardiomyocyte experiments do not account for ECM-based $F_{passive}$. More recently, functional experiments on small myocardial muscle strips allowed for the differentiation between cardiomyocyte- and ECM-based $F_{passive}$. In a HFpEF patient population undergoing coronary artery bypass surgery, force measurements were performed on epicardial biopsy samples obtained during surgery (29). In these patients, ECM-dependent stress was associated with elevated filling pressures and left atrial dilatation. At higher muscle and sarcomere lengths (SL), increases in ECM-based $F_{passive}$ account for more than two-thirds of total $F_{passive}$ in HFpEF (29), which suggests that collagen serves as a back-up mechanisms to prevent supraphysiological stretch (31). At lower SL, the titin-dependent $F_{passive}$ was also shown to be increased and to correlate with left atrial diameter (29). The relative contributions of titin and collagen to $F_{passive}$ are therefore SL dependent, but the actual SL operating range in HFpEF patients is unknown. The giant protein titin forms a unique filament network in cardiomyocytes, which engages in both mechanical and signalling functions of the heart and will be discussed in more detail later (32).

Of interest, the functional relevance of myocardial collagen on diastolic dysfunction may depend on the stage of the disease as illustrated in a ZSF1 (Zucker diabetic fatty/Spontaneously hypertensive heart failure F1 hybrid) rat model in which HFpEF is induced by obesity and DM on top of AHT (33). 20 week-old HFpEF rats had increased myocardial $F_{passive}$ compared to hypertensive controls (without a HFpEF phenotype). However, CVF was not increased in these animals compared to the control group and the increase in $F_{passive}$ was attributed to a stiffer cardiomyocyte compartment (33).

Finally, as previously mentioned, not only collagen quantity but also some qualitative aspects like the degree of collagen cross-linking (16) or the collagen type I: type III ratio, with collagen type I being stiffer than collagen type III (34), may influence collagen solubility and myocardial stiffness. In this regard, in hypertensive HF patients, collagen cross-linking but not CVF was associated with elevated filling pressures (Figure 3) (35). Moreover, an increase in insoluble collagen accounts for the increase in total collagen and ECM-based $F_{passive}$ in HFpEF

patients (29). On the other hand, whereas collagen type I expression was found to be increased in the myocardium of HFpEF patients and associated with the E:E' ratio, no significant changes were found in collagen type III expression (17).

CARDIOMYOCYTES IN HFPEF

Next to the observed ECM changes, cardiomyocytes also undergo changes in HFpEF. When compared to HFrEF, cardiomyocytes in HFpEF patients are larger and stiffer with higher $F_{passive}$ upon stretch (30). In cardiomyocytes, the giant protein titin operates as a bidirectional spring and gives stability to the other myofilaments (36). Titin determines the sarcomeric viscoelasticity, whereas actin and myosin mainly contribute to force generation (37). Titin is able to modulate cardiomyocyte based $F_{passive}$ by means of isoform switching, phosphorylation and oxidative modifications (32). In the adult human heart, titin exists as two isoforms: a longer and more compliant N2BA isoform and a shorter and stiffer N2B isoform. The N2BA:N2B ratio changes during the course of different heart diseases, but in general the ratio increases in eccentric remodeling and decreases in concentric remodeling (38). However, these changes probably take place gradually during the course of days to weeks and evolve during disease states, whereas phosphorylation and oxidative modifications occur much faster (32).

Titin can be divided into certain regions, and especially the I-band is known to contain two spring elements: the N2B-unique sequence (N2-Bus) and a region rich in proline, glutamate, valine, and lysine (PEVK) (32). Many serine and threonine residues of titin are already identified as phosphorylation sites for different protein kinases (PK), such as PKA (39), PKC (40), PKG (41), extracellular signal-regulated kinase-2 (ERK2) (42) and Ca2+/ calmodulin-dependent protein kinase-II (CaMKII) (43,44). Phosphorylation of specific titin-sites can alter its distensibility and hence stiffness. For example, in the first studies in human HFpEF biopsy samples, it was observed that *in vitro* administration of PKA decreased $F_{passive}$ in isolated cardiomyocytes, suggesting a titin phophorylation deficit in HFpEF (12). Indeed, relative hypophosphorylation of the stiff, N2B titin-isoform was confirmed in later human experiments, which could be corrected upon *in vitro* administration of PKA or PKG (45), but also in several small (ZSF1-obese rats) and large (old hypertensive dogs) HFpEF animal models (33,46). On the other hand, phosphorylation of the PEVK region by PKC increased $F_{passive}$ *in vitro* (40), but

in the ZSF1-obese rats these specific PEVK sites were not hyper-phosphorylated compared to controls (33). It was recently suggested that hypo-phosphorylation of the N2-Bus and hyper-phosphorylation of the PEVK domain can act complementary to elevate passive tension in failing human hearts (47). The clinical relevance of these finding for HFpEF needs to be studied in more detail.

For HFpEF, especially the relative hypophosphorylation of PKG-dependent titin sites is an interesting finding that offers potential therapeutic targets (Figure 4). Not only was titin relatively hypophosphorylated, also PKG activities were shown to be decreased in human HFpEF myocardium, in combination with decreased cyclic guanosine 3',5'-monophosphate (cGMP) concentration, which activates PKG (48). In the HFpEF paradigm proposed by Paulus and Tschöpe, decreased cGMP concentration and PKG activity are the final steps in a complex pathway, ultimately leading to increased myocardial $F_{passive}$ and cardiomyocyte hypertrophy (3). On top of this pathophysiological cascade are metabolic comorbidities such as obesity, DM and AHT that induce a chronic, inflammatory state, also affecting the coronary microvascular endothelium and leading to oxidative stress (3). Inflammation and oxidative stress also reduce nitic oxide (NO) bioavailability with subsequently less stimulation of soluble guanylate cyclase (sGC), which catalyzes the conversion of guanosine 5'-triphosphate (GTP) to cGMP (49). Also, direct oxidation of sGC leads to a dysfunctional, heme-free isoform which is unresponsive to NO (50). The finding that LV dysfunction and increased myocardial stiffness in diabetic mice are attenuated by the inhibition of dipeptidyl peptidase 4 also supports this paradigm, since these effects are potentially mediated by the stimulation of the cGMP-PKG pathway and the phosphorylation status of titin (51). This cascade is an important potential target for future HFpEF therapeutic strategies, which will be discussed in detail in another review in this series.

Besides indirect effects via decreased NO-sGC-cGMP-PKG signaling, oxidative stress can also have direct effects on titin-based stiffness (Figure 4). The N2Bus, containing 6 cysteines, has a potential to undergo disulfide bonding under conditions of oxidative stress. Indeed, in atomic force experiments it was demonstrated that in the absence of reducing agents, up to three titin-stabilizing disulfide bonds could be formed in N2Bus, leading to a shorter titin length and a secondary increase in $F_{passive}$ (52).

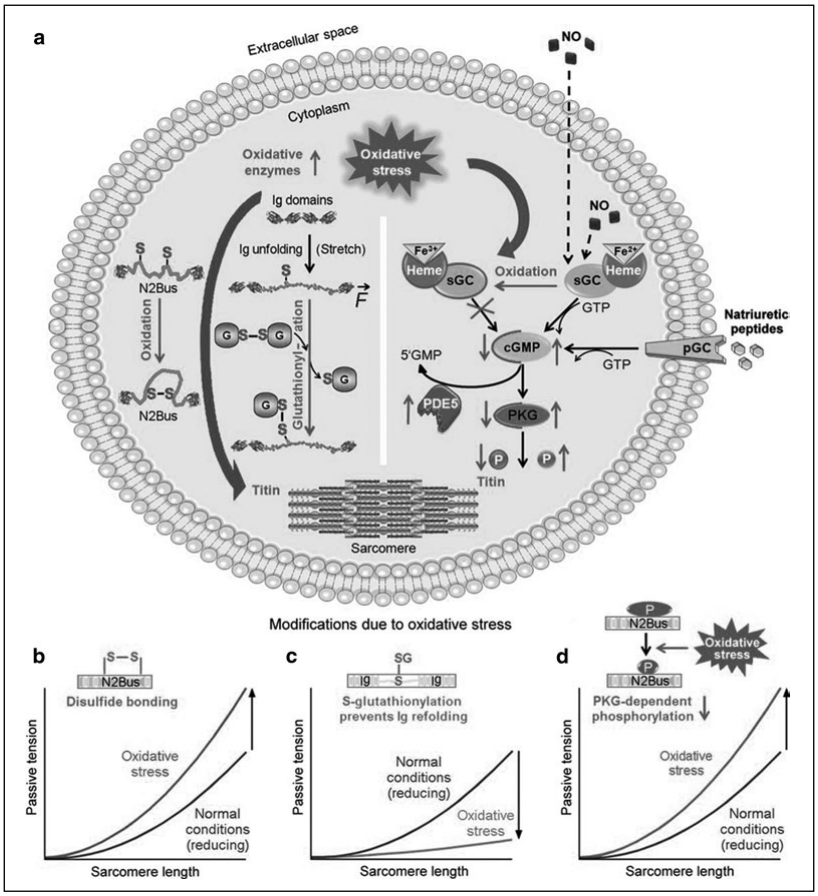

Figure 4. The effects of oxidative stress on titin and cardiomyocyte-based stiffness. **a** Oxidative stress induces post-translational modi - cations of titin, such as oxidation of cysteines in N2B-unique sequence of titin (N2-Bus) caus- ing disulphide bonding (*far left*), S-glutathionylation of cysteines in unfolded Ig domains inhibiting domain refolding (*left-middle*), and reduced cGMP-dependent protein kinase-G (PKG)–dependent N2-Bus phosphorylation, because of oxidation of the haeme moiety in soluble guanylyl cyclase (sGC) and the ensuing blockade of cGMP production (*right*). Graphs in B to D show oxidative stress–related effects on titin-based passive tension caused by S–S bonding within N2-Bus (**b**), S-glutathionylation of unfolded titin-Ig domains (**c**), or depressed cGMP-PKG pathway activation (**d**). *5'GMP* guanosine- 5'-monophosphate, *cGMP* cyclic guanosine monophosphate, *G* glutathione, *GSSG*, glutathione- disulphide, NO, nitric oxide, P, titin phosphorylation, PDE5, phosphodiesterase-5, pGC, par- ticulate guanylyl cyclase, PKG, cGMP-dependent protein kinase- G, and sGC, soluble guanylyl cyclase. (Used with permission from [62])

Another part of the I-band contains segments that are rich in immunoglobulin-like (Ig) domains, which make up the majority of elastic titin. Usually parts of these Ig-domains are folded into crypts and they can become unfolded and expose cryptic cysteines to disulfide bonding or S-glutathionylation during stretch (32). A recent study demonstrated that stretching with subsequent S-glutathionylation led to persistent unfolded states, which decreases the mechanical stability of the parent Ig domain as well as its ability to fold and as final result a more extensible state of titin (53). Especially when stretched cardiomyocytes were incubated with oxidized gluthathione, $F_{passive}$ decreased, whereas incubation with reduced glutathione increased $F_{passive}$, suggesting that also the redox state plays a modifying role in titin based stiffness that needs to be studied in more detail (Figure 4).

CARDIOMYOCYTE AND ECM CROSS-TALK

Cardiomyocytes and myocardial ECM are not two completely independent compartments and a close interaction can be expected. Both have been shown to contribute to myocardial stiffness (29) and to be associated with diastolic dysfunction (12,17,29). Interestingly the combination of CVF- and cardiomyocyte-dependent stiffness improved the association of both individual parameters with diastolic dysfunction (12). Myocardial cells and the ECM can interact at multiple levels. For instance, it has been recently shown that necrotic cardiomyocytes release damage associated molecular patterns (DAMPS) which induce fibroblast activation *in vitro* and myocardial inflammation and fibrosis *in vivo* (54). Since cardiomyocyte necrosis is more specific to HFrEF (3), future studies are needed to address a potential role for DAMPS in HFpEF. As hypothesized, the pathophysiology of HFpEF starts in the coronary microvasculature, where inflammation and oxidative stress trigger a cascade that affects both the ECM and cardiomyocytes (3). The importance of the endothelium in HFpEF pathophysiology is also stressed by the finding of coronary microvasculary rarefaction in HFpEF (13).

This interplay between endothelium, ECM and cardiomyocytes has several implications. Firstly, therapeutic options that target either the ECM (e.g. spinoronolactone) or the cardiomyocytes (e.g. sildenafil), cannot be expected to "cure" HFpEF. Secondly, changes in cardiomyocyte function due to inflammation or oxidative stress may trigger ECM-changes and, vice versa, ECM-changes caused by

chemical or oxidative endothelial-ECM signaling or by mechanical stress can be expected to disturb normal cardiomyocyte functioning (55). Thirdly, the relative importance of the ECM and the cardiomyocytes to HFpEF pathophysiology is expected to vary during the course of the disease. One could hypothesize that in the early phase of the disease, cardiomyocytes are more determinant of HFpEF pathophysiology and that ECM changes are more relevant in later stages. Indeed, oxidative stress has many very rapid effects on cardiomyocyte function due to the nature of reactive oxygen species (56). In contrast, the transition of fibroblasts into myofibroblasts that secrete collagen, the formation of more insoluble and stiffer collagen fibers may take longer before it has a significant effect. For instance ZSF1-obese rats developed a HFpEF phenotype with diastolic dysfunction and elevated filling pressures and an increased myocardial $F_{passive}$ without any change in ECM turnover at 20 weeks of age (33). On the other hand, in more advanced stages of HFpEF, the ECM seems to be more prominent and capillary rarefaction can be observed (13,29).

This hypothesis has important therapeutic implications. If cardiomyoctes predominate the initial phases of HFpEF, it's probably useful to improve cGMP-PKG signalling to reduce cardiomyocyte $F_{passive}$ (57). However, at more advanced stages of HFpEF, correcting cGMP-PKG signalling might have less effects and drugs targeting myocardial fibrosis (e.g. mineralocorticoid receptor antagonists) or more specific processes like collagen cross-linking (e.g. anti-lysyl oxidase) could prove more effective. This is illustrated by the results from the RELAX trial (58). In the RELAX trial, chronic treatment with sildenafil was used with the rationale that this would inhibit cGMP breakdown and increase its concentrations, leading to higher levels of PKG. However, sildenafil appeared to have no beneficial effects in this advanced HFpEF population. Therefore, future trials should test different therapeutic strategies based on different phases of HFpEF. Possibly, circulating ECM biomarkers and/or imaging techniques such as T1 mapping with CMR will help identifying which patient might benefit most from each therapy.

CONCLUSIONS

HFpEF is a complex disease of which the pathophysiology is gradually becoming unraveled. Both structural and functional alterations in cardiomyocytes and the ECM have been reported, leading to increased myocardial $F_{passive}$. However,

further mechanistic studies are necessary to elucidate the relative contribution and interplay between both mechanisms, which may depend on the etiology and comorbidities as well as on the stage of the disease.

On the other hand, it is essential to develop non-invasive biomarkers for the early identification of the alterations in these 2 components. For instance, non-invasive techniques such as CMR with T1 mapping and circulating ECM biomarkers are promising, although future studies are needed to assess their potential to truly diagnose, stage or predict outcome and response to therapeutic strategies in HFpEF.

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EDITOR'S COMMENT

Heart failure with preserved ejection fraction: current status and challenges for the future

J. van der Velden[1,2] · E.E. van der Wall[3] · W.J. Paulus[1]

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Over the past two decades it has become evident that more than 50% of all heart failure patients suffer of heart failure with preserved left ventricular (LV) ejection fraction (HFpEF), previously called diastolic heart failure because of important involvement of diastolic LV dysfunction. The advances and current status of HFpEF research are described in this special focus issue of the Netherlands Heart Journal. HFpEF is the most common heart failure phenotype in ageing societies, highly prevalent in elderly women and frequently accompanied by comorbidities which result from a detrimental lifestyle, such as obesity, metabolic syndrome, type 2 diabetes mellitus and salt-sensitive arterial hypertension [1]. Despite modern heart failure therapy, the prognosis of HFpEF has not improved over the last decades. By 2020, the prevalence of HFpEF is projected to exceed 8% of persons older than 65 years of age and because of the current pandemic of obesity, the prevalence of HFpEF in persons younger than 65 years of age is expected to rise exponentially.

A targeted strategy is needed to prevent the progression from obesity and type 2 diabetes towards diastolic LV dysfunction and HFpEF. Available heart failure therapies might exert beneficial effects at an early stage of HFpEF, which is, however, poorly recognised with many patients initially presenting with an advanced form of heart failure. Recognition of an early stage of diastolic dysfunction and initiation of heart failure therapy should be done in primary care, and novel staged therapeutic strategies should be tested to prevent or retard the progression from diastolic LV dysfunction to end-stage heart failure. To battle this life-threatening chronic disease, specialists from different areas of expertise need to join forces.

Based on recent findings in cardiac tissue samples from HFpEF patients, a novel paradigm on HFpEF pathogenesis was formulated [1]. This paradigm proposes that the different comorbidities initiate chronic systemic inflammation, which via perturbations of the microvasculature of the heart stiffens and damages cardiac muscle cells causing diastolic dysfunction. Impaired relaxation not only results from stiffened cardiac muscle cells, but also involves changes of the extracellular matrix and deposition of collagen [2]. The earliest stage of HFpEF should be detected by general practitioners as previously unknown heart failure was found in 27.7% of patients with type 2 diabetes aged 60 years or over, of which 83% involved HFpEF. Boonman-de Winter and colleagues [3] emphasise that general practitioners, internists and other healthcare professionals may be unaware of the high prevalence of heart failure in older diabetic patients and describe a disease-management program to detect heart failure in older patients with type 2 diabetes [3]. Also in the clinic, diagnosis of HFpEF remains a challenge with the currently used diagnostic tools. Accurate diagnosis of HFpEF and staging of this complex type of heart failure may require invasive stress testing and accurate assessment of right ventricular function by means of cardiac MRI [4]. Moreover, novel blood biomarkers may become available to better identify and stage HFpEF patients [5, 6]. As HFpEF clinical trials have provided neutral outcomes and because of the magnitude of this type of heart failure, basic scientists and clinicians need to join forces to develop novel treat-

✉ J. van der Velden
j.vandervelden@vumc.nl

[1] Department of Physiology, Institute for Cardiovascular Research (ICaR-VU), VU University Medical Center, Amsterdam, The Netherlands

[2] ICIN—Netherlands Heart Institute, Utrecht, The Netherlands

[3] Netherlands Society of Cardiology/Holland Heart House, Utrecht, The Netherlands

ment strategies to prevent and reverse cardiac dysfunction. Stiffening of the heart muscle may be reversed by targeting protein kinase G and phosphorylation of the giant protein titin [7], which is an important regulator of muscle cell stiffness and relaxation. Newly developed drug therapies should be tested in models which resemble the complex disease phenotype observed in humans [8]. During past years insights have been obtained into the complex pathogenesis of HFpEF, which are summarised in this special issue. The challenge for the future lies in raised awareness of this devastating disease, improved patient stratification and development of stage-specific therapies.

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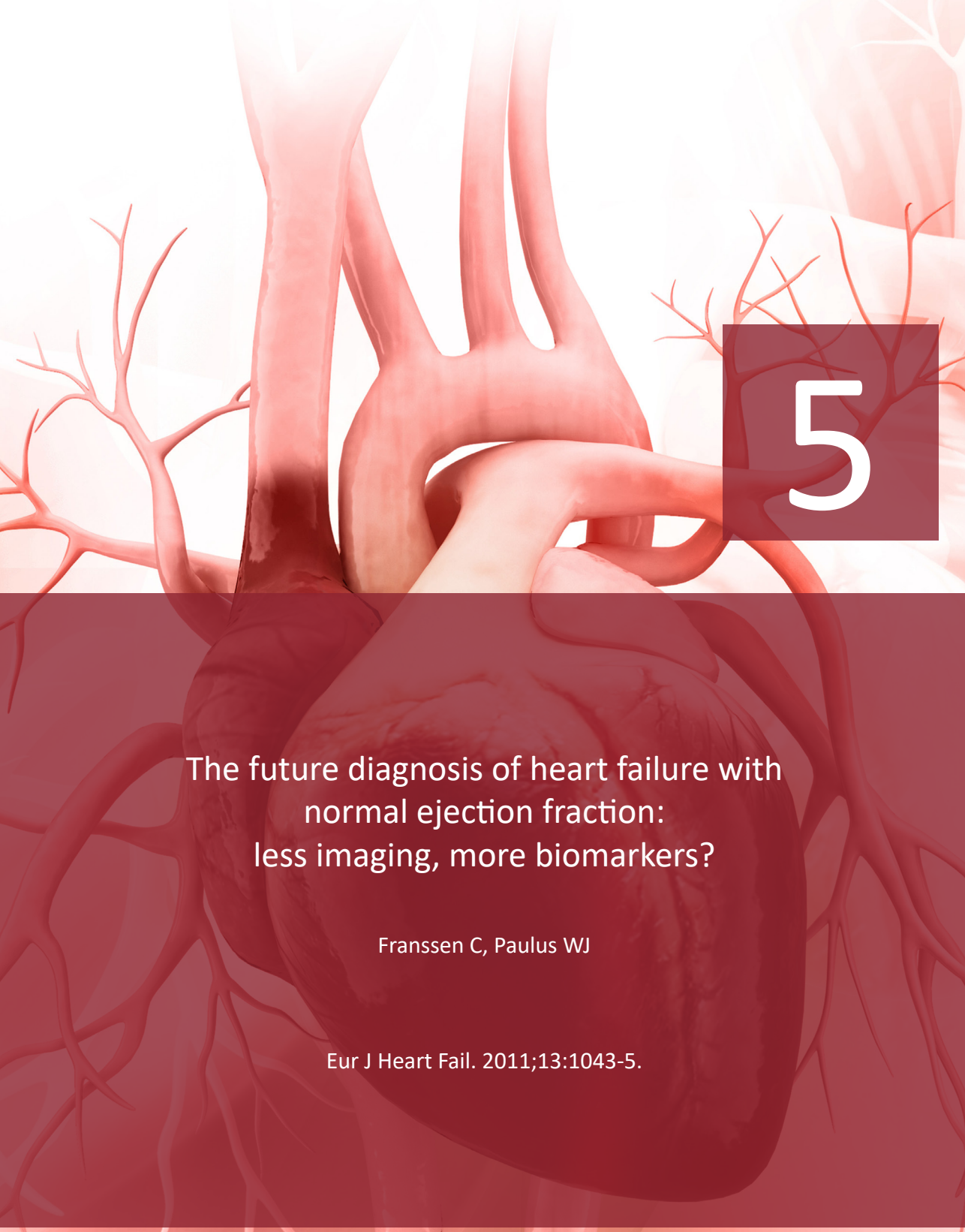

5

The future diagnosis of heart failure with normal ejection fraction: less imaging, more biomarkers?

Franssen C, Paulus WJ

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The future diagnosis of heart failure with normal ejection fraction: less imaging, more biomarkers?

by

Constantijn Franssen, Walter J. Paulus.

From

Department of Physiology, Institute for Cardiovascular Research,
VU University Medical Center, Amsterdam, The Netherlands.

INTRODUCTION

In contrast to heart failure with reduced ejection fraction (HFREF), which is easily diagnosed by clinical signs or symptoms of fluid over- load in the presence of a left ventricular ejection fraction (LVEF) <35%, the diagnosis of heart failure with normal ejection fraction (HFNEF) is cumbersome as it requires three conditions to be simultaneously satisfied.[1] These three conditions consist of (i) signs or symptoms of fluid overload; (ii) an LVEF >50% and an LV end- diastolic volume index (LVEDVI) <97 mL/m^2 ; and (iii) evidence of diastolic LV dysfunction derived either from cardiac catheterization or tissue Doppler imaging (TDI). The latter can eventually be implemented with measurements of mitral flow velocity, left atrial volume index (LAVI) or plasma natriuretic peptide levels. Due to their poor positive predictive value, natriuretic peptides are considered unable to provide stand-alone evidence for HFNEF and always need to be implemented with TDI evidence of diastolic LV dysfunction. Because of mounting evidence of TDI-derived indices failing to adequately reflect LV filling pressures[2] and because of emerging reports of several biomarkers being raised in HFNEF,[3–5] the future paradigm for the diagnosis of HFNEF will probably be diverted away from elaborate imaging techniques and oriented towards the integrated use of biomarkers. Such an integrated use of biomarkers for assessing HFNEF and risk of HFNEF development is exemplified in the current issue of the European Journal of Heart Failure by the article of Collier et al.[6]

LESS IMAGING

E/E' (ratio of early transmitral velocity to TDI mitral annular early diastolic velocity) suffers from shortcomings when used as an estimate of LV filling pressures in HFREF or in HFNEF.[2] The shortcomings mainly derive from questionable assumptions such as E' solely depending on LV relaxation pressure and E' not being affected by early diastolic load.[7] Because of the conceptual weakness of E/E', stand-alone diagnostic evidence for diastolic LV dysfunction is provided only by an elevated E/E' value (E/E'>15).[1] The vast majority of HFNEF patients present with an intermediate non-diagnostic E/E' value (8<E/E'>15) and thus require additional evidence of diastolic LV dysfunction such as abnormal mitral or pulmonary vein flow velocities, a high LAVI or elevated plasma natriuretic peptides.[1] These

drawbacks of TDI were nicely illustrated in a recent study, which evaluated several algorithms for the diagnosis of HFNEF.[8] In this study, E/E' >15 had limited sensitivity for HFNEF (35%) and a serial evaluation of E/E', mitral or pulmonary vein flow velocities and LAVI was needed to raise the diagnostic sensitivity of the echocardiographic work-up to 77%.

Apart from LAVI, which reflects chronic diastolic LV function, all imaging-derived indices provide an estimate of instantaneous LV end-diastolic distensibility relating LVEDVI to an estimate of LV end-diastolic pressure. These indices are sensitive to the volume status of the patient and will always carry the risk of a false-negative diagnosis following diuresis and a false-positive diagnosis following fluid overload. Only an LV diastolic pressure-volume relation fitted to two or more LV end-diastolic pressure-volume points, measures intrinsic diastolic myocardial stiffness and overcomes the confounding effects of volume status. This, however, requires an intervention such as transient balloon caval occlusion or exercise. Although preliminary studies on exercise stress testing provided encouraging results for the diagnosis of HFNEF, its applicability is limited as most HFNEF patients have poor exercise capacity due to high age and debilitating comorbidities. In contrast to imaging-derived indices of LV end- diastolic distensibility, fibrosis-related biomarkers reflect a chronic cardiac process occurring in HFNEF. This process consists of myocardial fibrosis as a result of concentric LV remodelling and is not affected by the patient's instantaneous volume status.

MORE BIOMARKERS

Only natriuretic peptides have so far been extensively evaluated for the diagnosis of HFNEF. Patients with HFNEF have lower plasma natriuretic peptide levels than HFREF patients[9] and the levels are especially low in HFNEF patients presenting in an outpatient clinic with complaints of limited exercise tolerance.[10] Natriuretic peptides were therefore judged to be of limited clinicalvalue for the diagnosis of HFNEF and their use recommended only for exclusion of HFNEF.[1] This impression was further confirmed in large HFNEF registries such as the DIAST-CHF (Diastolic Congestive Heart Failure) study[5] which observed a sensitivity of 65% for the diagnosis of HFNEF when using the recommended N-terminal- pro brain natriuretic peptide cut-off value of 220 pg/mL.[1] Even lower sensitivities (27 and

38%) were recently reported in a critical evaluation of diagnostic HFNEF algorithms.[8]

In contrast to natriuretic peptides, fibro-inflammatory biomarkers raise high expectations for HFNEF risk assessment and for HFNEF diagnosis as illustrated in this issue of the Journal by
the article of Collier *et al*.[6] This study reported HFNEF to be associated with increased circulating biomarkers of inflammation [interleukin 6 (IL6), interleukin 8 (IL8), and monocyte chemoattractant
protein 1 (MCP1)], of collagen metabolism [aminoterminal propeptide of collagen 3 (PIIINP), carboxy-terminal telopeptide of collagen 1 (CITP)], and of extracellular matrix turnover [matrix metalloproteinase 2 (MMP2) and matrix metalloproteinase 9 (MMP9)]. Apart from the inflammatory biomarkers, the present study is an extension of previous reports by the same authors[3] and by other investigators[4,5] evaluating the diagnostic use of fibrosis-related biomarkers in HFNEF. In a previous study, a cut-off value of 1585 ng/mL of serum MMP2 was shown to provide 91% sensitivity and 76% specificity for predicting HFNEF.[3] In the present study, these results were further refined as MMP9, tissue inhibitor of matrix metalloproteinase 1 (TIMP1) and the ratio of MMP9/ TIMP1 correctly identified patients with a high LAVI. The correct identification of an enlarged left atrium is an important finding because LAVI measures chronic diastolic LV function and its correct identification therefore supports the idea that fibrosis-related biomarkers reflect chronic concentric LV remodelling and are therefore unaffected by the patient's instantaneous volume status. The findings on TIMP1 are, however, at odds with a previous study which observed higher serum TIMP1 levels in HFNEF patients with elevated LV filling pressures[4] whereas the current study observed lower serum TIMP1 levels in patients with high LAVI. Encouraging results have also been reported with another fibrosis-related biomarker namely growth differentiation factor 15 (GDF-15), which is a distant member of the transforming growth factor β superfamily.[5] In this study, GDF-15 also related closely to LAVI again indicative of the biomarker reflecting chronic LV remodelling.

The most important finding of the present study probably relates to the significant elevation of inflammatory biomarkers in HFNEF. Inflammatory biomarkers IL6, IL8, and MCP1 were not only raised when HFNEF patients were compared with arterial hypertension patients but also elevated when arterial hypertension patients were compared with age-adjusted reference values. The latter was not observed for the fibrosis biomarkers, as PIIINP and CITP were raised

in HFNEF compared with arterial hypertension but similar to age-adjusted reference values in arterial hypertension. These findings support systemic inflammation to precede myocardial remodelling in HFNEF. A similar conclusion was also reached by the Health ABC study, which reported inflammatory biomarkers such as IL6, tumor necrosis factor α, and C-reactive protein to be strongly associated with risk of HFNEF development in older adults.[11] Myocardial inflammation was recently also observed in endomyocardial biopsies of HFNEF patients.[12] These biopsies contained inflammatory cells, whose number appeared to relate to collagen volume fraction and diastolic LV function. Because of production of transforming growth factor β, the inflammatory cells induced transdifferentiation of fibroblasts to myofibroblasts with high production of collagen and low expression of MMP-1. Although this study suggested the raised inflammatory biomarkers in HFNEF to result from myocardial inflammation, the reverse situation seems to be more likely with systemic inflammation instigating myocardial inflammation. Potential sources of systemic inflammation in HFNEF are obesity and diabetes mellitus. Obesity and diabetes are highly prevalent in HFNEF[13,14] and are able to induce expression of E-selectin,[15] a leukocyte-attracting protein, in the intramyocardial endothelial cells.

CONCLUSIONS

The diagnosis of HFNEF remains challenging especially when the patient presents in an outpatient setting without obvious signs of volume overload. Future diagnostic algorithms for HFNEF are likely to rely less on imaging and more on biomarkers. Most imaging indices, even those obtained with sophisticated tissue Doppler or speckle tracking imaging, provide an assessment of instantaneous diastolic LV distensibility and are therefore sensitive to the patient's volume status. As nicely demonstrated by the article of Collier et al.[6] in this issue of the Journal and by some other recent reports,[3–5] fibroinflammatory biomarkers have the advantage of reflecting the chronic myocardial remodelling process of HFNEF and are therefore unaffected by the patient's instantaneous volume status. Furthermore, fibroinflammatory bio- markers also provide a comprehensive picture of the initiating systemic and evolving myocardial processes occurring during HFNEF development.

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CONFLICT OF INTEREST

None declared.

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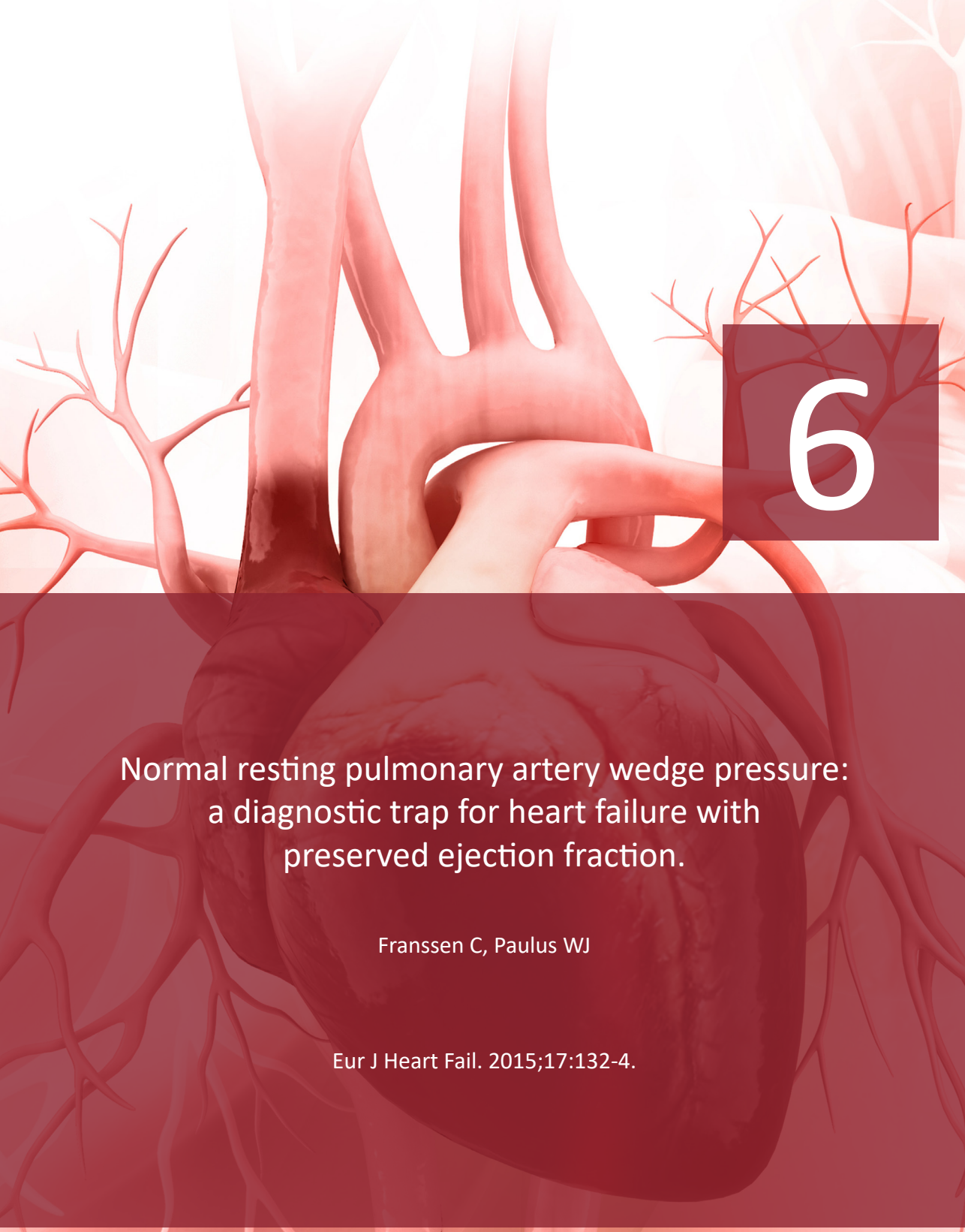

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Normal resting pulmonary artery wedge pressure: a diagnostic trap for heart failure with preserved ejection fraction.

Franssen C, Paulus WJ

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Normal resting pulmonary artery wedge pressure:
a diagnostic trap for heart failure with preserved ejection fraction.

by

Constantijn Franssen, Walter J. Paulus.

From

Department of Physiology, Institute for Cardiovascular Research,
VU University Medical Center, Amsterdam, The Netherlands.

INTRODUCTION

The diagnosis of heart failure with preserved ejection fraction (HFPEF) remains challenging. A correct diagnosis requires the presence of signs or symptoms of congestion, normal LV systolic function, and evidence of diastolic LV dysfunction.[1] Failure to establish the diagnosis of HFPEF correctly can be related to omission of evidence of diastolic LV dysfunction,[2] to exclusive reliance on elevated natriuretic peptides,[3] which are only modestly raised in HFPEF,[4] and to the fortuitous presence of a hypovolaemic status at the time of diagnostic evaluation,[5] which necessitates a repeat assessment during exercise[6] or saline infusion.[7] The latter was convincingly demonstrated in the current issue of the journal by the study of Maor et al., who performed a limited upper body exercise stress test mimicking daily living activities during right heart catheterization in patients with pulmonary hypertension (PHT) [mean pulmonary artery pressure (mPAP) >25 mmHg] and normal resting pulmonary artery wedge pressure (PAWP <15 mmHg).[8] Despite attaining a rise in heart rate of 10%, which was only 62% of the age-predicted maximal heart rate, one-third of the patients had a substantial rise of PAWP from 11.4±3.3 to 28.0±6.5 mmHg. Without exercise stress testing, their HFPEF-induced post-capillary (group 2) PHT would have remained unnoticed and these patients would have been erroneously classified as pre-capillary (group 1) PHT in accordance with the Dana Point PHT criteria.[9] Significant predictors of an exercise-induced rise in PAWP were a borderline resting PAWP (12<PAWP<15 mmHg), a high body mass index (BMI), presence of obesity, and a dilated left atrium.

THE PAWP 'GREY ZONE'

The use of the E/e' (the ratio of early transmitral diastolic flow velocity to tissue Doppler early mitral annular diastolic velocity) as Doppler echocardiographic evidence of diastolic LV dysfunction in HFPEF has been discredited by the presence of a wide 'grey zone' ranging from 8<E/e' <15. Only when E/e' exceeds 15 does it provide stand-alone diagnostic evidence of diastolic LV dysfunction.[1]
When 8<E/e' <15, secondary evidence of diastolic LV dysfunction is required, which can consist of transmitral diastolic flow velocities, combined transmitral and pulmonary flow velocities, left atrial size, LV hypertrophy, AF, or raised natriuretic peptides.[1] The presence of this wide 'grey zone' is considered to be a major

methodological shortcoming of Doppler echocardiographic imaging for the diagnosis of diastolic LV dysfunction. The study by Maor *et al*. in this issue sheds further light on this 'grey zone' as it suggests a similar 'grey zone' for a normal resting PAWP, which ranges from 12 to 15 mmHg.[8] In their study, 62% of patients, who had a substantial rise in exercise PAWP, had a resting PAWP in the range of 12–15 mmHg, and a resting PAWP within this range made it 4.5 times more likely to be in the highest tertile of exercise PAWP. The upper cut-off value of this range corresponds to the upper limit of normal resting PAWP proposed by the Dana Point PHT consensus classification,[9] whereas the lower cut-off value corresponds to the upper limit of normal resting PAWP proposed by the European HFPEF consensus document.[1] Hence, the study by Maor *et al*. and side by side comparison of the upper limits of normal resting PAWP proposed in both consensus documents suggest a 'grey zone' of normal resting PAWP ranging from 12 to 15mmHg similar to the 8<E/e' <15 'grey zone' of the Doppler evaluation of diastolic LV dysfunction. As patients with a resting PAWP in the range of 12 15mmHg are 4.5 times more likely to have HFPEF-induced post-capillary (group 2) PHT, a PAWP equalling 12mmHg is to be preferred as the upper cut-off value of a normal resting PAWP.[1]

Both the 8<E/e'<15 and 12<PAWP<15mmHg 'grey zones' are reflections of the physiological variability of volume status and not methodological shortcomings. The response of PAWP to shifts in volume status was recently compared between control subjects and HFPEF patients.7 Plots of PAWP (mmHg) vs. indexed volume of a rapid saline infusion (L/m^2) were constructed, and the slope of the relationship was twice as steep in HFPEF patients (25±12 mmHg/L/ m^2) than in both young and old control groups (12±3mmHg/L/m^2; 14± mmHg/L/ m^2). Based on these relationships, HFPEF patients appear to be exquisitely sensitive to volume status, with a small 0.6 L volume load already eliciting a 10mmHg PAWP rise. It is therefore no surprise that limited salt intake or use of a diuretic will cause many HFPEF patients to present with a low resting PAWP, as obvious from the study of Maor *et al*., which reported a resting PAWP of 11.4±3.3mmHg in the PHT patient group reclassified as HFPEF following invasive exercise stress testing. The exquisite sensitivity of PAWP to volume shifts was also evident in this patient group from the impressive 18mmHg mean PAWP rise elicited by limited upper body exercise.

CHARACTERISTICS OF THE MISCLASSIFIED PULMONARY HYPERTENSION PATIENTS

In the study by Maor *et al*., the PHT patients erroneously classified as group 1 PHT based on the Dana Point criteria and reclassified as HFPEF-induced group 2 PHT following exercise stress testing presented with interesting clinical, echocardiographic, and haemodynamic features.[8] Specific clinical features were high BMI (P =0.023) and obesity (P =0.035). A relationship was established between BMI and exercise-induced rise in PAWP, with each 5 kg/m^2 increase in BMI causing a 2.5±1.0mmHg increase in exercise PAWP. The prominent role for obesity fits into a recently proposed paradigm whereby metabolic co-morbidities drive LV remodelling and dysfunction in HFPEF[10] and into earlier observations that metabolic syndrome reinforces pulmonary venous hypertension.[11] The former was explained by deficient myocardial microvascular nitric oxide (NO)/cGMP signalling, [12] and the latter by excessive pulmonary venous endothelin-1-mediated vasoconstrictor tone.[13] Both derive from a deranged Yin–Yang between endothelial NO and endothelin-1 in metabolic disturbances.[14] The clinical characteristics of the misclassified PHT provide the readers of the journal with an important 'take-home' message, namely that overweight/obese patients with PHT and normal PAWP need to undergo invasive exercise testing or a volume infusion challenge before they can be classified as pre-capillary PHT group 1 patients.

The echocardiographic and haemodynamic features of the misclassified PHT patients are equally intriguing. Specific echocardiographic and haemodynamic features of the misclassified patients were higher resting mean PAWP (11.4±3.3 mmHg) (P =0.007), a large left atrial volume index (LAVI) (P =0.029), and a tall E wave (P =0.030). The higher resting mean PAWP is no surprise. In PHT group 1 patients, right ventricular dysfunction reduces filling of the left heart chambers and lowers intrinsically normal left-sided diastolic pressures. In the misclassified PHT patients, a fortuitous volume shift also reduces filling of the left heart chambers but lowers intrinsically elevated left-sided diastolic pressures because of high diastolic LV stiffness

In the misclassified PHT patients, the large LAVI (57±22 mL/m^2) was an important tip off for HFPEF-induced goup 2 PHT. The observed LAVI by far exceeded the previously proposed cut-off values for the diagnosis of HFPEF (40mL/m^2).[1] The large LAVI observed in the misclassified PHT patients provides another important message for the practising clinician, namely that patients with

PHT, normal PAWP, and a dilated left atrium need to undergo invasive exercise stress testing or a volume challenge before they can be classified as pre-capillary group 1 PHT patients. The tall E wave indicative of restrictive LV filling is of interest because it implies that raised end-diastolic stiffness is obligatory for HFPEF development and that slow LV relaxation is not sufficient. This was also evident from a previous study looking at the response to a volume challenge in young and old controls and in HFPEF patients.[7] Despite the slow LV relaxation in the old controls, their rise in PAWP was similar to that of the young controls and inferior to that of the HFPEF patients.

CONCLUSIONS

Patients with HFPEF are exquisitely sensitive to volume shifts, with a small 0.6 L saline infusion already eliciting a 10mmHg PAWP increase. A fortuitous reduction in fluid intake at the time of invasive testing can therefore lead to a normal PAWP and, as illustrated by the study of Maor *et al*.,[8] to the incorrect diagnosis of group 1 PHT instead of HFPEF-induced group 2 PHT. Exercise stress testing or a volume challenge at the time of invasive measurements unmask the erroneously reduced PAWP. These additional tests are definitely indicated for patients presenting with PHT and normal PAWP, who are obese or who have a dilated LA.

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CONFLICT OF INTEREST

None declared.

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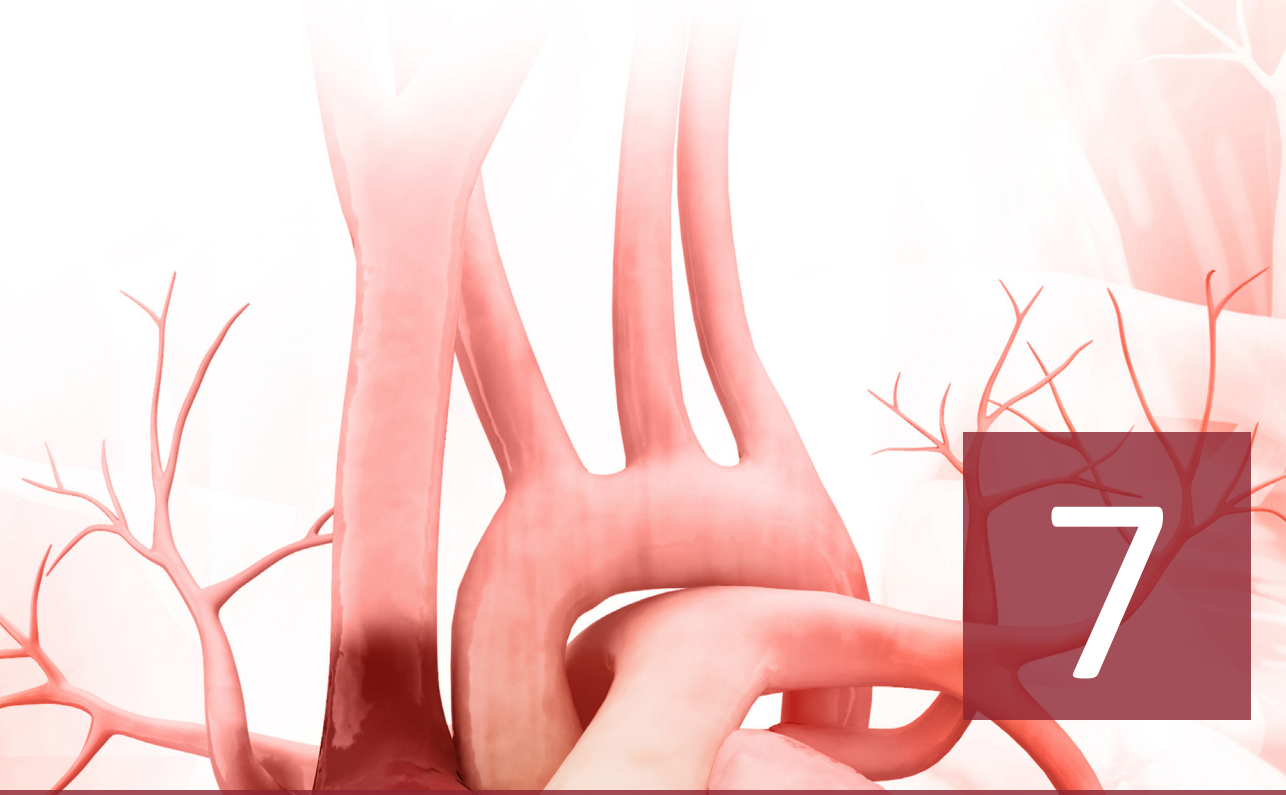

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Sarcomeric accumulation of α-B Crystallin reverses high diastolic stiffness of failing human cardiomyocytes

Franssen C, Kole J, Musters R, Hamdani N, Paulus WJ

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Sarcomeric accumulation of α-B Crystallin reverses high diastolic stiffness of failing human cardiomyocytes

by

Constantijn Franssen, Jeroen Kole, René Musters, Nazha Hamdani, Walter J. Paulus.

From

Department of Physiology, Institute for Cardiovascular Research,
VU University Medical Center, Amsterdam, The Netherlands.

ABSTRACT

Background: Cardiomyocytes (CM) with a less distensible titin and interstitial collagen (CG) contribute to the high diastolic stiffness of failing myocardium. Their relative contributions and mechanisms underlying loss of titin distensibility were assessed in failing human hearts.

Methods and results: Left ventricular tissue was procured in patients with aortic stenosis (AS, N=9) and dilated cardiomyopathy (DCM, N=6). Explanted donor hearts (N=8) served as controls.

Stretches were performed in myocardial strips and an extraction protocol differentiated between passive tension ($F_{passive}$) attributable to CM or to CG. $F_{passive}$-CM was higher in AS and DCM at shorter muscle lengths whereas $F_{passive}$-CG was higher in AS at longer muscle lengths and in DCM at shorter and longer muscle lengths.

CM were stretched to investigate titin distensibility. CM were incubated with alkaline phosphatase (AP), subsequently reassessed after a period of prestretch and finally treated with the heat shock protein α-B crystallin. AP shifted the $F_{passive}$-sarcomere length (SL) relation upward only in donor. Prestretch shifted the $F_{passive}$-SL relation further upward in donor and upward in AS and DCM. Alfa-B crystallin shifted the $F_{passive}$-SL relation downward to baseline in donor and to lower than baseline in AS and DCM. In failing myocardium confocal lasermicroscopy revealed α-B crystallin in subsarcolemmal aggresomes.

Conclusion: High CM stiffness contributed to stiffness of failing human myocardium because of reduced titin distensibility. The latter resulted from an absent stiffness lowering effect of baseline phosphorylation and from titin aggregation. High CM stiffness was corrected by α-β crystallin probably through relief of titin aggregation.

KEY WORDS

Heat shock proteins; Myocardial stiffness; Myocardial fibrosis

INTRODUCTION

High diastolic stiffness of failing human myocardium results from raised diastolic stiffness of cardiomyocytes and from excessive deposition of collagen[1]. Raised diastolic stiffness of cardiomyocytes has been reported in several heart failure phenotypes such as heart failure with preserved ejection (HFPEF)[2], heart failure with reduced ejection fraction (HFREF)[3] or aortic valve stenosis (AS)[4]. Several issues concerning the raised diastolic stiffness of failing human cardiomyocytes remain incompletely understood and are addressed in the present study. These issues are: 1) Does raised diastolic stiffness of failing human cardiomyocytes contribute to the overall myocardial stiffness? 2) Which modifications of titin, the giant cytoskeletal protein responsible for cardiomyocyte stiffness, account for the high intrinsic stiffness of failing human cardiomyocytes and 3) how can they be corrected?

Because of parallel alignment of cardiomyocytes and extracellular matrix, overall myocardial stiffness tracks stiffness of the most severely affected compartment. Raised cardiomyocyte stiffness will therefore contribute less to myocardial stiffness in the presence of severe myocardial fibrosis. Using a myofilamentary extraction technique and isolated left ventricular (LV) myocardial muscle strips[5], the relative contributions of cardiomyocyte and extracellular matrix stiffness have been assessed in mice following transverse aortic constriction[6] (TAC), in mice with a knock-out of immunoglobulin (Ig)-like domains of titin (Ig-KO)[7], in ZSF1 rats with HFPEF[8], in dilated cardiomyopathy (DCM)[9] patients and in HFPEF patients with coronary artery disease[10] (CAD). In TAC mice, in DCM patients with low N2BA/N2B titin isoform ratio and in HFPEF patients with CAD, both titin and collagen contributed to overall myocardial stiffness with equalization of their respective contributions at the outer limit of physiological sarcomere lengths whereas in Ig-KO mice and ZSF1-HFPEF rats titin remained the main determinant of overall myocardial stiffness even at the outer limit of physiological sarcomere lengths. The present study extends these observations to clinical heart failure by examining LV myocardial muscle strips procured from patients without CAD suffering either from DCM or from AS.

Raised diastolic stiffness of failing human cardiomyocytes was hitherto mainly attributed to posttranslational modifications of titin consisting of lack or excess phosphorylation at specific sites along the titin molecule[11,12]. Recently, altered diastolic stiffness was suggested to also originate from titin being damaged

by oxidative or physical stress[13,14]. S-glutathionylated cryptic cysteines of Ig domains were shown to decrease mechanical stability and refolding ability of titin[14]. Furthermore, prior exposure of cardiomyocytes to stretch and low pH caused a rise of cardiomyocyte stiffness, which was suppressed by small heat shock proteins such as HSP27 and α-B crystallin[15]. The protective role of α-B crystallin on titin distensibility was also evident from earlier studies in which α-B crystallin lowered the persistence length of the N2B-Us segment and reduced the unfolding probability of the Ig domains flanking the N2B-Us segment[16]. The relative importance of deranged phosphorylation and structural damage of titin for diastolic stiffness of failing human cardiomyocytes is currently unclear and therefore is also addressed in the present study.

METHODS

Human samples

AS patients (N=9) had symptomatic, severe AS without concomitant CAD. Biopsy specimens from this group were procured from endomyocardial tissue resected from the septum (Morrow procedure) during aortic valve replacement. DCM specimens were procured from LV biopsies (N=3) or from explanted hearts from end-stage heart failure patients (N=3). DCM patients had no significant coronary stenosis and their biopsies showed no inflammation or infiltration. Control samples were obtained from explanted donor hearts (N=8). The local ethics committee approved the study protocol. Written informed consent was obtained from all patients. Clinical characteristics and hemodynamic data of the different patient groups are shown in Table 1.

Force measurements in small muscle strips

Small muscle strips (150-450µm in diameter and 800-1900µm in length) were dissected from myocardial samples (n=24 for AS; n=16 for DCM; n=20 for Donor). After incubation for 1 hour in relaxing solution supplemented with 0.2 % TritonX-100 to remove all membrane structures, strips were attached between a force transducer and length motor in a relaxing solution (in mmol/L: free Mg, 1; KCl, 100; EGTA, 2; Mg-ATP, 4; imidazole, 10; pH 7.0). Strips were gently stretched till slack length, i.e. the minimal length at which passive tension ($F_{passive}$) is being build up. As a test of cell viability, each muscle strip was transferred from relaxing

to maximally activating solution (pCa4.5), at which isometric force developed. After stabilization for 5 minutes in relaxing solution, strips were stretched to 10, 15, 20 and 25% length relative to slack length with return to slack length in between each lengthening step. $F_{passive}$ was measured at each stage of muscle strain.

Table 1: Baseline characteristics and hemodynamics of patients

	Donors	AS	DCM
Age, y	51±4	65.3±1.6	60.0±2.1
Male, %	-	47	70
Hypertension, %	-	58	16
DM, %	-	26	30
BMI, kg/m^2	-	28.1±0.6	27.5±0.8
GFR, mL/ min/1.73 m^2	-	68.9±18.5	63.5±15.7
Atrial fibrillation, %	-	2	33
Medication, %			
ACE-I/ARB	-	43	81
β-blocker	-	61	63
Diuretic	-	54	72
Aldosterone receptor antagonist	-	7	74
Digoxin	-	2	33
Statin	-	46	21
Metformin	-	3	14
Bronchodilators	-	9	19
Hemodynamics			
HR, beats/min	80±16	74±2	82±4
LVPSP, mmHg	135±15	223±4	120±3
LVEDP, mmHg	13±4	22.8±1.4	22.3±1.4
LVEDVI, ml/m^2	78±23	55±2	127±5
LVEF, %	73±3	64.0±1.2	29.4±1.5

AS indicates aortic stenosis; DCM, dilated cardiomyopathy; DM, diabetes mellitus; BMI, body mass index; ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; GFR; glomerular filtration rate; HR, heart rate; LVPSP, left ventricular peak-systolic pressure; LVEDP, left ventricular end-diastolic pressure; LVEDVI, left ventricular end-diastolic volume index; LVEF, left ventricular ejection fraction.

Subsequently, thick and thin filaments were extracted by immersing the preparation in relaxing solution containing 0.6 mol/L KCl (45 minutes at 20°C)

followed by relaxing solution containing 1.0 mol/L KI (45 minutes at 20°C)[5,8,10]. After the extraction procedure, the muscle bundles were stretched again and the $F_{passive}$ remaining after KCl/KI treatment was ascribed to the extracellular matrix (E-matrix). At each stage of muscle strain, $F_{passive}$ following extraction was subtracted from baseline value to yield $F_{passive}$ attributable to cardiomyocyte-stiffness, or titin.

Force measurements in isolated cardiomyocytes

Force measurements were performed on single demembranated cardiomyocytes (n ranged from 9 to 15 for each group and experimental protocol). Cardiomyocytes were isolated from donor, AS, and DCM hearts. Briefly, samples were defrozen in relaxing solution (in mmol/L: free Mg, 1; KCl, 100; EGTA, 2; Mg-ATP, 4; imidazole, 10; pH 7.0), mechanically disrupted and incubated for 5 min in relaxing solution supplemented with 0.5% Triton X-100. The cell suspension was washed 5 times in relaxing solution. Single cardiomyocytes were selected under an inverted microscope and attached with silicone adhesive between a force transducer and a piezoelectric motor. Cardiomyocyte $F_{passive}$ was measured in relaxing buffer at room temperature within a sarcomere length (SL) range between 1.8 and 2.4µm. The stretch protocol consisted of an uninterrupted stepwise increase in SL without return to slack length[14,15]. Force values were normalized to cardiomyocyte cross-sectional area calculated from the diameter of the cells, assuming a cylindrical shape. As a test of cell viability, each cardiomyocyte was also transferred from relaxing to maximally activating solution (pCa4.5), at which isometric force developed. Once a steady state force was reached, the cell was shortened within 1 ms to 80% of its original length to determine baseline force. Only cells developing active forces >20 mN/mm^2 were included in the analysis. Thereafter, cardiomyocytes were incubated in relaxing solution supplemented with alkaline phosphatases (AP) (0.2 U/µL; New England Biolabs), 6 mmol/L dithiothreitol (MP Biochemicals) for 40min at room temperature and $F_{passive}$ was measured at SL 1.8-2.4µm. The stretch protocol again consisted of an uninterrupted stepwise increase in SL without return to slack length[14,15]. Subsequently, cardiomyocytes were stretched to ~2.6µm SL, incubated in relaxing buffer with pH 6.6, and held in the stretched state for 15 min (prestretch). Thereafter, cardiomyocytes were returned to slack length and stabilized for 5 minutes before recording the $F_{passive}$ with an identical stretch protocol from SL 1.8-2.4µm in the low pH buffer. Finally, the pH 6.6 buffer was supplemented with recombinant human α-B crystallin at 0.1 mg/ml and $F_{passive}$ was measured in the presence of α-B crystallin

again at SL 1.8-2.4µm with an identical stretch protocol. In a second set of experiments, single AS cardiomyocytes underwent either incubation at pH 6.6 alone or a prestretch in relaxing buffer with pH 6.6 to ~ SL 2.6µm, followed by the same stretch protocol from SL 1.8-2.4µm. Finally, in a third set of experiments, $F_{passive}$ was measured in single AS cardiomyocytes from SL 1.8-2.4µm before and after incubation with α-B crystallin, before and after incubation with α-B crystallin and a prestretch to ~ SL 2.6µm, and before and after incubation with α-B crystallin, a prestretch to ~2.6µm SL and incubation at pH 6.6.

Immunofluorescence staining and confocal scanning laser microscopy

Frozen human heart tissue was sectioned to a thickness of 5 micrometers using a cryostat (Leica). The sections were fixed with 3% paraformaldehyde, permeabilized with 0.05% Tween 20 and immunostained using goat anti-alpha B-crystallin (Santa Cruz) diluted 1 in 100 in PBS + 1% BSA (immunohistochemical grade, Vector Laboratories). Anti-goat conjugated to Alexa 555 (Thermofisher) was used to visualize α-B crystallin. Membranes were stained using WGA conjugated with Alexa 647 (Thermofisher) diluted 1 in 100 in PBS. Nuclei were visualized using Picogreen reagent (Thermofisher) diluted 1 in 10.000 in PBS. Confocal scanning laser microscopy was performed on a Leica TCS SP8 STED 3X (Leica Microsystems). Picogreen, Alexa 555 and Alexa 647 were irradiated with a pulsed white light laser at 502nm, 553nm and 631nm respectively. A 63x oil objective with NA 1.4 was used to image the sample. Detection of the fluorescent signal was performed with gated Hybrid Detectors. Finally, the images were deconvolved using Huygens Professional (Scientific Volume Imaging).

Statistical analysis

Comparisons between DCM and donor and between AS and donor were analysed using ANOVA followed by a multiple comparison procedure. Differences within groups were measured by repeated measures ANOVA. A 2-tailed test with a *P* value <0.05 was considered significant. All analyses were performed using Prism software (GraphPad Software Inc, version 6.0).

RESULTS

Force measurements in muscle strips

Initial stretches with intact muscle strips, in which both cardiomyocytes and E-matrix contribute to $F_{passive}$, showed that DCM and AS strips developed higher forces during all stretches compared to donor (Figure 1A). The extraction protocol decreased $F_{passive}$ in all groups (Figure 1B). $F_{passive}$ caused by E-matrix stiffness was higher in DCM strips than in donors for stretches ranging from 10 to 20% slack length (Figure 2). $F_{passive}$ caused by E-matrix was higher in AS than in donors for stretches exceeding 20% slack length (Figure 2). $F_{passive}$ caused by cardiomyocyte stiffness, which is attributable to titin, was higher in DCM than in donors for stretches ranging from 10 to 20% slack length and higher in AS than in donors for stretches ranging from 10 to 15% slack length (Figure 3).

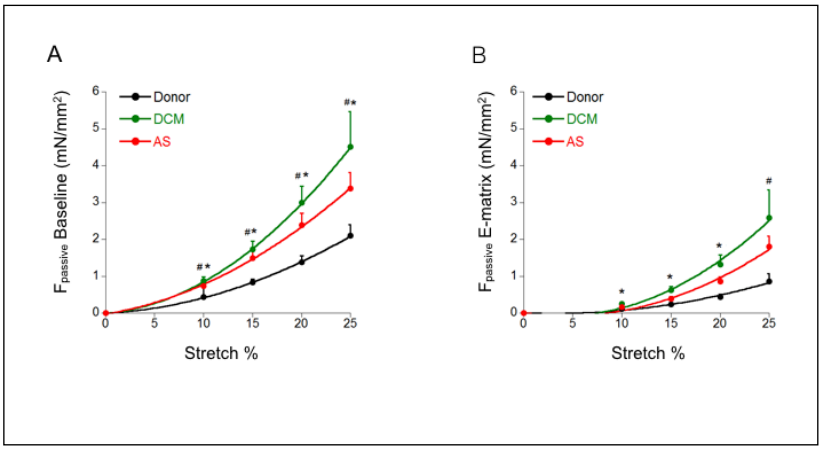

Figure 1: *$F_{passive}$ in myocardial strips.* At baseline, $F_{passive}$ was significantly higher in DCM and AS strips compared to donor (**A**). After extracting thick and thin filaments, the remaining $F_{passive}$ can be attributed to the E-matrix (**B**). * P<0.05 DCM vs Donor; # P<0.05 AS vs Donor.

The higher $F_{passive}$ caused by cardiomyocyte stiffness cannot be explained by isoform shifts of titin because both DCM and AS express more of the compliant N2BA titin isoform[3,4,17]. To further analyze the difference in $F_{passive}$ related to cardiomyocytes, we subjected isolated cardiomyocytes 1) to treatment with AP to discern the contribution of titin phosphorylation and 2) to conditions prevailing in failing myocardium such as high stretch and hypoperfusion-related acidosis[15].

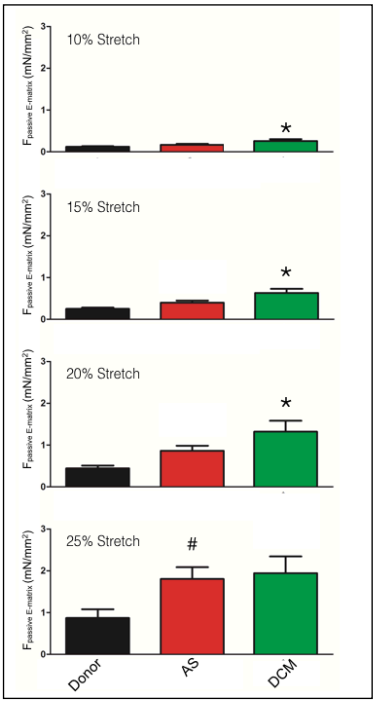

Figure 2: *$F_{passive}$ caused by the E-matrix.* AS strips had significantly higher E-matrix based $F_{passive}$ than donor when stretched to 25% slack length. DCM strips had significantly higher E-matrix based $F_{passive}$ for stretches ranging from 10 to 20% slack length. * P<0.05 DCM vs Donor; # P<0.05 AS vs Donor.

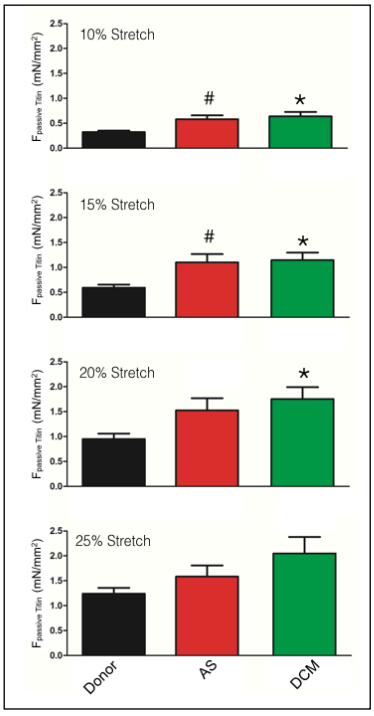

Figure 3: *$F_{passive}$ caused by titin.* In AS strips, titin-based $F_{passive}$ was higher than in donor strips for stretches ranging from 10 to 15% slack length. In DCM strips, titin-based $F_{passive}$ was higher than in donor for stretches ranging from 10 to 20% slack length. * P<0.05 DCM vs Donor; # P<0.05 AS vs Donor.

Force measurements in isolated cardiomyocytes

In donor cardiomyocytes (Figure 4A), the $F_{passive}$-SL curve shifted upward after administration of alkaline phosphatase (AP), which dephosphorylates titin. In contrast, the $F_{passive}$-SL curve failed to shift in AS and DCM cardiomyocytes (Figure 4B-C). This suggests that prior in-vivo phosphorylation status did not correct high stiffness of AS and DCM cardiomyocytes. The $F_{passive}$-SL curve of donor cardiomyocytes after AP was still lower ($p<0.05$) than the $F_{passive}$-SL curve after AP in AS and DCM cardiomyocytes. Mechanisms other than phosphorylation status therefore contribute to the high diastolic stiffness of AS and DCM cardiomyocytes.

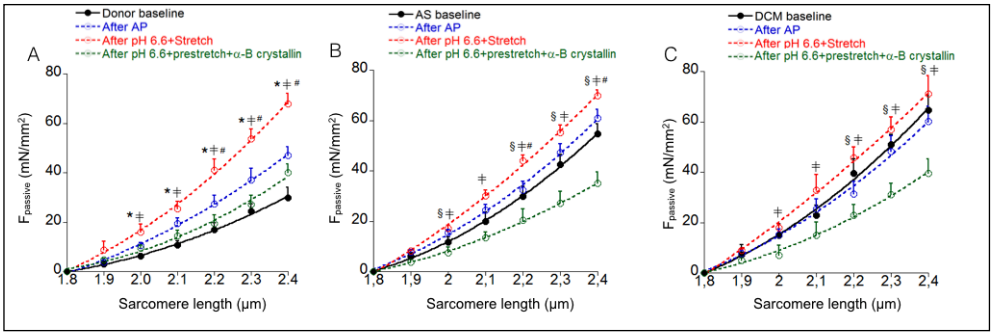

Figure 4: *$F_{passive}$ in single myocytes.* **A**: In donor cardiomyocytes, $F_{passive}$ significantly increased after administration of alkaline phosphatase and increased further after performing a prestretch in an acidic environment. After in vitro administration of α-B crystallin, $F_{passive}$ fell to a position slightly lower than after AP. **B**: In AS cardiomyocytes, no significant change in $F_{passive}$ was observed after incubation with AP. After the prestretch in pH 6.6, $F_{passive}$ increased significantly compared to baseline, but in vitro treatment with α-B crystallin lowered $F_{passive}$ to a level significantly lower than baseline. **C**: In DCM cardiomyocytes, incubation with AP had no effect on $F_{passive}$, but performing a prestretch in an acidic environment significantly increased passive stiffness. After in vitro treatment with α-B crystallin, $F_{passive}$ fell to a level significantly below baseline. * P<0.05 AP vs Baseline; # P<0.05 pH 6.6 + prestretch vs AP; ǂ P<0.05 α-B crystallin vs pH 6.6 + prestretch; § P<0.05 α-B crystallin vs baseline.

The effects of prestretch and acidic pH were subsequently investigated. The $F_{passive}$-SL curve shifted further upward in donor cardiomyocytes after performing a prestretch and imposing an acidic pH. After administration of α-B crystallin, the curve returned to a position slightly lower than after AP. In AS cardiomyocytes (Figure 4B), no significant change in the $F_{passive}$-SL curve was observed after incubation with AP. After imposition of prestretch and pH 6.6, the $F_{passive}$-SL curve shifted upward compared to baseline. After treatment with α-B crystallin, the

$F_{passive}$-SL curve fell to a position which was significantly lower than baseline and comparable to the baseline position of donor cardiomyocytes. This finding implies presence at baseline of prior stretch- and pH-induced changes in AS myocardium, which could be corrected by administration of α-B crystallin.
The same series of experiments in single DCM cardiomyocytes showed similar findings (Figure 4C) as in AS cardiomyocytes: incubation with AP had no effect on the $F_{passive}$-SL curve, but performing a prestretch in an acidic environment shifted the curve significantly upward. After in vitro treatment with α-B crystallin, the $F_{passive}$-SL curve fell to a position that was significantly below baseline and comparable to the baseline position of donor cardiomyocytes. This again implies presence at baseline of prior stretch- and pH-induced changes in DCM cardiomyocytes, which could be corrected by administration of α-B crystallin.

Effects of prestretch, pH and α-B crystallin in single AS cardiomyocytes
The relative importance of prestretch and pH 6.6 were analyzed in single AS cardiomyocytes. In the absence of prior administration of AP, lowering the pH to 6.6 had no effect on $F_{passive}$ (Figure 5A), but addition of a prestretch to ~2.6µm SL significantly shifted the $F_{passive}$-SL curve upward (Figure 5B). Incubation with α-B crystallin shifted the $F_{passive}$-SL curve significantly downward compared to baseline (Figure 6A). Incubation with α-B crystallin shifted the $F_{passive}$-SL curve to a similar position after prestretch or prestretch in combination with pH 6.6 (Figures 6B-C).

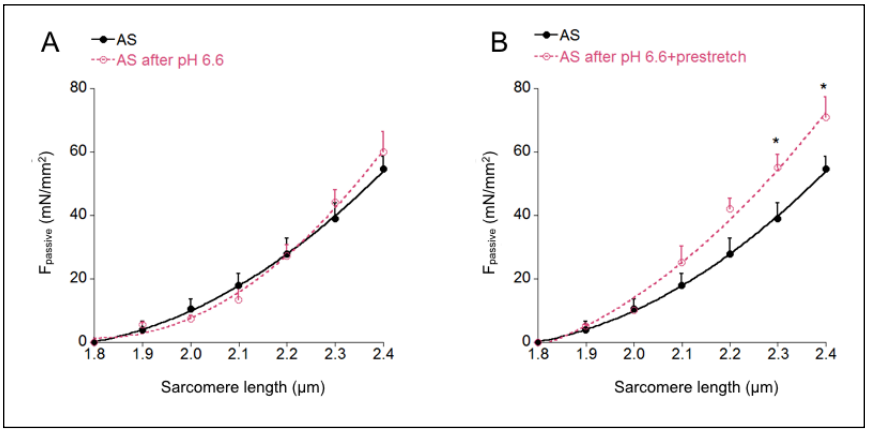

Figure 5: *Effects of acidosis and prestretch on $F_{passive}$ in single AS myocytes*. Incubating single AS cardiomyocytes in pH 6.6 did not change $F_{passive}$ (**A**). Performing a prestretch to ~2.6µm SL in pH 6.6 increased $F_{passive}$ (**B**). * P<0.05 vs AS baseline.

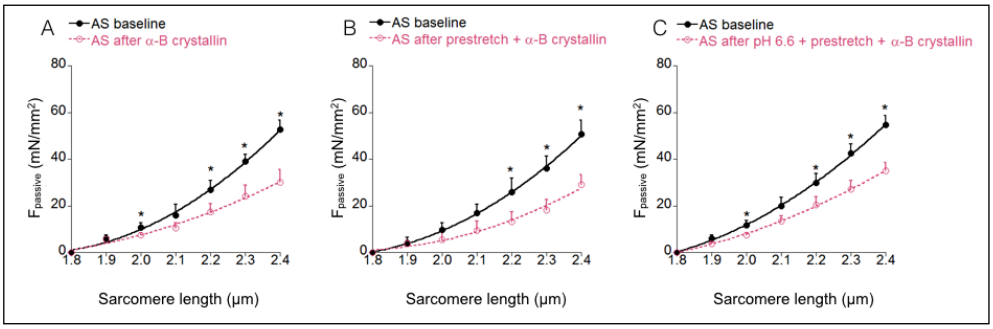

Figure 6: *Effects of α-B crystallin at baseline and after acidosis and prestretch on $F_{passive}$ in single AS cardiomyocytes.* Incubation with α-B crystallin decreased $F_{passive}$ significantly (**A**). In single AS cardiomyocytes that underwent prestretch alone (**B**) or a prestretch in combination with pH 6.6 (**C**), α-B crystallin also decreased $F_{passive}$ significantly. * P<0.05 vs AS baseline.

Immunofluorescence staining and confocal scanning laser microscopy

Confocal lasermicroscopical images were obtained from LV myocardium of donor and AS patients with immunohistochemical visualization of cell membranes, nuclei and α-B crystallin (Figure 7). In myocardium of AS patients, intensity of α-B crystallin

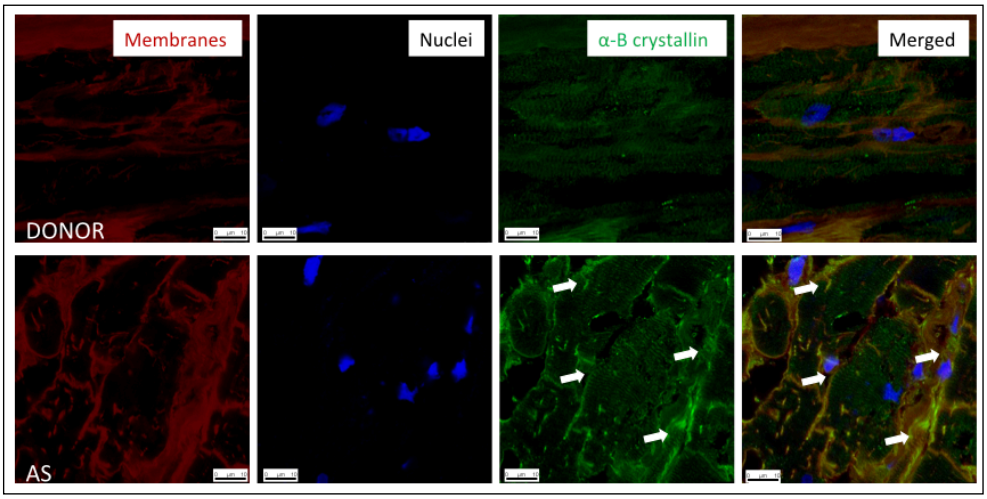

Figure 7: *Confocal lasermicroscopy for α-B crystallin in donor and AS.* Confocal lasermicroscopical images were obtained from LV myocardium of donor and AS patients with immunohistochemical visualization of cell membranes (A), nuclei (B) and α-B crystalline (C) . In myocardium of AS patients, intensity of α-B crystallin expression (C) was higher than in donor with visualization in the merged images (D) of subsarcolemmal aggresomes especially in the vicinity of capillaries (white arrows).

expression was higher than in donor with appearance of α-B crystallin containing aggresomes[18] (Figure 7), which were especially prominent in the subsarcolemma close to the capillaries (white arrows in Figure 7). The latter suggests signals originating from the microvascular endothelium to be involved in the subsarcolemmal mobilization of α-B crystallin in failing cardiomyocytes.

DISCUSSION

The present study investigated high diastolic stiffness of failing human myocardial strips and cardiomyocytes and observed the following: 1) High diastolic stiffness of cardiomyocytes significantly contributes to the overall stiffness of LV myocardial strips of AS and DCM patients; 2) Dephosphorylation with AP shifts the diastolic $F_{passive}$-SL relation upward in donor but not in AS or DCM cardiomyocytes; 3) Following dephosphorylation, exposure to prestretch causes an upward shift of the diastolic $F_{passive}$-SL relations in AS and DCM cardiomyocytes and a further upward shift of the diastolic $F_{passive}$-SL relation in donor cardiomyocytes; 4) Subsequent administration of α-B crystallin shifts the diastolic $F_{passive}$-SL relations downward in donor, AS and DCM cardiomyocytes to a position which coincides with the baseline diastolic $F_{passive}$-SL relation of donor cardiomyocytes. This finding is consistent with α-B crystallin providing protection against stretch-induced damage to titin in failing AS and DCM cardiomyocytes.

Cardiomyocyte versus E-matrix Stiffness

High diastolic stiffness of failing human cardiomyocytes was a significant contributor to overall stiffness of LV myocardial strips of AS and DCM patients up to 20% stretch in AS and up to 25% stretch in DCM. Use of dissected myocardial strips precluded visualization of sarcomeres and strip lengthening was therefore expressed as % stretch with respect to slack length, i.e. the minimal length at which $F_{passive}$ started to develop (Figure 1). At 25% stretch, the contribution of cardiomyocyte stiffness to overall stiffness no longer differed between donor and AS cardiomyocytes but continued to differ between donor and DCM cardiomyocytes (Figure 3). This could relate to less constraint by the extracellular matrix in DCM despite raised collagen volume fraction in both AS and DCM[3,4]. The latter could be consistent with different distribution and homeostasis of myocardial fibrosis in AS and DCM: in DCM there is focal replacement fibrosis

whereas in AS there is diffuse reactive fibrosis[3,4] and, as reflected by plasma biomarker elevations, fibrinolytic mechanisms are present in DCM in contrast to mainly profibrotic mechanisms in AS[19]. These findings illustrate the importance of concentric versus eccentric remodeling for the constraint imposed by the E-matrix on the cardiomyocytes.

Cardiomyocyte Stiffness and Titin Dephosphorylation

Altered cardiomyocyte stiffness can result from isoform shifts of the giant cytoskeletal protein titin, from posttranslational modifications of titin such as phosphorylation[2,11,20–24], formation of disulfide bonds[13], carbonylation[25] and s-glutathionylation[14] or from stretch-induced titin modification[15]. Because of higher expression of the compliant N2BA isoform in AS[4] and DCM[9,26], titin isoform shifts do not contribute to the observed rise of cardiomyocyte stiffness observed in AS and DCM cardiomyocytes in the present study. Because of altered activity in failing myocardium of different kinases such as protein kinase A[2], protein kinase C[23], protein kinase G[21], calcium/calmodulin-dependent kinase II (CaMKII)[22] and extracellular signal-regulated kinase (ERK)[24], altered phosphorylation of titin by these kinases was suspected to be involved in the raised stiffness of failing human cardiomyocytes. The present study confirms this involvement as it observed treatment with AP to raise diastolic stiffness in donor cardiomyocytes but not in AS and DCM cardiomyocytes (Figure 8). This implies a preexisting imbalance of titin phosphorylation in AS and DCM cardiomyocytes with either reduced phosphorylation of sites that increase titin elasticity or increased phosphorylation of sites that decrease titin elasticity.

Posttranslational modifications of titin other than phosphorylation have recently been implicated in altered cardiomyocyte stiffness. These mechanisms include among others modification of the titin molecule induced by excessive physical stretch. A prior study indeed showed stretch-induced mechanical unfolding of Ig domains of titin to expose cryptic cysteines to S-glutathionylation which interfered with the ability of titin to refold and left titin in a more extensible state[14]. In acidic pH, the reverse was observed, namely a prestretch-induced reduction of titin extensibility[15]. This is especially relevant to failing myocardium, which is exposed to both high filling pressures and jeopardized coronary perfusion. The present study therefore imposed prestretch and acidic pH on failing human cardiomyocytes.

Cardiomyocyte Stiffness and Prestretch

In the present study, cardiomyocytes were subjected to a prestretch protocol, which consisted of a 15 minutes stretch period at 2.6µm followed by a 5 minutes stabilization period at slack length. This prestretch protocol was executed in pH 6.6 in donor, AS and DCM cardiomyocytes. Following dephosphorylation with AP, exposure to prestretch caused an upward shift of the diastolic $F_{passive}$-SL relations in AS and DCM cardiomyocytes and a further upward shift of the diastolic $F_{passive}$-SL relation in donor cardiomyocytes (Figure 4). The identical position of all diastolic $F_{passive}$-SL relations following prestretch argues in favor of prior stretch-induced damage being involved in the baseline elevation of diastolic stiffness of AS and DCM cardiomyocytes. The prestretch-induced upward shift of the diastolic $F_{passive}$-SL relations in AS and DCM cardiomyocytes was indeed smaller than the prestretch induced upward shift of the diastolic $F_{passive}$-SL relation in donor cardiomyocytes (Figure 8). This yielded an identical position of all diastolic $F_{passive}$-SL relations because in AS and DCM cardiomyocytes a smaller shift was

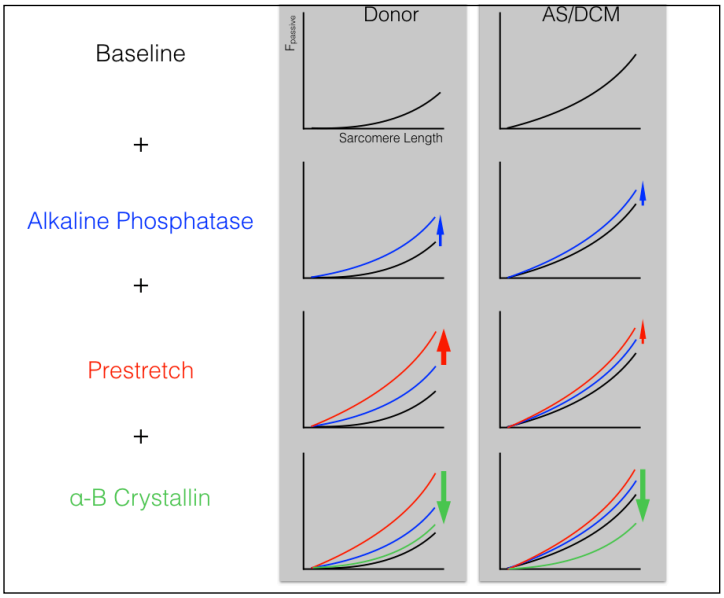

Figure 8: Diagram showing respective responses of donor and AS/DCM cardiomyocytes to administration of alkaline phosphatase, prestretch and α-B crystallin.

superimposed on baseline stretch-induced damage whereas in donor cardiomyocytes prestretch elicited a larger shift because of absent baseline stretch-induced damage.

The origin of the baseline stretch-induced damage in AS and DCM cardiomyocytes is uncertain and could relate to external stretch on

cardiomyocytes or to internal stretch within cardiomyocytes. The former obviously relates to elevated LV filling pressures at rest or during exercise. The latter is consistent with either a modified Z-disc structure or with the previously observed widening of the Z-disc[27]. Z-disc widening results from reduced elasticity of cytoskeletal proteins[28,29], which from both sides pull at and open up adjacent Z lines. In AS and DCM cardiomyocytes, internal stretch and stretch-induced damage could have resulted from the aforementioned imbalance of titin phosphorylation.

In contrast to a previous study[14], separate imposition of pH 6.6 failed to induce an upward shift of the diastolic $F_{passive}$-SL relation (Figure 5A). The upward shift of the diastolic $F_{passive}$-SL relation following combined administration of prestretch and acidic pH therefore appeared to be solely related to preceding sarcomere stretch. Furthermore, omission of prior treatment with AP also did not influence the combined effect of prestretch and acidic pH (Figure 5B).

Cardiomyocyte Stiffness and α-B crystallin

Beneficial myocardial effects of α-B crystallin were previously reported in ischemia-reperfusion experiments[30,31] and attributed to interaction between α-B crystallin and titin[16,32]. Recently, the small heat shock proteins HSP27 and α-B crystallin were shown to protect cardiomyocytes against stretch-induced damage in acidic pH[15]. In these experiments administration of small heat shock proteins to donor cardiomyocytes corrected the combined effects of prestretch and acidic pH on the diastolic $F_{passive}$-SL relation. This result was confirmed in the present study (Figure 4). The present study also administered α-B crystallin to AS and DCM cardiomyocytes. In contrast to donor cardiomyocytes, α-B crystallin not only corrected the combined effects of prestretch and acidic pH but also reversed the baseline upward displacement of the diastolic $F_{passive}$-SL relation (Figure 8). This finding was consistent with baseline involvement of prior stretch-induced titin damage in AS and DCM cardiomyocytes and was confirmed in a separate series of experiments, in which α-B crystallin shifted the diastolic $F_{passive}$-SL relation downward without any prior or concomitant intervention (Figure 6). In these experiments, the magnitude of the downward displacement of the diastolic $F_{passive}$-SL relation was similar in the absence (Figure 6A) or presence of foregoing interventions (Figures 6B,C).

In AS and DCM cardiomyocytes α-B crystallin lowered diastolic stiffness well below baseline values as previously reported following administration of PKA or PKG[2,4,20,21]. This supports overlapping effects of titin phosphorylation and

stretch-induced titin aggregation possibly because of preexisting stretch-induced titin aggregation obstructing phosphorylation at sites that specifically increase titin elasticity. This finding has important therapeutic implications as it implies limited efficacy of drugs that increase PKA or PKG activity for treatment of diastolic LV dysfunction related to high cardiomyocyte stiffness and could relate to the failure of dobutamine to improve diastolic LV dysfunction[33] and of phosphodiesterase 5 inhibitors (PDE5I) to improve exercise tolerance or hemodynamics in HFPEF[34,35].

The present study observed upregulation and subsarcolemmal localization of α-B crystallin in AS and DCM cardiomyocytes. Because of the close vicinity of capillaries (white arrows in Figure 7), the localization of α-B crystallin in subsarcolemmal aggresomes was consistent with signals from the microvascular endothelium being involved in their formation. The subsarcolemmal localization also suggested that endogenous α-B crystallin was diverted from the sarcomeres and therefore failed to exert its protective action on titin distensibility, which was however restored following administration of exogenous α-B crystallin. The latter finding supports future therapeutic efforts to raise concentration of α-B crystallin in failing myocardium through direct administration of α-B crystallin, through administration of α-B crystallin analogues or through administration of heat shock protein inducing drugs such as geranylgeranylacetone (GGA) or NYK9354.

LIMITATIONS

The limited size of donor and patient populations reduced statistical power and precision of estimates. The study failed to address titin isoform shifts in the AS and DCM samples and presumed larger expression of the compliant N2BA titin isoform in both conditions based on previous evidence obtained in the author's[3,4] and in other[9] laboratories. A potential contribution of altered titin isoform expression could therefore not be excluded. Furthermore, the effects of AP administration on $F_{passive}$ in donor, AS and DCM samples were not confirmed by measures of overall titin phosphorylation.

CONCLUSIONS

High cardiomyocyte stiffness significantly contributed to overall myocardial stiffness in AS and DCM. High cardiomyocyte stiffness resulted from titin phosphorylation failing to improve cardiomyocyte stiffness and from prior stretch-induced aggregation of titin, both of which were corrected by administration of α-B crystallin. Diastolic LV dysfunction in heart failure could therefore benefit from treatment with α-B crystallin.

DISCLOSURES

None

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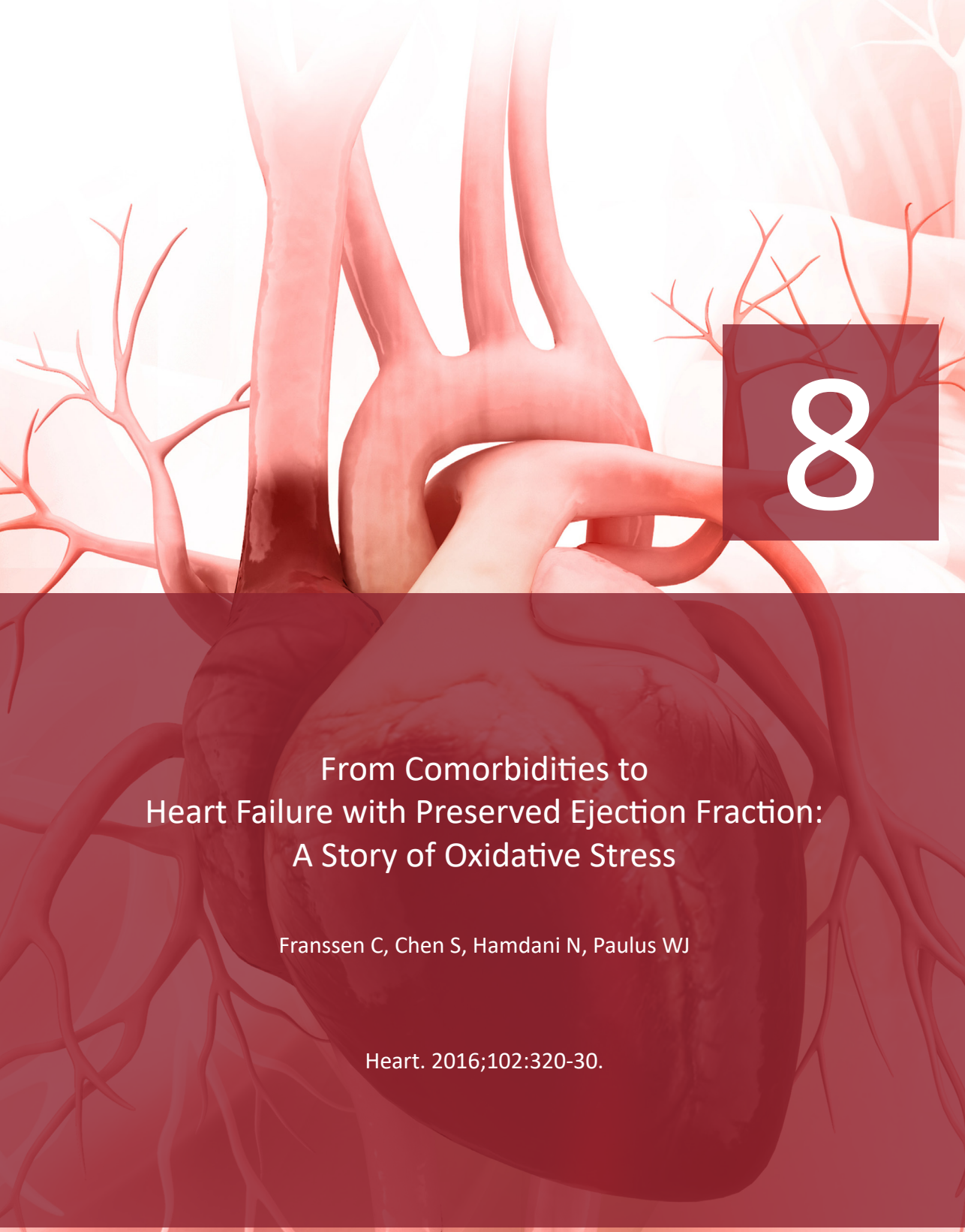

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From Comorbidities to Heart Failure with Preserved Ejection Fraction: A Story of Oxidative Stress

Franssen C, Chen S, Hamdani N, Paulus WJ

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From Comorbidities to
Heart Failure with Preserved Ejection Fraction:
A Story of Oxidative Stress

by

Constantijn Franssen, Sophia Chen, Nazha Hamdani, Walter J. Paulus.

From

Department of Physiology, Institute for Cardiovascular Research,
VU University Medical Center, Amsterdam, The Netherlands.

Running Title: Microvascular Inflammation in HFpEF

INTRODUCTION

Heart failure (HF) with reduced ejection fraction (HFrEF) and HF with preserved ejection fraction (HFpEF) currently account for roughly equal proportions of HF[1]. The incidence of HFpEF increased rapidly during the past decades and is becoming the dominant form of HF[2]. Recently this was rappraised and it was shown that the incidence of HF decreased, but this was more pronounced for HFrEF than in HFpEF[1,3,4]. Also, since HFrEF patients benefit from therapeutic progress, many of these patients shifted to HFpEF for which there is no specific treatment[1]. Although various HFpEF trials conducted to date could be criticised for methodological shortcomings[5], a more serious weakness is our incomplete understanding of the pathophysiology of HFpEF[6].

HFpEF is characterised by a high incidence of non-cardiac comorbidities such as obesity, diabetes mellitus (DM), chronic obstructive pulmonary disease (COPD) and arterial hypertension, all of which are of prognostic importance and lead to increased morbidity and mortality in this elderly population[1]. This marked association suggests that comorbidities play a key role in HFpEF pathophysiology.

HFpEF is characterized by concentric left ventricular (LV) remodelling and diastolic dysfunction[7]. In addition, impairments in chronotropic reserve, atrial function, systemic and pulmonary vasculature, vasodilatation and many other factors are known to be involved[8]. Identifying and understanding these underlying mechanisms is essential to develop treatment strategies for HFpEF. In this review, we will provide an overview of HFpEF from diagnosis to pathophysiology, and we will highlight the importance of comorbidities in endothelial inflammation and oxidative stress as underlying pathophysiological mechanisms. Finally, we will touch upon some of the many treatment strategies that have been and will be studied in HFpEF.

DIAGNOSIS OF HFPEF

Diagnosing HFpEF remains challenging and requires signs or symptoms of congestion, preserved or mildly abnormal LV systolic function (EF>50%, end-diastolic volume index <97 ml/m^2) and diastolic LV dysfunction (Figure 1)[9]. Diastolic LV dysfunction is defined as the inability of the ventricle to fill to a normal

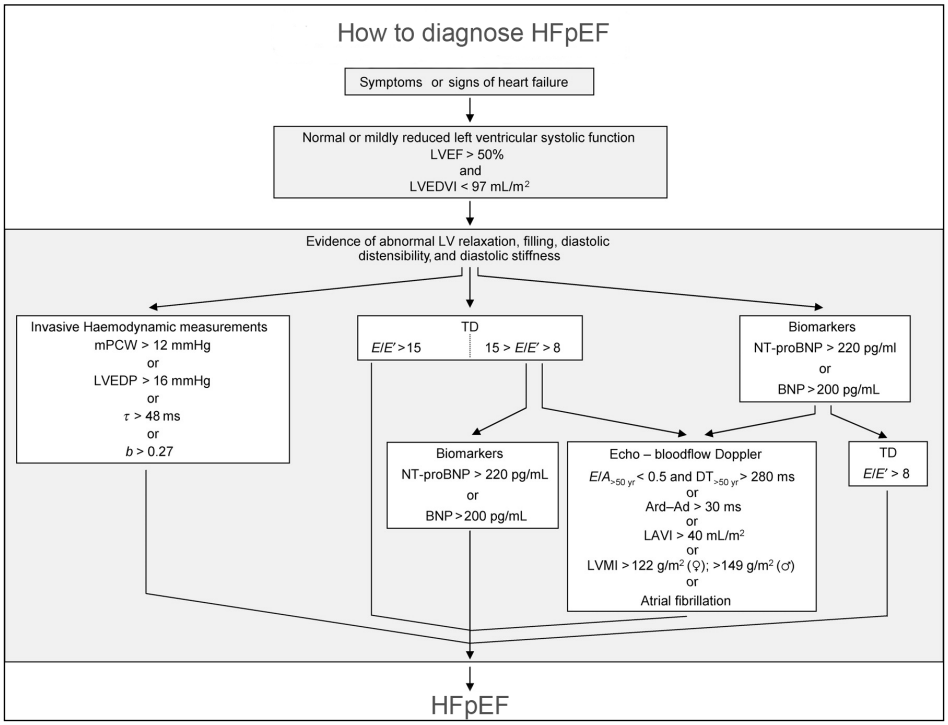

Figure 1. Diagnostic flowchart on 'How to diagnose HFpEF' in a patient suspected of HFpEF. LVEDVI, left ventricular end-diastolic volume index; mPCW, mean pulmonary capillary wedge pressure; LVEDP, left ventricular end-diastolic pressure; *τ*, time constant of left ventricular relaxation; b, constant of left ventricular chamber stiffness; TD, tissue Doppler; *E*, early mitral valve flow velocity; *E'*, early TD lengthening velocity; NT-proBNP, N-terminal-pro brain natriuretic peptide; BNP, brain natriuretic peptide; *E*/*A*, ratio of early (*E*) to late (*A*) mitral valve flow velocity; DT, deceleration time; LVMI, left ventricular mass index; LAVI, left atrial volume index; Ard, duration of reverse pulmonary vein atrial systole flow; Ad, duration of mitral valve atrial wave flow. Future improvements of this diagnostic algorithm should focus on comorbidity profile, exercise hemodynamics and new biomarkers. Adapted with permission from Paulus et al[9].

preload volume at low pressures. It can be diagnosed invasively by measurement of an increased pulmonary capillary wedge pressure (PCWP), LV end-diastolic pressure or prolonged LV isovolumic relaxation[9]. Doppler echocardiography guides non-invasive diagnosis with an E/E' >15 (ratio of early transmitral diastolic flow velocity to tissue Doppler early mitral annular diastolic velocity). When E/E' is 8-15, other echocardiographic parameters (left atrial size, transmitral and pulmonary flow velocities, LV hypertrophy), atrial fibrillation and natriuretic

peptides (NP) may yield secondary evidence of diastolic dysfunction[9]. Future efforts to improve this diagnostic algorithm should focus on comorbidity profile, exercise hemodynamics (as discussed below) and new biomarkers.

However, many HFpEF patients only develop symptoms during (limited) exercise and are asymptomatic at rest - when echocardiography or cardiac catheterization are usually performed. It has been demonstrated that HFpEF patients are very sensitive to volume status and even a relatively small volume load with 0.6l of saline can make PCWP rise sharply[10]. Since many patients are on fluid and/or salt restriction before undergoing catheterization, measured values might underestimate actual pressures outside the clinical situation. Also, normal levels of natriuretic peptides do not rule out HFpEF[11]. Importantly, exercise during invasive measurements made filling pressures rise at even limited workloads, probably more accurately reflecting activities of daily living[12–14]. Moreover, exercise testing was whown to be more sensitive than saline loading to detect hemodynamic derangements indicative of HFpEF[15]. Furthermore, invasive exercise testing enables the clinician to detect mechanisms limiting exercise tolerance, such as prompt elevation of filling pressures, chronotropic incompetence[16], paradoxical rise of pulmonary vascular resistance[17] and inappropriate widening of arterio-venous oxygen content difference[18].

The cardiac and cardiovascular mechanisms that impair exercise tolerance in HFpEF, such as ventricular diastolic and systolic reserve function, heart rate reserve and rhythm, atrial dysfunction, stiffening of the ventricles and vasculature, impaired vasodilatation, pulmonary hypertension, endothelial dysfunction and peripheral abnormalities were recently extensively reviewed elsewhere[8].

PATHOPHYSIOLOGICAL FINDINGS IN HFPEF

HFpEF is characterized by concentric LV remodeling, diastolic dysfunction and increased myocardial stiffness[7,19]. These findings are associated with structural and functional changes in myocardial samples from patients and animal models. The most important findings are discussed below.

Structural changes in HFpEF myocardium: the extracellular matrix

The changes in the extracellular matrix (ECM) known to occur in HFpEF patients are largely determined by collagen and consist of an increased collagen volume

fraction (CVF), a relative abundance of the stiff collagen type 1 and more collagen cross-linking[20,21]. Inflammatory markers (such as interleukins 6 and 8), endothelial adhesion molecules [intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1], and markers of ECM turnover [matrix metalloproteinase 9 (MMP9), tissue inhibitor of metalloproteinase 1 (TIMP1)] are all increased in HFpEF[22]. As recently hypothesized, microvascular inflammation is probably the driving mechanism leading to proliferation of fibroblasts and myofibroblasts as a consequence of decreased NO bioavailability, which induces profibrotic effects and coronary microvascular rarefaction[6,23].

Besides changes in the ECM, structural differences between HFpEF and HFrEF cardiomyocytes were observed[24]. Cardiomyocyte diameters were larger in HFpEF than in HFrEF in absolute values and at corresponding levels of CVF[24]. This and other recent evidence from HFpEF biopsy samples suggest that, on top of the observed structural changes, cardiomyocytes functionally contribute to HFpEF pathophysiology[23,25]. Additionally, no changes in ECM structure were observed in a novel rat model, in which a HFpEF phenotype with diastolic dysfunction, pulmonary congestion and increased myocardial stiffness was induced by metabolic risk factors such as obesity and DM on top of arterial hypertension[26]. These findings indicate that functional changes in cardiomyocytes are important in HFpEF.

Functional changes in HFpEF cardiomyocytes

As already mentioned, cardiomyocyte diameters are typically larger typically in HFpEF than in HFrEF[24]. Also, cardiomyocyte stiffness was higher in HFpEF than in HFrEF and highest in HFpEF patients with DM[24,27]. Upon administration of protein kinase A (PKA), cardiomyocyte stiffness declined more in HFpEF than in HFrEF cardiomyocytes, suggesting that a phosphorylation deficit contributes to myocardial and diastolic stiffness in HFpEF.

The giant protein titin is the main determinant of myocardial stiffness in cardiomyocytes supporting diastolic distensibility. Titin modulates cardiomyocyte stiffness, via either phosphorylation or oxidation[28]. Titin can, for example, be phosphorylated by protein kinase A (PKA), upon -adrenergic stimulation, or by protein kinase G (PKG), upon activation by nitric oxide (NO) or natriuretic peptides (NP). PKA and PKG phosphorylation acutely increase titin compliance and potentially contributes to enhanced cardiac filling[28]. The reduced myocardial

stiffness upon titin phosphorylation by PKA and PKG suggests that the chronic phosphorylation deficit observed in HFpEF is most likely due to impaired PKA and PKG signalling. Indeed, both PKA and/or PKG could correct the high cardiomyocyte stiffness of HFpEF patients biopsy samples and animal models of HFpEF[24,26,27,29,30]. However, a HFpEF dog model showed no change in PKA activity, suggesting that PKG may be more important[31]. Indeed, PKG activity and cGMP concentration were reduced in LV myocardium of HFpEF patients and this was associated with increased myocardial nitrotyrosine levels[29]. Elevated nitrotyrosine is a marker of nitrosative stress as will be discussed below. Together, these findings suggest that downregulated cGMP-PKG-signalling is an important finding in HFpEF and cGMP-enhancing therapy could be useful.

Next to the cGMP-PKG-signalling pathway, recent studies suggest a role for proteotoxicity in cardiac dysfunction, as it was previously demonstrated in neurological diseases such as Alzheimer's and Huntington's disease[32]. The basis for this proteotoxicity resides in the failing quality-control and/or repair mechanisms to correct for aggregated or damaged proteins as a consequence of inflammation and oxidative stress, but it is also part of normal (cardiac) aging[32,33] and it can affect mitochondrial dysfunction. Knowledge about the role for proteotoxicity and mitochondrial dysfunction in relation to aging, inflammation and oxidative stress in HFpEF is still premature, but might explain the absence of benefit from different treatment strategies so far[34].

COMORBIDITIES IN HFPEF PATIENTS

Prevalence

Several comorbidities are highly prevalent in HFpEF patient populations. Coronary artery disease (CAD) has been reported in HFpEF, is associated with increased mortality and its prevalence ranges from 25% to 68%, depending on the methodology applied in two recent studies[35,36]. Unfortunately, non-invasive detection is still problematic with many false-positive and false-negative exercise tests[35]. Also, the proportion of patients reporting angina did not differ between patients with or without significant CAD[35], suggesting the presence of microvasculatory dysfunction with subsequent angina. Prospective studies are needed to define how to diagnose CAD in HFpEF patients in the absence of an

acute coronary syndrome, but for now, coronary angiography should probably be considered in case of recurrent or worsening HF[36].

Although waist circumference and overweight patients are often not reported in studies, one third of patients has a body mass index ≥30kg/m^2 [37,38]. DM prevalence ranges from 37 to 45% in various registries, while arterial hypertension shows an even higher prevalence, ranging from 76 to 96%[37–40]. Furthermore, around 1/3 of HFpEF patients suffer from COPD and 26-52% of patients have chronic kidney disease[38,40]. Moreover, many patients have more than one comorbidity and the number of comorbidities correlates with the prognosis[3,4]. Non-cardiac comorbidities induce a chronic, low-grade inflammatory state and oxidative stress, as will be discussed next.

Inflammation

The association between chronic, low-grade inflammation and the development of HF has been a topic of extensive research. Diverse inflammatory markers are associated not only with the development and diagnosis of HFpEF, but also with the prognosis of HFpEF patients[22,41]. Obesity with increased visceral adipose tissue induces several pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), monocyte chemoattractant protein 1 (MCP-1) and other chemokine ligands, all of which lead to monocyte recruitment and macrophage activation[42]. Increased peripheral inflammation, monocytosis, and monocyte differentiation to anti-inflammatory/pro-fibrotic M2 macrophages was shown to be associated with HFpEF in a population with a very high prevalence of metabolic comorbidities[43]. In addition, perivascular adipose tissue plays a major role in mediating vascular tone and (endothelial) inflammation through the interaction of perivascular adipocytes, immune cells, vascular endothelium and smooth muscle cells[44], effects also mediated by reduced expression of endothelial NO synthase (eNOS) and thus decreased NO synthesis, leading to reduced vasorelaxation[42]. In addition, obesity-associated inflammation also induces insulin resistance (IR)[42], an early step in the development of DM. In HFpEF patients, inflammatory changes could also occur in skeletal muscle and account for the impaired diffusion of oxygen to skeletal muscle during exercise[45] and the blunted systemic vasodilator response[46].

Oxidative stress

Inflammatory cytokines induce the production of the reactive oxygen species (ROS) superoxide (O_2^-) by NADPH oxidases (NOX), enzymes that are widespread in the human body (e.g. in macrophages, endothelial cells, vascular smooth muscle cells and cardiomyocytes)[47]. Increased myocardial activation of NOX was previously described in patients with dilated and ischemic cardiomyopathy[48,49] . Aortic banding in rats led to LV hypertrophy and HF and resulted in increased NOX expression in cardiomyocytes and endothelium[50].

At low concentrations, ROS play a physiological role in signaling cascades[51]. However, when ROS production increases or availability of antioxidants is insufficient, oxidative stress results with detrimental effects. Increased concentrations of ROS induce nonspecific damage to cellular membranes, proteins, and DNA, and is especially harmful in mitochondria since mitochondrial DNA and enzymes are highly susceptible to oxidative damage[52]. In the microvasculature, ROS are known to 'uncouple' endothelial nitric oxide synthase (eNOS, also known as NOS3)[53]. eNOS is found in the cardiovascular system and normally produces NO in its dimer state. However, the uncoupled monomer generates superoxide rather than NO and exacerbates the oxidative stress. Moreover, the NO generated reacts rapidly with superoxide to generate peroxynitrite ($ONOO^-$), which is a toxic, reactive nitrogen species with the ability to nitrate tyrosine residues and form nitrotyrosine[51]. The resultant decreased NO bioavailability was recently proposed to be essential in HFpEF development and is further discussed next[6] (Figure 2).

DECREASED NO BIOAVAILABILITY DRIVES HFPEF DEVELOPMENT

NO is essential in cardiovascular physiology and its function as an endothelial-derived relaxant of smooth muscle was described almost three decades ago[54]. NO regulates the activity of the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA), which adjusts Ca^{2+} re-uptake into the SR, and hence inotropy[55]. NO also regulates the ryanodine receptors, responsible for the cytosolic Ca^{2+}-influx from the SR, and decreased NO bioavailability can change contractility[56].

Besides effects on contractile properties, NO can induce earlier LV relaxation and reduce end-diastolic stiffness[57]. This last mechanism functions via stimulation of cardiac soluble guanylate cyclase (sGC) receptors, which catalyze

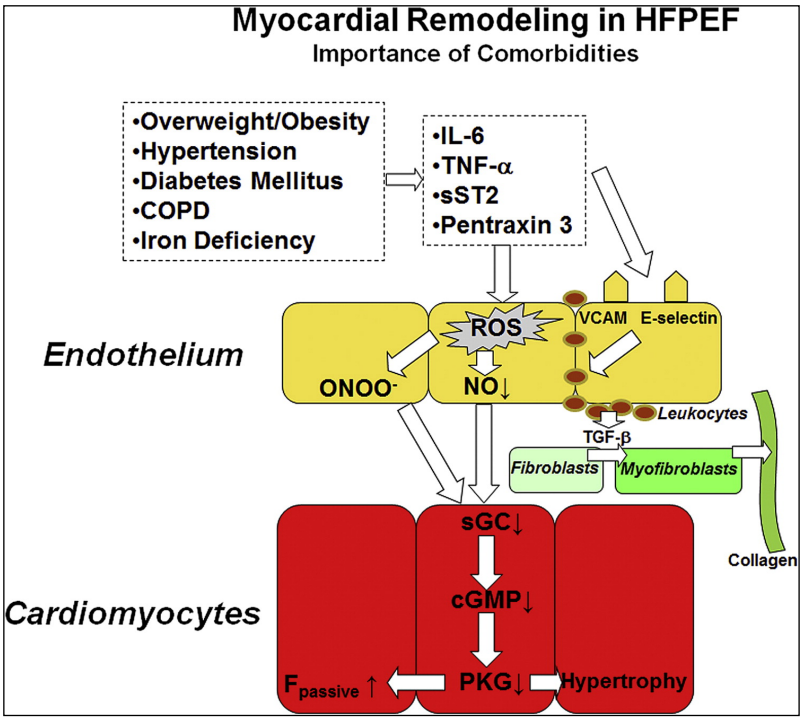

Figure 2.Comorbidities Drive Myocardial Dysfunction and Remodeling in HFpEF. Comorbidities induce a systemic proinflammatory state with elevated plasma levels of interleukin (IL)-6, tumor necrosis factor (TNF)-α, soluble ST2 (sST2), and pentraxin 3. Coronary microvascular endothelial cells reactively produce reactive oxygen species (ROS), vascular cell adhesion molecule (VCAM), and E-selectin. Production of ROS leads to formation of peroxynitrite ($ONOO^-$) and reduced nitric oxide (NO) bioavailability, both of which lower soluble guanylate cyclase (sGC) activity in adjacent cardiomyocytes. Lower sGC activity decreases cyclic guanosine monophosphate concentration and protein kinase G (PKG) activity. Low PKG activity increases resting tension ($F_{passive}$) of cardiomyocytes because of hypophosphorylation of titin and removes the brake on prohypertrophic stimuli inducing cardiomyocyte hypertrophy. VCAM and E-selectin expression in endothelial cells favors migration into the subendothelium of monocytes. These monocytes release transforming growth factor β (TGF-β). The latter stimulates conversion of fibroblasts to myofibroblasts, which deposit collagen in the interstitial space. COPD = chronic obstructive pulmonary disease; HFpEF = heart failure with preserved ejection fraction. Reprinted with permission from Paulus and Tschöpe[6].

the conversion of guanosine 5’-triphosphate (GTP) to cyclic guanosine 3’,5’-monophosphate (cGMP)[58]. NPs such as brain natriuretic peptide (BNP) increase cGMP via stimulation of particulate guanylate cyclase (pGC)[59].

cGMP is an ubiquitous intracellular second-messenger, vital to endothelial, vascular smooth muscle, and cardiomyocyte function[6,60]. It exerts its actions through cGMP-gated cation channels, cGMP-dependent protein kinases (PKG), and cGMP-regulated phosphodiesterases (PDEs), which in turn hydrolyze cGMP and other cyclic nucletides[60].

In HFpEF, where NO bioavailability is low due to inflammation and oxidative stress, cGMP concentration and PKG activity decrease. Also, oxidative stress itself shifts sGC toward an oxidized, dysfunctional heme-free form that is unresponsive to both endogenous and exogenous NO[61]. Indeed, cGMP concentration and PKG activity were shown to be lower in myocardial samples from HFpEF patients compared to patients with HFrEF or AS and *in vitro* PKG normalized the increased stiffness of HFpEF cardiomyoctes[29]. As already mentioned, HFpEF animal models confirmed titin hypo-phosphorylation associated with stiff cardiomyocytes, which was normalized upon *in vitro* administration of PKG[26,30,31]. As a consequence, this disrupted NO-sGC-cGMP-PKG pathway in HFpEF might yield therapeutic targets in HFpEF[61], as discussed below (Figure 3).

THERAPEUTIC STRATEGIES

The past

The management of patients with HFrEF has improved considerably over the past decades and current guidelines from the joint European Society of Cardiology (ESC) and American College of Cardiology Foundation (ACCF) and the American Heart Association (AHA) on the management of HF provide the clinician with guidance[62,63]. In stark contrast, the guidelines sections related to HFpEF are sparse and are limited to symptomatic treatment of congestion with diuretics and the control of (metabolic) comorbidities such as arterial hypertension and DM. No treatment strategy studied to date in large HFpEF trials has proven to improve disease progression and survival, including betablockers[64], angiotensin-converting enzyme inhibitors[65], angiotensin receptor blockers[66,67], and digoxin[68]. An exhaustive enumeration of past trials in HFpEF was recently tabulated[69].

The present: PDE5 inhibitors and aldosterone antagonists in HFpEF

cGMP breakdown can be inhibited by the PDE5 inhibitor sildenafil with potential beneficial effects such as improved cardiac relaxation and LV reverse remodelling[70] (Figure 3). PDE5 inhibition is integrated in the guidelines for pharmacologic treatment of pulmonary arterial hypertension in adults, where it improves exercise capacity, functional status and hemodynamics [71,72]. When treated with sildenafil, pulmonary vascular resistance and right heart pressures decreased in HFrEF patients with secondary pulmonary hypertension (PHT) and long-term treatment improved functional status, exercise tolerance, LV diastolic function and cardiac geometry[73–75]. Many HFpEF patients develop PHT due to elevated left-sided filling pressures[76] and, vice versa, patients with unexplained PHT appear to have HFpEF[77]. In a small clinical study in HFpEF patients with PHT, treatment with sildenafil for 12 months improved LV diastolic function and reduced hypertrophy and pulmonary pressures[78]. However, these effects were not reproduced in a larger, long-term (24 weeks) trial of sildenafil (RELAX) in HFpEF patients. In this study, exercise capacity and clinical status did not improve and sildenafil failed to increase plasma cGMP concentrations or yield hemodynamic benefits[79]. The authors suggested these disappointing results were attributable to the relatively low right-sided heart pressures in their patient population compared with the earlier studies in HFrEF. In addition, plasma levels of N-terminal pro-BNP (NT-proBNP) and prevalence of atrial fibrillation were high, indicating that patients in the RELAX trial were at an advanced stage of HFpEF and therefore less likely to benefit from a limited strategy involving only inhibition of cGMP breakdown[61]. Since decreased myocardial cGMP concentration is an important finding in HFpEF, stimulation of cGMP production is an interesting therapeutic strategy in HFpEF as will be discussed in more detail below.

In patients with HFrEF, regardless of the cause, the aldosterone antagonists spironolactone and epleronone reduce total and cardiovascular mortality[80–82] and are recommended in current HF guidelines[62,63]. Aldosterone plays an important role in the pathophysiology of all forms of HF and is implicated in vascular dysfunction, endothelial inflammation, increased sodium retention and volume load, hypertrophy and fibrosis[83,84]. Specific to HFpEF, elevated plasma aldosterone levels have been associated with more pronounced concentric LV hypertrophy[85]. A clinical trial in which HFpEF patients were administered spironolactone or placebo for 12 months demonstrated an

improvement of diastolic function (E/E') on echocardiography, but did not increase maximal exercise capacity (peak VO_2)[86]. The relevance of the observed improvement in E/E' is unknown. A large multicenter, randomized, double-blind trial (TOPCAT) included almost 3500 patients and they were assigned to spironolactone or placebo, with a mean follow-up of 3.3 years[87]. Overall, spironolactone did not significantly reduce the composite primary endpoint of death from cardiovascular causes, aborted cardiac arrest, or hospitalization for HF. However, a subanalysis revealed important regional variation in the study[88]. Patients recruited in Russia and Georgia appeared to have fewer comorbidities and were younger,but had experienced more myocardial infarctions, had more hospitalizations for HF, lower LVEF and higher diastolic blood pressure compared with patients enrolled in American countries. In Russia and Georgia, there were less clinical events and spironolactone was uneffective whereas in America, with a four times higher event rate, spironolactone reduced rates for the primary outcomes cardiovascular death or hospitalization for HF. In conclusion, aldosterone antagonists may have an indication in HFpEF, but the appropriate selection of patients is still a topic for further research.

The future: targeting inflammation, oxidative stress and cGMP?

Overweight and obesity induce inflammatory and oxidative stress with reduced signalling via the NO-sGC-cGMP-PKG pathway, associated with titin hypophosphorylation and increased myocardial diastolic stiffness. Weight loss is therefore expected to yield beneficial effects. Specific effects of weight loss on the heart include decreased LV mass and improved diastolic function[89–91]. As a preclinical demonstration of the beneficial effects of improving NO-based signalling via weight loss, an obese rat model for erectile dysfunction (where the same sGC-cGMP pathway is affected) demonstrated increased NOS expression and lower oxidative stress following bariatric surgery[92]. Although weight loss is often difficult to realize in clinical practice, even in older (>65 years of age) obese adults, lifestyle interventions with caloric restriction and exercise were associated with decreased concentrations of inflammatory molecules and metabolic markers, together with improved muscle quality and physical function[93]. However, larger studies with longer follow-up are needed to evaluate potential effects on the prevention or treatment of HFpEF. Also, it can be considered common sense to treat the underlying cause of the disease before any further treatment can be expected to have optimal effects, in line with treating HFrEF caused by ischemia,

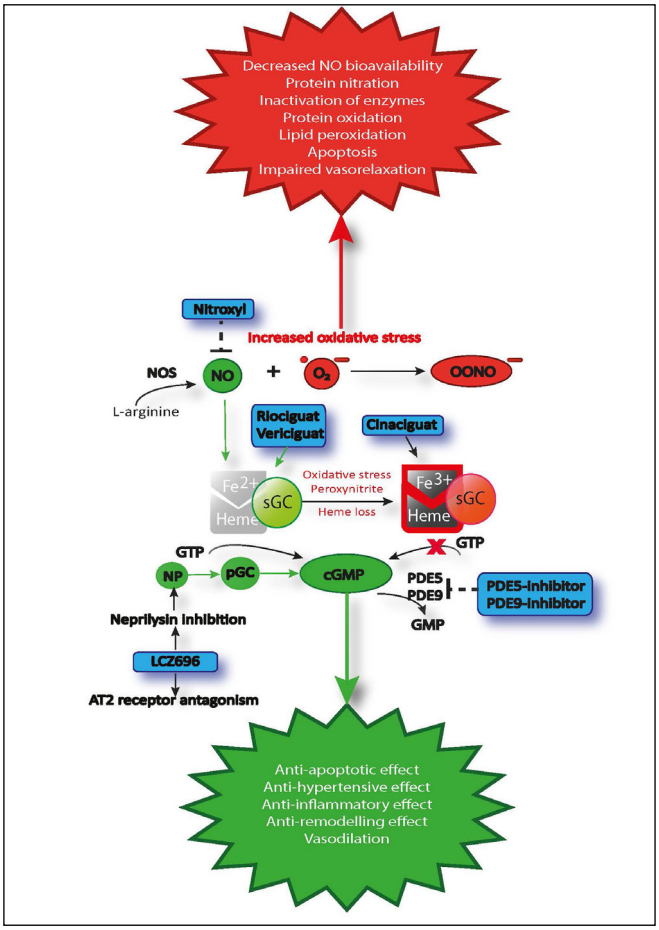

Figure 3. cGMP-PKG pathway modulation. In the normal situation, NO is generated from L-arginine by eNOS. NO stimulates sGC to catalize the formation of cGMP from GTP. However, cGMP can also be generated by stimulation of pGC by NPs. cGMP exerts its effects via PKG and both have diverse beneficial cardiovascular effects (green). In the situation of inflammation and/or increased oxidative stress, NO reacts rapidly with $O_2^{-\cdot}$ to form the toxic $ONOO^-$ with many deleterious cardiovascular effects (red). Moreover, inflammation and oxidative stress uncouple the NO producing eNOS dimer into $O_2^{-\cdot}$ generating monomers, further exacerbating oxidative stress. Another effect of oxidative stress and $ONOO^-$ is oxidation of sGC, which leads to loss of its heme-group, making sGC dysfunctional and unresponsive to NO and blocking cGMP formation. Diverse therapeutic strategies are shown in blue. Nitroxyl is better tolerated and more stable than nitrates and stimulates sGC. pGC can be stimulated by increasing the availability of NPs, which can be achieved with the combined neprilysin/angiontensin receptor blocker LCZ696. sGC in its inactive, NO resistant heme-free form can be targetted by cinaciguat, a sGC activator. Riociguat and vericiguat mimick NO and are able to directly stimulate sGC. Finally, cGMP can be inbitited by blocking PDEs, such as PDE5 and the (probably) myocardial specific PDE9. Abbreviations: NO, nitric oxide; eNOS, endothelial NO synthase; sGC, soluble guanylate cyclase; cGMP, cyclic guanosine 3',5'-monophosphate; GTP, guanosine 5'-triphosphate; pGC, particulate GC; NP, natriuretic petptide; PKG, protein kinase G; $O_2^{-\cdot}$, superoxide; $ONOO^-$, peroxynitrite; PDE, phosphodiesterase. Adapted and modified with permission from Hobbs and Stasch[61].

tachyarrhythmias or cardiotoxic drugs. In the case of HFpEF, an equivalent approach would be to treat overweight/obesity and related complications and achieve optimal control of glucose levels and blood pressure, as these maintain inflammatory processes and oxidative stress.

The role for exercise training in HFpEF is still not as clearly defined as in HFrEF. A recent meta-analysis demonstrated that exercise training improves cardiorespiratory fitness and quality of life in HFpEF patients without significant changes in systolic or diastolic LV function [94]. However, most studies only used E/A ratio (the ratio of peak early to late diastolic filling velocity) as a parameter to assess diastolic function, whereas one study showed improved exercise capacity and quality of life to be associated with atrial reverse remodeling and decreased E/E' in HFpEF patients after an exercise training program for 3 months[95]. Although these results are promising, the timing of a training program and the question whether intensity or duration of exercise training is more important still needs to be investigated and is studied an ongoing trial (NCT02078947).

Other therapeutic strategies target one or more steps of HFpEF pathophysiology, some of which will be briefly discussed next. Tetrahydrobiopterin (BH4) is an essential cofactor for eNOS function and prevents generation of superoxide release from uncoupled eNOS[96]. Although experimental data was promising, clinical studies with BH4 supplementation have been limited and have produced disappointing results so far[97,98]. A class of drugs with proven anti-inflammatory effects are statins, which can also limit oxidative stress in the endothelium[99]. In a recent meta-analysis, statin therapy was associated with a trend towards improved survival in HFpEF, but these findings require confirmation in randomized controlled trials[100]. Resveratrol, an antioxidant and constituent of red wine, berries and peanuts, has been studied extensively in animal models in which it decreased vascular inflammation and oxidative stress and improved endothelial function[101]. However, data from clinical studies are still not sufficiently robust to justify supplementation at this time[102].

Besides the previously discussed weight loss and the PDE5 inhibitor sildenafil, other pharmaceuticals are available that increase cGMP content (Figure 3 and Table 1). cGMP concentrations are regulated via the sGC receptor by NO or via the pGC receptor by NPs, as discussed above[58,59]. Stimulation of these receptors increases cGMP-PKG signalling. Whereas tolerance is a general issue for NO donors, nitroxyl (HNO, a reduced form of NO) is both better tolerated and more stable. Indeed, HNO increased cGMP concentrations and had NOX

suppressing and antihypertrophic actions in rat cardiomyocytes[103]. A recent study with a more translational focus demonstrated that HNO can reduce left and right ventricular filling pressures and systemic vascular resistance in diverse animal models and patients with HFrEF[104]. However, effects in HFpEF still need to be determined. Neprilysin is the enzyme responsible for the breakdown of natriuretic peptides and a new compound, LCZ696, is a combined angiotensin II receptor antagonist -neprilysin inhibitor[105]. LCZ696 was recently shown to be superior to enalapril in reducing the risks of death and hospitalization for HF in a large HFrEF population with LVEF ≤ 40%[106]. LCZ696 was well tolerated in a phase II trial in HFpEF and produced lower levels of NT-proBNP, smaller left atrial volumes, improved functional class and glomerular filtration rates compared to valsartan at 12 weeks. These effects were independent of a reduction in systolic blood pressure[107,108]. Whether these outcomes will translate to an improved HFpEF prognosis has to be awaited and is currently studied in a multicenter (NCT01920711).

sGC can also be activated and stimulated directly. Preclinical studies with the direct sGC activator, cinaciguat, unloaded the heart, increased cardiac output and renal blood flow, and preserved glomerular filtration rate and sodium and water excretion without further neurohumoral activation[109]. However, a phase II trial of intravenous administration of cinaciguat was terminated early due to hypotension even at low doses, but also failed to show an effect on dyspnea and cardiac index[110]. sGC can also be directly stimulated via compounds that mimic NO. The concept of direct sGC stimulation is based on its relative resistance to NO in situations of increased oxidative stress[61]. In phase III trials in patients with pulmonary arterial hypertension and chronic thromboembolic PHT, the oral sGC stimulator riociguat significantly improved symptoms, exercise capacity, pulmonary vascular resistance and NT-proBNP levels[111,112]. Riociguat was also well-tolerated in patients with PHT caused by systolic left ventricular dysfunction, and resulted in improved symptoms, cardiac index and pulmonary and systemic vascular resistance[113]. Currently, the oral sGC stimulator vericiguat (BAY 1021189) is in phase II trial to study its pharmacodynamic effects, safety and tolerability at 12 weeks compared with placebo in patients with HFrEF and in patients with HFpEF[114].

In addition to PDE5, PDE9 was recently shown to be expressed in the mammalian heart (including human) and to be upregulated in hypertrophy and HF. PDE9 regulates NP rather than NO-stimulated cGMP in cardiomyocytes and its

inhibition protects against pathological responses to neurohormones *in vitro* and sustained pressure overload *in vivo*[115]. PDE9 expression is increased in the LV myocardium of patients with hypertrophy due to AS (pressure overload), and even more so in patients with HFpEF. These data suggest that inhibition of PDE9 activated PKG and might blunt pathological stress responses. Both PDE5 and PDE9 regulate cGMP-PKG activity, and in combination they could be beneficial in the treatment of HFpEF and thus need to be considered for future research.

CONCLUSION

Metabolic comorbidities are highly prevalent in HFpEF and are probably the driving force in the underlying pathophysiology, causing a chronic, low-grade endothelial inflammation that generates oxidative stress. As a consequence of oxidative stress NO bioavailability is reduced, leading to a subsequent downregulation of the cGMP-PKG pathway and titin hypophosphorylation (a known characteristic of increased myocardial passive stiffness). Many promising treatment strategies that could potentially restore this pathway are currently under study or under development. However, since no pharmacological therapy has yet proven successful in HFpEF, in the meantime a top-down approach of stringent control of comorbidities would probably be the most pragmatic strategy.

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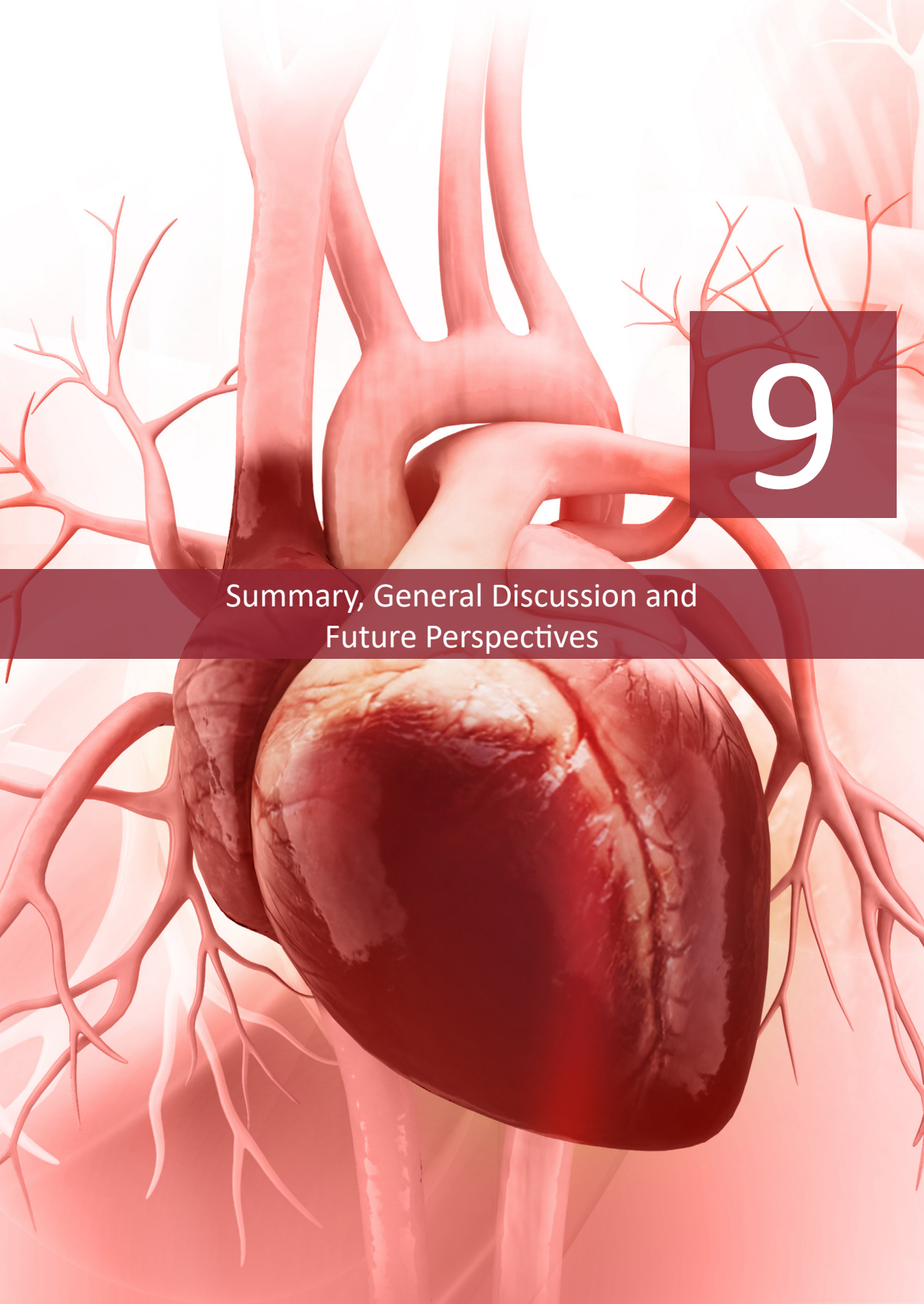

9

Summary, General Discussion and Future Perspectives

SUMMARY, GENERAL DISCUSSION AND FUTURE PERSPECTIVES

HFpEF pathophysiology

In the current thesis we explored the pathophysiological mechanisms underlying heart failure with preserved ejection fraction (HFpEF) and tried to define future diagnostic and therapeutic options based on this knowledge.

In **Chapter 2**, a ZSF1-rat model for HFpEF is characterized.[1] ZSF1-rats are hybrids based on a cross between a ZDF female and a SHHF male rat. This leads to a leptin resistant, spontaneously hypertensive rat that develops obesity and diabetes mellitus (DM). Interestingly, only obese rats developed the HFpEF-phenotype (further named ZSF1-HFpEF rats).[1] Lean, hypertensive ZSF1-rats without leptin resistance did not develop a HFpEF-phenotype.[1] This clearly indicates the importance of metabolic comorbidities in the development of HFpEF.[2] Post-mortem analysis on ZSF1-HFpEF rats demonstrated increased passive stiffness ($F_{passive}$) of myocardial muscle strips and single cardiomyocytes. The increased $F_{passive}$ was predominantly attributable to titin hypophosphorylation on specific sites of the N2Bus and PEVK segments of titin.[1] In contrast, there was no increased myocardial fibrosis or collagen deposition in ZSF1-HFpEF rats.

The study described in **Chapter 3** explores how metabolic comorbidities lead to increased myocardial $F_{passive}$ via systemic, low-grade inflammation.[3] In the myocardium of HFpEF patients and ZSF1-HFpEF rats, markers for inflammation and oxidative stress were measured. E-selectin and intercellular adhesion molecule (ICAM)-1 expression levels were upregulated in both human and rat HFpEF samples, indicating microvascular inflammation. Also, NADPH oxidase 2 expression as a marker of oxidative stress was raised in HFpEF. However, NADPH was only increased in macrophages and endothelial cells but not in cardiomyocytes. These findings indicate that the *primum movens* in HFpEF pathophysiology seems to be endothelial inflammation and activation and not a cardiomyocyte related problem. In HFpEF myocardium, endothelial nitric oxide synthase (eNOS) was shown to be uncoupled. eNOS-uncoupling was associated with reduced myocardial nitrite/nitrate concentration, cyclic guanosine monophosphate (cGMP) content, and protein kinase G (PKG) activity.[3] In other words, a reduction of NO-dependent signalling from endothelial cells to cardiomyocytes was observed. This reduced

signalling can contribute to the high cardiomyocyte $F_{passive}$ and hypertrophy observed in HFpEF.

Titin hypophosphorylation leading to increased cardiomyocyte $F_{passive}$ was reported in previous studies to be a key finding in HFpEF.[1,4–7] However, manipulation of titin phosphorylation state could not explain all the changes observed in cardiomyocyte based passive stiffness, as demonstrated in **Chapter 7**. Dephosphorylation of single cardiomyocytes from explanted donor hearts increased passive stiffness. However, $F_{passive}$ in aortic stenosis (AS) or dilated cardiomyopathy (DCM) cardiomyocytes still exceeded $F_{passive}$ in dephosphorylated donor cardiomyocytes. Incubation in an acidic environment and, more important, performing a prestretch, raised $F_{passive}$ in donor cardiomyocytes to values observed in diseased hearts. Interestingly, *in vitro* incubation with the small heat shock protein α-B crystallin decreased $F_{passive}$ to donor baseline levels in donor as well as in diseased cardiomyocytes. The mechanism behind this is supposed to be aggregation of titin due to (myocardial) stretch, present in conditions such as heart failure. Stretch unfolds immunoglobulin-like (Ig) domain-containing regions of titin, which can aggregate, especially under acidic conditions.[8] This aggregation is prevented by α-B crystallin.[8]

These findings raise the hypothesis that there is a continuum in HFpEF pathophysiology. This pathophysiology might start with (metabolically induced) endothelial inflammation and activation and leads to decreased NO-bioavailability. Subsequently, the cardiomyocytes develop a relative hypophosphorylation of titin, which leads to increased passive stiffness. These processes probably set in rapidly.[9] In a more advanced phase, where wall stress and cardiomyocyte stretch increase, titin aggregation can be expected to play a more prominent role in causing increased passive stiffness. In a last stage, the HFpEF myocardium is characterized by increased collagen deposition and fibrosis, as reviewed in **Chapter 4**.[10] These stages in HFpEF pathophysiology are probably the basis for future diagnostic and therapeutic strategies, as will be discussed next.

HFpEF diagnosis

The diagnosis of HFpEF remains challenging and requires signs or symptoms of congestion, preserved or mildly abnormal LV systolic function (EF>50%, end-diastolic volume index <97 mL/m^2) and diastolic LV dysfunction.[11] Diastolic LV

dysfunction is defined as the inability of the ventricle to fill to a normal preload volume at low pressures. It can be diagnosed invasively by measurement of an increased pulmonary capillary wedge pressure (PCWP), LV end-diastolic pressure or prolonged LV isovolumic relaxation.[11] Doppler echocardiography guides non-invasive diagnosis with an E:E' >15 (ratio of early transmitral diastolic flow velocity to tissue Doppler early mitral annular diastolic velocity). When E:E' is in the "grey zone" of 8–15, secondary evidence for diastolic dysfunction is needed. This evidence can be based on echocardiographic parameters (left atrial size, transmitral and pulmonary flow velocities, LV hypertrophy), the presence of atrial fibrillation or increased natriuretic peptides.[11]

As reviewed in **Chapter 8**, the current diagnostic criteria for HFpEF have some limitations.[12] First of all, many patients only develop symptoms during exercise, but the current diagnostic criteria use measurements at rest. Diastolic stress-testing will probably play an important role in the near future in HFpEF-diagnosis. Ideally echocardiography is a cornerstone in the diagnosis, since it is readily availably and non-invasive. However, limitations of echocardiography in HFpEF diagnosis are recently recognized both at rest and during exercise. For example, even despite high values of invasively measured PCWP, E:E' can sometimes be misleading.[13] Even more important, within individual patients E:E' does not reliably track changes in left-sided filling pressures (PCWP), making its usefulness debatable in HFpEF.[14] Many protocols and parameters have been tested during exercise. A change in E:E' was the most frequently used measurement, but currently there is insufficient evidence to use this or any other specific parameter or protocol for routine use when evaluating patients suspected of having HFpEF.[15]

At the moment, exercise testing with measurement of invasive hemodynamics seems a promising approach in HFpEF diagnosis since already a limited workload induces a significant increase in PCWP (≥25mmHg) in HFpEF patients.[16–18] Of interest is also the finding that invasive exercise hemodynamics unmasked diastolic dysfunction in symptomatic patients with a tentative diagnose of primary pulmonary hypertension and normal PCWP at rest.[19] This reclassifies these pulmonary hypertension patients as HFpEF patients as discussed in **Chapter 6**.[20] Also, since patients are usually under fluid restriction at the time of conventional testing, exercise hemodynamics may identify HFpEF, especially in obese patients with a dilated left atrium and

presenting with pulmonary hypertension.[20] These findings indicate the importance of exercise testing with invasive hemodynamic measurements in patients in whom HFpEF is suspected.

A second pitfall in the current diagnostic criteria for HFpEF is the use of natriuretic peptides as biomarkers. It is known that normal levels of natriuretic peptides do not rule out HFpEF.[13] As discussed in **Chapter 5**, many recent studies focused on biomarkers reflecting the inflammatory and profibrotic changes in the myocardium and extracellular matrix.[21] This is a potential advantage above echocardiography. Although none of these biomarkers has a place in clinical practice yet, MMP9 and TIMP1, for example, were shown to have a potential role in predicting the development of heart failure in patients with asymptomatic LV diastolic dysfunction[22]. This might help to identify a population of patients at risk for HFpEF that would benefit most from cardiovascular risk reduction strategies.

Currently, especially ST2 and galectin-3 predict prognosis in HFpEF.[23] Besides diagnosis and prognosis, biomarkers might identify patients that potentially benefit most from certain treatment options. For example, higher levels of soluble ST2 and galactin-3 correlated with more reduction in left atrial size in patients treated with LCZ696 (valsartan/sacabutril).[24] The finding that biomarkers may identify patients that benefit more from specific therapies than other patients brings us to future therapeutic options, for which this "staging of HFpEF" will be of great help.

HFpEF treatment

In contrast to heart failure with reduced ejection fraction (HFrEF), the current guidelines on heart failure from the Heart Failure Association (HFA) of the European Society of Cardiology (ESC) lack evidence based treatment options for HFpEF and advice the clinician to reduce congestion with diuretics and to treat comorbidities.[25] As reviewed in **Chapter 8**, no treatment strategy studied to date in large HFpEF trials has proven to improve disease progression and survival, including betablockers,[26] angiotensin-converting enzyme inhibitors,[27] angiotensin receptor blockers,[28,29] and digoxin.[30] A subanalysis of the TOPCAT trial[31] (which had a neutral outcome) suggested that spironolactone may be more beneficial in older patients with more advanced HFpEF, comorbidities and a higher BNP than in younger patients with fewer comorbidities, less

severe HFpEF and a lower BNP[32]. This again that a staging strategy in HFpEF may be helpful.

Based on the expanding knowledge about HFpEF pathophysiology, newer treatment options aiming to restore the soluble guanlyate cyclase (sGC)-cGMP-PKG pathway were more recently explored and they are reviewed in **Chapter 8**. Unfortunately, chronic treatment with the phosphodiesterase-5 inhibitor sildenafil did not improve exercise capacity and clinical status in the RELAX trial.[33] Also, sildenafil failed to increase plasma cGMP concentrations or yield hemodynamic benefits.[33] In this study, plasma levels of N-terminal pro-BNP (NT-proBNP) and prevalence of atrial fibrillation were high. This indicates that patients were at an advanced stage of HFpEF and therefore less likely to benefit from a limited strategy involving only inhibition of cGMP breakdown.[34] Direct stimulation of sGC with oral vericiguat is currently under investigation in HFpEF (NCT01951638).

The first-in-class angiotensin receptor inhibitor neprilysin inhibitor (ARNI) is a combination of the angiotensin receptor blocker valsartan and the neprilysin inhibitor sacubitril. Neprilysin inhibition leads to increased stimulation of particulate GC via natriuretic peptides and hence to upregulation of cGMP/PKG. In HFrEF, valsartan/sacubitril was superior to enalapril in reducing the risks of death and of hospitalization for heart failure.[35] In HFpEF, valsartan/sacubitril reduced NT-proBNP to a greater extent than did valsartan at 12 weeks and was well tolerated in a Phase II-study (PARAMOUNT).[36] A PARAMOUNT-substudy demonstrated that biomarkers that reflect collagen homeostasis, such as soluble ST2 and galectin 3, correlated with the presence and severity of HFpEF.[24] Furthermore, higher levels of soluble ST2 and galectin 3 predicted a more pronounced decrease in left atrial volumes after treatment with valsartan/sacubitril, suggesting that patients with more advanced HFpEF might benefit most from this new drug.[24]

Although these recent studies have some promising results, the phenotypic diversity of HFpEF patients is increasingly recognized and probably personalized therapeutic strategies with combinations of treatment modalities are needed.[37] An approach that requires further research would be to classify patients into different stages of HFpEF based on disease severity. Subsequently, various treatment strategies can be studied according to their HFpEF-stage. For example, anti-inflammatory treatments aimed at restoring the NO-sGC-cGMP-PKG-pathway might be more effective in early stages. These NO-sGC-cGMP-

PKG restoring therapies will probably be less successful when myocardial fibrosis or atrial fibrillation suggest a more advanced HFpEF, as suggested by the negative results of the RELAX-trial.[34] Later stages might benefit more from therapeutic efforts to raise concentrations of α-B crystallin, which is able to decrease cardiomyocyte stiffness *in vitro*, as demonstrated in **Chapter 7**. This might be achieved through direct administration of α-B crystallin, through administration of α-B crystallin analogues or through administration of heat shock protein inducing drugs such as geranylgeranylacetone (GGA) or NYK9354. The most advanced stages of HFpEF are probably characterized by marked remodeling of the extracellular matrix. In an advanced stage patients might benefit more from treatment with valsartan/sacubitril or spironolactone, as suggested by the PARAMOUNT and TOPCAT substudies, respectively.[24,32]

CONCLUSION

The pathophysiological mechanisms in HFpEF are gradually becoming unraveled and non-cardiac comorbidities drive a systemic inflammatory state that also affects the coronary microvasculature. This coronary microvascular inflammation induces oxidative stress and leads to reduced NO-dependent signaling to cardiomyocytes with a subsequent PKG-deficit and increased passive stiffness and diastolic dysfunction. In probably more advanced stages, titin aggregation and myocardial fibrosis develop, further aggravating passive stiffness. Future therapeutic options will need to target the different aspects contributing to increased myocardial stiffness. Personalized treatment strategies are required and in order to make this feasible, a classification system for HFpEF is needed. Whether optimal classification is based on phenotypic characteristics, invasive hemodynamic findings, biomarkers or a combined approach, will be topic of future studies.

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10

Nederlandse samenvatting voor niet-ingewijden

Dankwoord

Curriculum vitae

List of publications

List of active congress participations

NEDERLANDSE SAMENVATTING VOOR NIET-INGEWIJDEN

In deze thesis zijn wij op zoek gegaan naar de pathofysiologische processen die aan de basis liggen van hartfalen met bewaarde linker ventrikel ejectiefractie (HFpEF). Bij dit type van hartfalen, is de pompkracht van de linker ventrikel bewaard, maar is de hartspier stijver en als gevolg hiervan de druk in het hart hoger. Dit uit zich bij de patiënt met name als kortademigheid.

Om meer over de pathofysiologie te weten te komen, maakten we onder andere gebruik van een diermodel waarbij ratten met arteriële hypertensie geen verzadigingsgevoel kenden en bleven eten. Op deze manier ontwikkelden deze dieren niet alleen obesitas en diabetes mellitus, maar ook HFpEF. Een opvallende bevinding was dat alleen dieren met obesitas en diabetes mellitus HFpEF ontwikkelden, wat het belang van deze metabole comorbiditeiten onderstreept. Kleine stukjes hartspierweefsel van deze ratten werden onderzocht op hun stijfheid in een proefopstelling waarbij de kracht gemeten werd die de spier ontwikkelde wanneer deze uitgerekt werd. De spiertjes van ratten met HFpEF bleken een hogere kracht te ontwikkelen bij uitrekken en dus stijver te zijn dan bij ratten zonder HFpEF.
Stijfheid van de hartspier wordt bepaald door een samenspel van de hartspiercellen en het bindweefselnetwerk (collageen) eromheen. In het gebruikte diermodel bleek duidelijk dat de stijfheid voornamelijk veroorzaak werd door de hartspiercellen, terwijl het bindweefsel nauwelijks een rol speelde. Verder bleek dat de stijfheid van de hartspiercellen in de proefopstelling kon normaliseren door te corrigeren voor een fosforylatietekort met proteïne kinase G (PKG). PKG grijpt aan op titine, het grootste eiwit van ons lichaam en verantwoordelijk voor de stijfheid van hartspiercellen.

Een vervolgonderzoek in deze HFpEF ratten toonde aan dat de metabole comorbiditeiten (obesitas en diabetes mellitus) tot een ontstekingsreactie leiden. Wij verkregen daarnaast bewijs dat dit ook de bekleding aan de binnenkant van de kleine bloedvaatjes (capillair endotheel) van het hart treft en daar leidt tot oxidatieve stress met een verminderde beschikbaarheid van stikstofmono-oxide (NO). NO is een zeer belangrijk signalisatiemolecule in het menselijk lichaam en stimuleert onder andere de aanmaak van PKG. Een tekort aan NO was inderdaad gekoppeld aan een verminderde PKG-activiteit. Deze bevindingen werden bevestigd in menselijke HFpEF-biopten.

In een laatste studie bestudeerden we de precieze mechanismen waardoor titine stijver wordt. Aangezien in hartspierbiopten van mensen met hartfalen de toegenomen stijfheid niet volledig normaliseerde met PKG, maar ook niet toe te schrijven was aan collageen, werd gezocht naar een andere factor. Met toediening van een klein heat-shock eiwit (α-B Crystallin) dat beschermende effecten uitoefent als reactie op stress-condities bleek de stijfheid wel volledig te normaliseren. Dit suggereert dat verschillende titinemoleculen door forse uitrekking in een zieke hartspier aan elkaar vast kunnen komen te zitten, aangezien α-B Crystallin juist dit voorkomt.

Voorlopig is er voor HFpEF nog geen wetenschappelijk onderbouwde behandeling. De huidige kennis van de pathofysiologie biedt echter verschillende therapeutische aanknopingspunten, maar een juiste selectie van patiënten en hun ziektestadium lijkt essentieel om in verschillende studies de juiste doelgroep en farmaca aan elkaar te koppelen. Hiervoor zal in de toekomst een verandering in de diagnostiek van HFpEF noodzakelijk zijn waarbij de focus lijkt te verschuiven naar het stadiëren van patiënten. Inspanningstesten tijdens invasieve hemodynamicastudies kunnen bijvoorbeeld patiënten in een vroeg stadium identificeren, terwijl verschillende biomarkers voor verhoogde collageenvorming vooral latere stadia kunnen markeren. Door therapeutische opties te ontwikkelen die gebaseerd zijn op de specifieke kennis van HFpEF pathofysiologie en dit in de juiste patiënten te onderzoeken, lijkt een behandeling voor HFpEF in de toekomst een reële mogelijkheid.

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CURRICULUM VITAE

Constantijn Franssen, de auteur van dit proefschrift, werd op 9 september 1980 geboren in Heerlen en groeide met zijn oudere broer Gaston op in Langraaf. Na het behalen van het gymnasium diploma aan het voormalige College Rolduc in Kerkrade, begon Constantijn aan de Geneeskunde opleiding in België. De eerste 3 jaar van deze opleiding vonden plaats aan het Limburgs Universitair Centrum van Diepenbeek, de huidige Universiteit Hasselt. Na het behalen van het Kandidaat Arts diploma, vervolledigde hij zijn opleiding cum laude aan Universiteit Antwerpen in 2005. In 2005 begon Constantijn aan de specialisatie tot cardioloog bij Prof. Dr. C. Vrints en de erkenning als cardioloog behaalde hij in 2011.

Tijdens de opleiding was er al een duidelijke interesse in wetenschappelijk onderzoek en participeerde Constantijn al vrijwillig aan verschillende klinische en preklinische projecten. Deze interesse verklaart dan ook waarom hij in 2011 startte aan een PhD-project bij Prof. Dr. W.J. Paulus op de afdeling Fysiologie van het VUmc in Amsterdam. Dit project vond plaats binnen een beurs van de Europese Commissie: FP7-Health-2010; MEDIA (MEtabolic Road to DIAstolic Heart Failure; 261409).

Naast het PhD-project bleef Constantijn zijn klinische activiteiten onderhouden. Initieel vond dit gedurende enkele jaren als niet-invasieve cardioloog plaats in het Universitair Ziekenhuis Brussel. Sinds december 2015 is Constantijn weer werkzaam in het Universitair Ziekenhuis Antwerpen, waar hij binnen het hartfalen en harttransplantatieteam werkt.

Ondanks dat de opleiding, het PhD-project en het werk veel tijd vragen, heeft Constantijn altijd veel gezwommen, hardgelopen en gefietst. Hij woont samen met Sophie Van Malderen in het Belgische Wintam en samen zijn zij de trotse ouders van hun oogappel Mira.

LIST OF PUBLICATIONS

1. Three-dimensional look at internal cardiodefibrillator-leads and right atrial thrombus. Franssen C, Conraads V, Vrints CJ. Europace. 2011;13:1427.

2. The future diagnosis of heart failure with normal ejection fraction: less imaging, more biomarkers? Franssen C, Paulus WJ. Eur J Heart Fail. 2011;13:1043-5.

3. Heart Failure with Preserved Ejection Fraction. Franssen C, Paulus WJ. Neth J Crit Care. 2012;16:125-32.

4. Molecular and cellular basis for diastolic dysfunction. Van Heerebeek L, Franssen C, Hamdani N, Verheugt FW, Somsen GA, Paulus WJ. Curr Heart Fail Rep. 2012;9:293-302.

5. Myocardial titin hypophosphorylation importantly contributes to heart failure with preserved ejection fraction in a rat metabolic risk model. Hamdani N*, Franssen C*, Lourenço A*, Falcão-Pires I, Fontoura D, Leite S, Plettig L, López B, Ottenheijm CA, Becher PM, González A, Tschöpe C, Díez J, Linke WA, Leite-Moreira AF, Paulus WJ. Circ Heart Fail. 2013;6:1239-49.

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6. Normal resting pulmonary artery wedge pressure: a diagnostic trap for heart failure with preserved ejection fraction. Franssen C and Paulus WJ. Eur J Heart Fail. 2015;17:132-4.

7. Myocardial Microvascular Inflammatory Endothelial Activation in Heart Failure With Preserved Ejection Fraction**.** Franssen C, Chen S, Unger A, Korkmaz HI, De Keulenaer GW, Tschöpe C, Leite-Moreira AF, Musters R, Niessen HWM, Linke WA, Paulus WJ, Hamdani N. JACC Heart Fail 2016;4:312-24.

8. Communication lessons in Heart Failure, Cell-Cell communication, mini-reviews series on the HFA Winter Research Meeting 2014. Bang C, Antoniades C, Antonopoulos AS, Eriksson U, Franssen C, Hamdani N, Lehmann L,

Moessinger C, Mongillo M, Muhl L, Speer T, Thum T. Eur J Heart Fail, 2015;17:1091-103.

9. From Comorbidities to Heart Failure with Preserved Ejection Fraction: A Story of Oxidative Stress. Franssen C, Chen S, Hamdani N, Paulus WJ. Heart. 2016;102:320-30.

10. The role of titin and extracellular matrix remodelling in HFpEF. Franssen C, González Miqueo A. Neth Heart J. 2016;24:259-67.

11. Sarcomeric accumulation of α-B Crystallin reverses high diastolic stiffness of failing human cardiomyocytes. Franssen C, Kole J, Musters R, Hamdani N, Paulus WJ. In Revision, Circ Heart Fail.

12. Nieuwe ontwikkelingen in hartfalen met bewaarde linker ventrikel ejectiefractie – licht aan het eind van een lange tunnel? Franssen C, Vrints C. Tijdschrift voor Cardiologie. 2016;1:23-31.

13. Ten Years after early-onset preeclampsia; cardiovascular assessment. Bokslag A, Teunissen PW, Franssen C, Van Kesteren F, Kamp O, Ganzevoort W, Paulus WJ, De Groot CJM. In revision, American Journal of Obstetrics & Gynaecology.

LIST OF ACTIVE CONGRESS PARTICIPATIONS

10/2012 Oral presentation: ICaR-VU Physiology meeting, Amsterdam

11/2012 Poster presentation: Dutch Physiological Society ("Papendal symposium"), Amsterdam

01/2013 Poster presentation: Heart Failure Association Winter Research Meeting, Les Diablerets

03/2013 Poster presentation: American College of Cardiology, San Francisco

05/2013 Moderated Poster presentation: ESC Heart Failure Congress, Lisbon

11/2013 Poster presentation: European Muscle Conference, Amsterdam

11/2013 Oral presentation: American Heart Association Scientific Sessions, Dallas

12/2013 Oral presentation: Annual FP7-Media consortium meeting, Leiden

01/2014 Oral presentation: Heart Failure Association Winter Research Meeting, Les Diablerets

02/2014 Oral presentation: ICaR-VU Physiology meeting, Amsterdam

03/2014 Poster presentation: American College of Cardiology, Washington

05/2014 Poster presentation: ESC Heart Failure Congress, Athens

11/2014 Poster presentation: American Heart Association Scientific Sessions, Chicago

11/2014 Moderated poster presentation: American Heart Association Scientific Sessions, Chicago

01/2015 2 Poster presentations: Heart Failure Association Winter Research Meeting, Les Diablerets

05/2015 Invited presentation ESC Heart Failure Congress, Seville

05/2015 Poster presentation: ESC Heart Failure Congress, Seville

12/2015 Oral presentation: Annual FP7-Media consortium meeting, Leiden

12/2015 Oral presentation: LOK-meeting Cardiology University Hospital Antwerp

03/2016 Oral presentation: Symposium Cardiology University Hospital Antwerp

05/2016 Oral presentation: Hesperis course of the ESOT, Rome

05/2016 Poster presentation: ESC Heart Failure Congress, Florence