UCLouvain

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# Heart failure with preserved ejection fraction: from comorbidities to endothelial dysfunction, exploring the road through inflammation and oxidative stress.

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# THESIS OUTLINE

Heart failure with preserved ejection fraction (HFpEF) affects millions of people worldwide, with significant repercussion on their life expectancy and quality of life. Despite major scientific interest, its pathophysiology is still incompletely understood and its diagnosis often missed, owing to a great heterogeneity in clinical presentations. In this context, our main objective was to characterize patients with HFpEF regarding clinical, biological and imaging data, and to analyse the impact of metabolic comorbidities (obesity and diabetes mellitus) on phenotype and prognosis. Then, we dug further into the role played by oxidative stress, endothelial dysfunction and nitric oxide imbalance in the development of the disease.

**Chapter 1** introduces the notion of HFpEF. Typical symptoms, cardiac and extra-cardiac findings, and guidelines for diagnosis and treatment are described from the perspective of the clinician. Then, we dive into cellular and molecular mechanisms incriminated in the development of the disease, with a special focus on inflammation, oxidative stress, endothelial dysfunction and nitric oxide imbalance. We finally address translational aspects and outline results of clinical trials targeting aforementioned mechanisms. After stating the aims of the thesis in **Chapter 2,** the methods and results are structured in **Chapter 3** comprising three manuscripts. Article 1 describes our Belgian cohort of patients with HFpEF, compares their characteristics with populations from the literature and underlines the impact of body mass index on presentation and prognosis. Article 2 focuses on particularities of diabetic patients with HFpEF. Article 3 (under review) explores oxidative stress (myeloperoxidase levels) and vascular function. The last part of Chapter 3 contains unpublished data on the state of nitric oxide. Finally**, Chapter 4** summarizes the main findings of this work and lays out future directions.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| AF | Atrial fibrillation |
| AGE | Advanced glycation end-products |
| AI | Augmentation index |
| AR | Adrenergic receptor |
| BH2 | Dihydrobiopterin |
| BH4 | Tetrahydrobiopterin |
| BMI | Body mass index |
| BNP | Brain natriuretic peptide |
| CBF | Coronary blood flow |
| CFR | Coronary flow reserve |
| CMD | Coronary microvascular dysfunction |
| CRP | C-reactive protein |
| ED | Endothelial dysfunction |
| EPR | Electron paramagnetic resonance |
| ESC | European society of cardiology |
| FMD | Flow mediated dilation |
| sGC | soluble Guanylate cyclase |
| GFR | Glomerular filtration rate |
| cGMP | cyclic Guanosine monophosphate |
| HBP | Hexosamine biosynthesis pathway |
| HF | Heart failure |
| HFA | Heart failure association |
| HFPEF | Heart failure and preserved ejection fraction |
| IL | Interleukin |
| IRE1α | Inositol-requiring protein 1α |
| LA | Left atrium |
| LV | Left ventricle |
| LVEF | Left ventricular ejection fraction |
| MBF | Myocardial blood flow |
| MCHC | Mean cell hemoglobin concentration |
| MPO | Myeloperoxidase |
| NAD+ | Nicotinamide adenine dinucleotide |
| L-NAME | L-NG-Nitro arginine methyl ester |

| | |
|---|---|
| NLR | Neutrophile to lymphocyte ratio |
| NO | Nitric oxide |
| NOS | Nitric oxide synthase |
| NYHA | New York Heart Association functionnal status |
| PAT | Pulse amplitude tonometry |
| PBMC | Peripheral blood mononuclear cellls |
| RT-qPCR | Reverse transcription quantitative polymerase chain reaction |
| PDE | Phosphodiesterase |
| PKG | Protein kinase G |
| RHI | Reactive hyperemia index |
| ROS | Reactive oxygen species |
| SGLT | Sodium glucose linked transporter |
| SIRT | Sirtuin |
| TGF-β | Transforming growth factor beta |
| TNF-α | Tumor necrosis factor alpha |
| UPR | Unfolded protein response |
| URAT1 | Uric acid transporter 1 |
| XBP1s | X-box-binding protein 1 spliced |

# 1. INTRODUCTION

## 1.1 HFpEF, the clinical perspective

### 1.1.1 Definition

Heart failure (HF) is defined as "the inability of the heart to pump blood to the body at a rate commensurate with its needs, or to do so only at the cost of high filling pressure."[1] Although this definition does not include left ventricular ejection fraction (LVEF), the first clinical trials on heart failure patients only involved patients with a reduced ejection fraction.[2] Over the years, the repeated observations that, despite the same signs and symptoms, a proportion of patients with HF did not have reductions in systolic function, led to the recognition that HF could also be the consequence of abnormalities in diastole. Rather than a reduction in the contraction capacity, a myocardial process impairing relaxation is at stake. First called "diastolic HF"[3,4], "heart failure with preserved ejection fraction" (HFpEF) became the most common term after the publication of a major outcome trial using that terminology (CHARM-preserved).[5] Ventricular relaxation depends on an active process of pressure decay during early diastole and on passive visco-elastic properties of the cardiomyocytes, the extracellular matrix, and the pericardium. Both active and passive components are affected in HFpEF.[3] Active relaxation relies on myofilament dissociation and calcium reuptake, and on elastic restoring forces built up during systole, generating an intraventricular pressure gradients needed for early diastolic suction. The passive component of relaxation was originally thought to be determined predominantly by collagen quantity and the qualities of the extracellular matrix. However, studies in the past decade pointed to the importance the cardiomyocyte itself. Cardiomyocyte hypertrophy was highly prevalent in HFpEF[6] and alteration in the phosphorylation state of the sarcomeric

macromolecule titin was demonstrated.[7-10] In HFpEF, increased fibrosis in the extracellular space and modifications within cardiomyocytes contributes to reduced ventricular compliance.[6,11] This reduction in compliance increases the slope of the ventricular end-diastolic pressure-volume relationship (EDPVR) and results in greater end-diastolic pressure (Figure 1.1)[3]. Pressure rising in the left ventricle is soon transmitted to the left atrium and the pulmonary vasculature. This can lead to pulmonary congestion and the main symptom of HFpEF: dyspnea.[12]

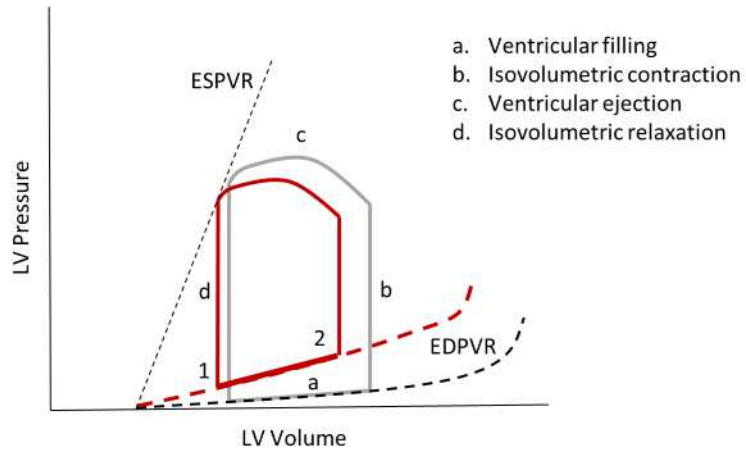



**Figure 1.1 Pressure – volume loops in the normal heart (grey) and in HFpEF (red)**
1. Impaired relaxation with incomplete decrease in LV pressure before ventricular filling occurs 2. Reduction in ventricular compliance, increases the slope of the ventricular EDPVR, resulting in less ventricular filling (decreased end-diastolic volume) and greater end-diastolic pressure.
LV: left ventricle, EDPVR: end-diastolic pressure-volume relationship, ESPRV: end-systolic pressure-volume relationship

The pathophysiology of HFpEF is more complex than merely an increase in myocardial stiffness and diastolic dysfunction (Figure 1.2). Although ejection fraction is within the normal range, around 30% of HFpEF hearts show some degree left ventricular hypertrophy and/ or of subtle systolic dysfunction with altered global longitudinal strain. Besides structural alterations, patients with HFpEF commonly suffer from chronotropic incompetence leading to reduced cardiac

output reserve on exertion[13,14] and from arrhythmias, mainly atrial fibrillation (AF). Left atrial enlargement due to elevated filling pressure forms a perfect substrate and the two disorders share common risk factors, which causes AF and HFpEF to develop in parallel.[15,16] Right ventricular (RV) dysfunction is also frequently described in HFpEF, present in at least 20% and up to 30 to 50% of patients.[17] Passive backward transmission of LV filling pressures induces an increase in venous pulmonary pressure and RV afterload. A precapillary component to pulmonary hypertension has also been incriminated, with local modification of the pulmonary vasculature secondary to the systemic proinflammatory state associated with HFpEF or to concomitant comorbidities (i.e., chronic obstructive pulmonary disease and sleep apneas).[18,19]

Renal dysfunction and HFpEF often co-exist and the progression of one disease aggravates the other. Venous congestion secondary to HFpEF causes decreased renal blood flow and renal perfusion pressure, leading to a reduction in glomerular filtration rate. In turn, renal impairment causes metabolic and systemic derangements in circulating factors contributing to an activated systemic inflammatory state and endothelial dysfunction, implicated in HFpEF pathophysiology. Hence, HFpEF might lead to renal dysfunction and vice versa, while the presence of common denominators cause both HFpEF and chronic kidney disease.[20,21]

Increased stiffness is not only seen in the heart, but also in large arteries such as the aorta. Invasive measurement of arterial waveforms shows reduced arterial compliance and higher arterial elastance at rest in HFpEF patients, independently of blood pressure.[22] In the microvasculature, the regulation by endothelial cells of reactive vasodilation in response to shear stress to meet tissular oxygen demands is impaired, and altered vasodilation is associated with poor prognosis.[13,23] Finally,

at the muscular level, HFpEF is associated with a reduction in slow oxidative type 1 fibers (rich in mitochondria and responsible for long lasting muscular tension) and a lower capillary to fiber ratio, which likely contributes to exercise intolerance.[24]

Overall the typical picture of HFpEF is an old patient, suffering from multiple interrelated diseases and risk factors, presenting with shortness of breath and functional limitations whose precise origin is difficult to determine.

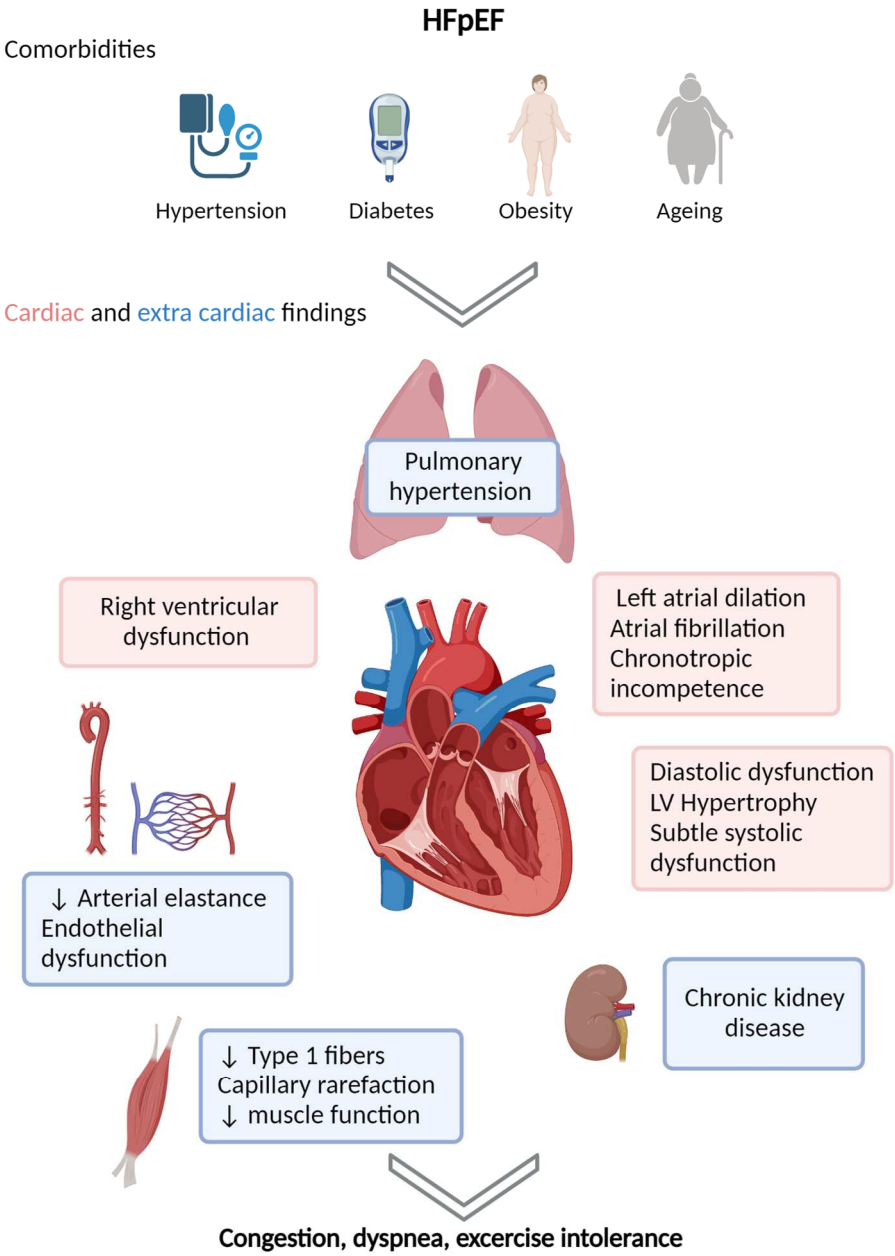



**Figure 1.2 Comorbidities, cardiac and extracardiac findings in HFpEF**
HFpEF typically occur in elderly patients with multiple comorbidities and is not limited to diastolic dysfunction but also include some degree of systolic dysfunction, repercussion on the pulmonary vasculature and on the right heart and alteration in vascular, muscle and renal function.

### 1.1.2 Epidemiology

The prevalence of heart failure is approximately 1–3% of the adult population in developed countries, rising to ≥10% among people older than 70 years.[25] The proportion of patients with a preserved ejection fraction ranges from 22 to 73%, depending on the definition applied and the clinical setting (primary care, hospital clinic, hospital admission). Compared with HFrEF, patients with HFpEF are older, predominantly women and have commonly a history of hypertension and atrial fibrillation, whereas a history of myocardial infarction is less common.[2,26,27]

Heart failure carries a poor prognosis, with a 5-year mortality rate of 40-75% after a first hospitalization for decompensated heart failure.[28,29] Epidemiological studies and clinical trials suggest that the incidence of cardiovascular mortality is lower, and non-cardiovascular mortality higher in HFpEF than in HFrEF. In contrast, rates and duration of hospitalization, and impairments in patient-reported outcomes such as quality of life appear similar in HFpEF and HFrEF.[2]

Due to aging of the population and increasing incidence of cardiovascular risk factors, the prevalence of HFpEF is expected to steadily increase in the coming decades.[25] This, together with the paucity of treatment, make HFpEF one of the greatest challenges in 21st-century cardiology.[30]

### 1.1.3 Diagnosis

The diagnosis of HFpEF remains challenging and a validated gold standard is still missing. Signs and symptoms of HF (Table 1.1) are often non-specific, especially in the typical elderly patient with co-morbidities presenting with dyspnea but no obvious signs of fluid overload. Therefore, the diagnosis of HFpEF relies on the presence of those signs and symptoms in combination with biological and echocardiographic findings. Most recent recommendations, based on an expert consensus from the Heart Failure Association (HFA) of the European society of

Cardiology (ESC), propose a step-wise approach with the HFA-PEFF (Pre-test assessment, Echocardiography & natriuretic peptide, Functional testing, Final etiology) algorithm[31] (Figure 1.3). Major (2 points) and Minor (1 point) criteria are derived from two domains of echocardiographic findings (functional and morphological), and on natriuretic peptide levels. A score higher than 5 points implies definite HFpEF; whereas a score less or equal to 1 makes HFpEF unlikely. Intermediate scores (2–4 points) indicate diagnostic uncertainty, in which case functional testing is recommended, with echocardiographic stress tests or invasive haemodynamic assessment of filling pressures [pulmonary capillary wedge pressure (PCWP) ≥15 mmHg or left ventricular end diastolic pressure (LVEDP) ≥16 mmHg]. Finally, an etiologic work up is recommended to establish a possible specific cause of HFpEF or alternative explanations.

**Table 1.1 : Signs and symptoms typical of heart failure**

| Symptoms | Signs |
|---|---|
| Typical | More specific |
| Breathlessness, orthopnoea<br>Paroxysmal nocturnal dyspnoea<br>Reduced exercise tolerance, fatigue<br>Ankle swelling | Elevated jugular venous pressure<br>Hepatojugular reflux<br>Third heart sound (gallop rhythm), laterally displaced apical impulse |
| Less typical | Less specific |
| Nocturnal cough<br>Wheezing<br>Bloated feeling<br>Loss of appetite<br>Confusion (especially in the elderly)<br>Depression<br>Palpitations<br>Dizziness<br>Syncope<br>Bendopnea | Weight gain (>2 kg/week)<br>Weight loss, cachexia (in advanced HF)<br>Cardiac murmur<br>Peripheral oedema, ascites<br>Pulmonary crepitations<br>Reduced air entry (pleural effusion)<br>Tachycardia, Irregular pulse<br>Tachypnoea<br>Cheyne Stokes respiration Hepatomegaly<br>Cold extremities<br>Oliguria<br>Narrow pulse pressure |
| Adapted from Ponikowski P, Voors AA, Anker SD, Bueno H, Cleland JG, Coats AJ, et al. 2016 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure: The Task Force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC). European journal of heart failure. 2016;18(8):891-975.[26] | |

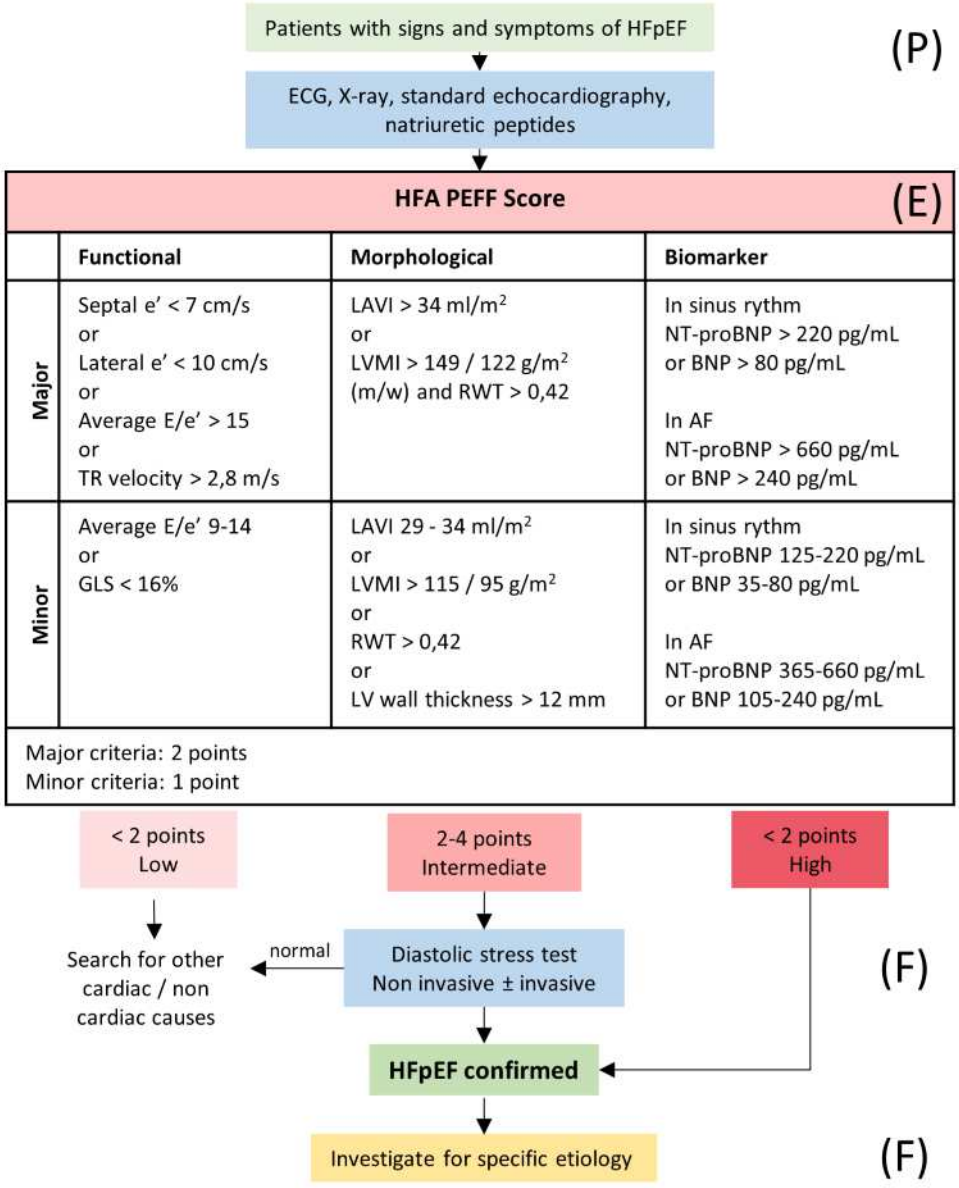



**Figure 1.3 The HFA PEFF diagnostic algorythm** is a stepwise approach including a prestest assessment (P), a diagnostic work up with echocardiography and natriuretic peptide (E), functional testing in case of uncertainty (F) and final aetiological work up (F)

Adapted from Pieske B, Tschöpe C, de Boer RA, Fraser AG, Anker SD, Donal E, et al. How to diagnose heart failure with preserved ejection fraction: the HFA-PEFF diagnostic algorithm: a consensus recommendation from the Heart Failure Association (HFA) of the European Society of Cardiology (ESC). European heart journal. 2019;40(40):3297-317.

### 1.1.4 Treatment

Up until last year, many articles on HFpEF started with "there is currently no treatment consistently improving prognosis in those patients". Indeed, clinical trials had been generally disappointing, with no benefit on mortality and marginal benefits on HF hospitalizations.[32] Symptomatic relief of congestion with diuretics and adequate control of comorbidities (in particular hypertension, diabetes mellitus and atrial fibrillation) were recommended. Recent results of clinical trials with sodium–glucose cotransporter 2 inhibitors (SGLT2i) will dramatically change the face of HFpEF therapeutics in the coming years. This class of medication is already recommended (2A) for HFpEF management in the American guidelines and it won't be long before they appear in the European guidelines as well. This is supported by the results of large randomized controlled trials, namely EMPEROR-Preserved, where Empalglifozin led to a 21% lower relative risk of cardiovascular death or hospitalization for heart failure (mainly driven by the reduction of hospitalizations).[33] This benefit was confirmed with Dapaglifozin in the lately published DELIVER trial.[34] Importantly, reduction of adverse events with Dapaglifozin was consistent for all ejection fractions. This contrasts with other treatments that had shown benefit only for patients at the lower end of the ejection fraction spectrum questioning the use of current cut offs of LVEF for patients' classification. Subgroup analysis of PARAGON showed a reduction in HF hospitalizations in those with an LVEF <57% (and in women) taking Sacubitril/valsartan[35], and in TOPCAT a significant reduction in the primary endpoint of cardiovascular death and HF hospitalization was observed for those with an LVEF <55% taking Spironolactone[36]. Hence these molecules may be considered in selected patients, while SGLT2 inhibitors can be used regardless of LVEF. Mechanisms of actions of SGLT2i in HFpEF will be discussed later in the manuscript (paragraph 1.3.1)

## 1.2 HFpEF, pathophysiology

### 1.2.1 Overview

Current understanding of molecular mechanisms underlying HFpEF links coexisting **comorbidities** to myocardial remodelling and dysfunction, via a systemic pro inflammatory state (Figure 1.4).[37] The higher prevalence of comorbidities in HFpEF compared to HFrEF led to the hypothesis that they play a causal role in the development of the disease.[38,39] The most important: **hypertension, obesity, diabetes mellitus**, chronic obstructive pulmonary disease, anemia, and chronic kidney disease all have the ability to induce **a systemic inflammatory state**. During inflammation, microvascular endothelial cells produce reactive oxygen species (hence **oxidative stress**), that react with nitric oxide (NO), forming peroxynitrite and limiting NO bioavailability. In the coronary microcirculation, low NO bioavailability predispose to reduced cyclic guanosine monophosphate (cGMP) production by soluble guanylate cyclase (sGC) for adjacent cardiomyocytes, leading to a decrease in protein kinase G (PKG) activity.[40] Both low cGMP content and low PKG activity were demonstrated in myocardial homogenates of HFpEF patients.[8] PKG is responsible for the phosphorylation of a vast number of target proteins involved in excitation contraction coupling, calcium homeostasis, suppression of hypertrophic signalling and stimulation of LV relaxation and LV distensibility through troponin I (TnI) and the giant titin protein. Furthermore, in the cardiomyocytes, high levels of peroxynitrite, increase diastolic calcium content, thereby delaying relaxation. Microvascular inflammation also directly favours proliferation of fibroblasts and myofibroblasts, leading to increased collagen deposit and fibrosis. Stiff cardiomyocytes with delayed relaxation, and increased collagen deposits cause diastolic LV dysfunction, the major functional deficit in HFpEF.[37]

An additional mechanism contributing to diastolic dysfunction, incriminating an **imbalance in nitric oxide signalling**, was proposed by Schiattarella et al[41] in 2019. They demonstrated an overexpression of the inducible NO synthase (iNOS, induced by inflammation) in a mouse model of HFpEF and in human myocardial biopsies. High concentration of NO produced by iNOS increases S-nitrosation of the endonuclease inositol-requiring protein 1α (IRE1α), leading to defective XBP1 splicing and reduced unfolded protein response (UPR). Accumulation of poorly folded proteins contributed to increased myocardial rigidity (Figure 1.4).

As the pro-inflammatory state in HFpEF is systemic, **endothelial dysfunction** is not confined to the heart but is present throughout the vasculature. An important function of normal blood vessels is to vasodilate on exertion, to meet the increased oxygen (O2) demands of the skeletal muscles. This reactive vasodilation is regulated by shear stress on the endothelial cells and is impaired in almost half of HFpEF patients.[13] A system-wide reduction in NO bioavailability could explain several pathophysiological findings, including reduced exercise-induced peripheral vasodilation, reduced vasoreactivity and vascular remodeling in the pulmonary arteries, reduced capillary density in the heart and skeletal muscle, and reduced renal blood flow.[30,42]

This understanding of the disease explains both cardiac diastolic dysfunction and other organ failure associated with HFpEF by placing inflammation, oxidative stress and endothelial function at the centre of the pathophysiology. More recently, another piece of the puzzle was added with the discovery that dysregulation of NO synthases activity and consequent dysregulation of NO signalling played an important role.[41,43,44]

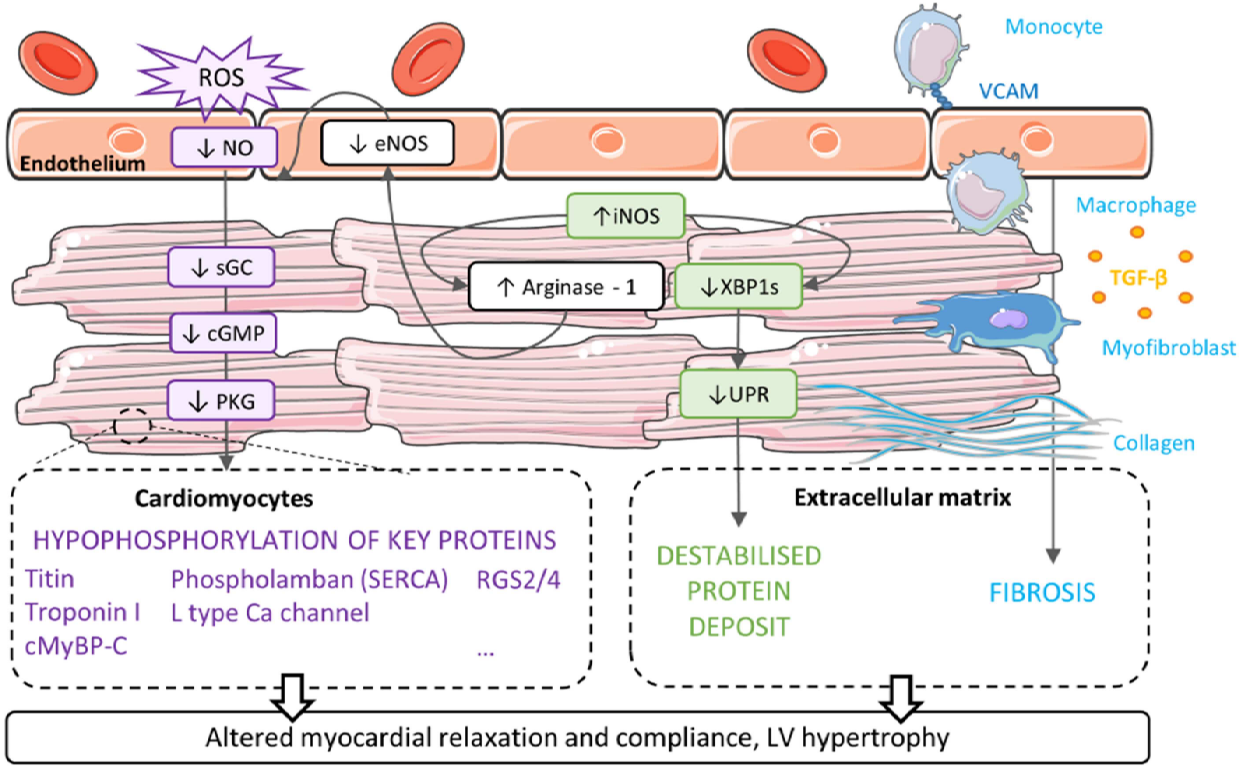



**Figure 1.4 Main mechanisms incriminated in HFpEF pathophysiology**.
Comorbidities induce a systemic proinflammatory state, with as consequences (1) production of reactive oxygen species (ROS) and hypophosphorylation of proteins including titin trough decrease in soluble guanylate cyclase (sGC), cyclic guanosine monophosphate (cGMP) and protein kinase G (PKG) activity; (2) activation of the inducible nitric oxide synthase (iNOS) with reduction in the unfolded protein response (UPR) and accumulation of destabilised protein and (3) recruitement of inflammatory cells through increase in vascular cells adhesion molecules (VCAM) leading to increased collagen deposition and fibrosis.

### 1.2.2 Comorbidities-induced inflammation

The most prevalent comorbidity in HFpEF is hypertension, which is present in the large majority of patients (over 90%) across epidemiological and registry studies, hence HFpEF was initially considered an expression of advanced hypertensive heart disease.[2] Arterial hypertension induces an increase in LV afterload with consequent neuro-humoral-induced myocardial fibrosis and concentric LV hypertrophy.[45,46] However, hypertension do not differentially predict incident HFpEF versus HFrEF and its prevalence increases in late life, also in people without HF.[47] This led to the conclusion that hypertension alone is not sufficient to cause HFpEF.

Over the last 10 years, a causal link between obesity, diabetes mellitus and alterations in cellular and molecular mediators of inflammation has been recognized. This metabolism-induced, chronic low-grade inflammatory response has been termed 'meta-inflammation'[48]. The role of meta-inflammation in the development of HFpEF was first established in a swine model where the induction of arterial hypertension, diabetes mellitus, and hypercholesterolemia led to diastolic dysfunction and HF, while EF was preserved.[49]

**Obesity**

Obesity is one of the main risk factors for HFpEF[42,50], and is more strongly associated with HFpEF than HFrEF.[51] Obesity and associated metabolic dysfunction have been proposed as major drivers of systemic inflammation. Visceral adipose tissue (VAT) in particular has been incriminated to cause insulin resistance and to release inflammatory cytokines.[52] In physiologic conditions, adipocytes secrete adiponectin that modulates local vascular tone by increasing NO bioavailability. In obesity and metabolic syndrome, this property of adipocytes is reduced.[48,53] Increase in visceral fat is also associated with excess release of free fatty acid into the circulation with deleterious effects on the heart. Finally, the presence of increased epicardial

adipose tissue plays a dual role at the local level: mechanically through increase in external constraint, and paracrinally through the release of pro-inflammatory cytokines in the direct environment of cardiomyocytes.

As a consequence, in HFpEF patients, body mass index (BMI) is positively correlated with inflammatory markers (hs-CRP).[54] Obese HFpEF subjects display plasma volume expansion, increased LV concentric remodelling, greater RV dysfunction and impaired pulmonary vasodilation.[55] They also suffer more severe clinical manifestations of HF, including higher symptom burden and functional class, worse maximal and submaximal exercise capacity, greater burden of congestion and poorer quality of life.[54]

**Diabetes mellitus**

Inflammation is clearly present in diabetes mellitus and is fed by oxidative stress (reactive oxygen species, ROS) in a vicious circle. Mouse and rat models of diabetes display systemic inflammation early in disease progression. Increased inflammatory biomarkers have also been evidenced in human studies.[56] A comprehensive network analyses found that specific pathophysiologic processes in patients with HFrEF and diabetes were associated with inflammation and neutrophil degranulation, not present in patients without diabetes.[57] Numerous pathophysiologic processes in diabetes mellitus alter the myocardium resulting in less effective relaxation and contraction: alterations in substrate metabolism, direct effect of glucotoxicity, disorders in calcium transport and mitochondrial dysfunction.[56,58,59] Insulin resistance leads to decreased glucose uptake and increased free fatty acid utilization by cardiac myocytes, causing production of toxic lipid intermediates and increased ROS, contributing to oxidative stress. Hyperglycemia is directly incriminated (glucotoxicity) through formation of advanced glycation end-products (AGE) and maladaptive hexosamine biosynthesis

pathway (HBP), both involved in inflammation and mitochondrial dysfunction. Hence diabetes and obesity contribute synergistically to the presence of a pro-inflammatory and pro-oxidant environment (Figure 1.5).

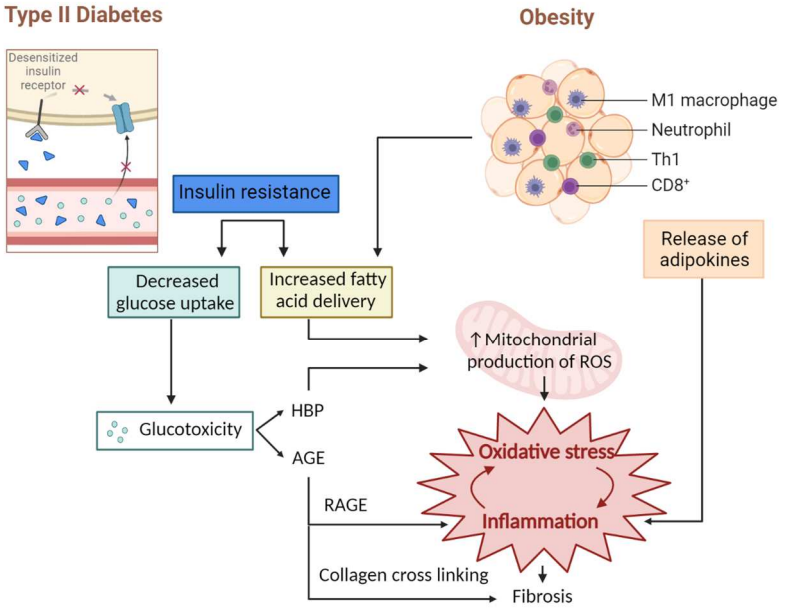



**Figure 1.5 Contribution of type 2 diabetes and obesity to systemic pro-inflammatory state and oxidative stress**. HBP: hexoamine biosynthesis pathway, AGE:advanced glycation end product, R: receptor, ROS: reactive oxygen species

Furthermore, decreased arterial compliance, renal angiopathy, and autonomic dysfunction associated with diabetes mellitus can also accelerate the progression of HFpEF.[56] In particular, hyperglycemia up-regulates the sodium-glucose cotransporter-2 (SGLT-2) contributing to increased proximal renal sodium absorption, volume expansion, and decreased responsiveness to diuretics.[60-62] Sub studies of large clinical trials (RELAX-HF[63], I-PRESERVE[64], CHARM-PRESERVED[65] and TOPCAT[66]) comparing diabetic and nondiabetic patients showed that HFpEF patients with diabetes mellitus were younger, more obese, exhibited higher LV masses and higher levels of myocardial fibrosis[64,67,68] and had a worse prognosis.

**Ageing**

Heart failure with preserved ejection fraction is a disease of the elderly. Mean age in most clinical trial is above 70 years. Ageing is also characterized by chronic, low-grade inflammation[69] and interacts with traditional cardiovascular risk factors to exacerbate their deleterious effects, sharing a number of signalling pathways and molecular effectors.[70] Hallmarks of inflammation related with ageing include chronic activation of the innate immune system and increased circulating levels of pro-inflammatory mediators, such as interleukin (IL)-1β, IL-6 and TNF-α. In mice, the introduction of metabolic stress (high fat diet) in senescence-accelerated animals led to cardiovascular inflammation, endothelial dysfunction and HFpEF-like features.[71]

**Evidence for inflammation as a key driver of HFpEF**

The first argument making a case for a causal role of inflammation in the development of HFpEF emerged from a study among older patients without prevalent HF, reporting that inflammatory markers were predictive of incident HFpEF.[72] Network analysis were then conducted to infer the most prevalent pathophysiological pathways involved in HFpEF and HFrEF based on circulating biomarker levels and, in HFpEF, those biomarkers were specifically related to inflammation and extracellular matrix reorganization.[73,74] Recently, a mechanistic study specifically incriminated neutrophils-mediated release of pro-inflammatory cytokines, especially in patients with HFpEF and diabetes.[75]

Metabolic-driven inflammation likely occurs before the aging of the population, these events synergically acting to facilitate HFpEF. Incriminated in both processes (inflammation driven by ageing and metabolic stress) is the NLRP3 inflammasome. Inflammasomes are intracellular multiprotein complexes that promote the maturation and release of highly inflammatory cytokines, ie, interleukin (IL)-1β and

IL-18. It can be activated by a wide range of molecules reflecting cellular damage and metabolic stress, such as extracellular adenosine triphosphate (ATP), cholesterol crystals, angiotensin II, saturated fatty acids, and glucose.[76] A recent study created a novel HFpEF mouse model that integrated aging, obesity (high-fat diet), and hypertension (desoxycorticosterone pivalate stimulation). Among the prominent features of this model were systemic inflammation as measured overproduction of IL-1β and IL-18 and NLPR3 inflammasome activity.[77] Future studies are necessary to determine the interplay between senescence mechanisms and metabolic-induced inflammation for the HFpEF pathogenesis.

### 1.2.3 Oxidative Stress

In physiologic conditions, there is a delicate balance between the formation of free radicals (reactive oxygen species, ROS) and their inactivation via the antioxidant systems. When a certain threshold of ROS formation and impaired ROS degradation is reached, protein oxidation, lipid peroxidation, and DNA damage take place and contribute to cellular dysfunction.[78] This process is called oxidative stress.[79] ROS production is enhanced in inflammatory conditions and mediates a vicious circle that could be the mechanistic link between comorbidities and related complications.[80,81] Besides increased ROS production, antioxidant defences are lower in obese patients than in normal-weight counterparts, and their levels correlate inversely with central adiposity. In diabetes mellitus, metabolic abnormalities cause mitochondrial superoxide overproduction in endothelial cells of both large and small vessels, as well as in the myocardium.[82] Oxidative stress is also implicated in age-related changes in the heart.[83]

Oxidative stress and inflammation contribute synergistically to the pathophysiology of HFpEF. Several transcription factors that regulate the expression of pro-

inflammatory cytokines are activated under oxidative stress. Reciprocally, pro-inflammatory cytokines induce the generation of ROS, thus creating a potential vicious cycle of oxidation and inflammation.[84] An illustrative example is myeloperoxidase (MPO), a leukocyte-derived enzyme released during inflammation and producing hypochlorous acid, a potent pro-oxidant and pro-inflammatory molecule. Animal studies showed the association of elevated MPO levels with collagen accumulation in matrix remodeling after myocardial infarction[85] and its contribution to the pathophysiology of atrial fibrillation through atrial accumulation and fibrosis.[86] In human HFrEF studies, increasing levels of MPO were associated with restrictive diastolic stage, right ventricular systolic dysfunction and tricuspid regurgitation.[87,88] These results offer insight into the role of MPO-mediated oxidative stress in the progression of restrictive filling pattern, myocardial fibrosis and atrial fibrillation. MPO could play a part in the development of HFpEF where diastolic dysfunction, atrial fibrillation and fibrosis are major components.[88]

### 1.2.4 Endothelial dysfunction

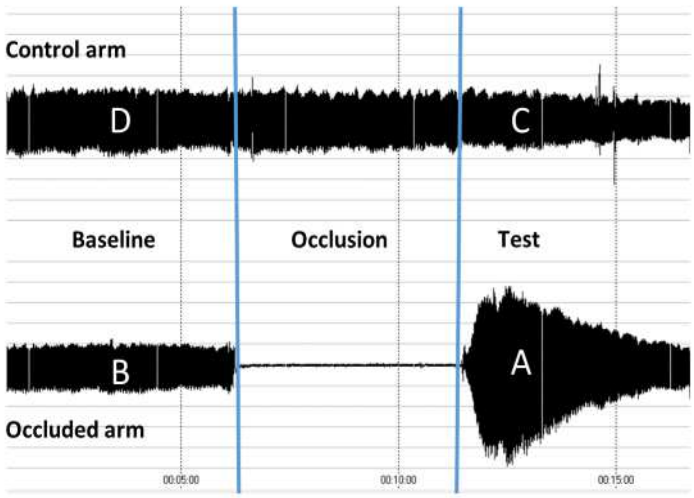
The endothelium is the innermost layer of the blood vessels, present from the smallest capillary to the aorta, including in the coronary circulation. A similar tissue is present in the cardiac chambers (endocardium). More than just a protective layer between the blood and extravascular tissues, endothelial cells are dynamic, highly interactive cells that regulate vascular function and homeostasis. The healthy endothelium prevents platelet aggregation and leukocyte adhesion, inhibits smooth muscle proliferation, and regulates vascular tone through release of vasoactive substances.[89] The vasodilatory response to shear stress adapting oxygen delivery to tissues' needs is largely dependent on the endothelium and on its main

effector molecule nitric oxide (NO).[90,91] Pro-inflammatory conditions in combination with oxidative stress lead to the disturbance of NO homeostasis and result in endothelial dysfunction.[92,93]

**Assessment of endothelial function**

Techniques to measure endothelial function rely on the change in flow or in artery diameter after pharmacological or mechanical stimulation of the endothelium. Due to its non-invasive approach, flow-mediated vasodilatation of the arm arteries (FMD) has become the most widely used technique. This technique relies on the measurement by ultrasound of the change in diameter of the brachial or radial artery during reactive hyperemia after a 5 minute occlusion of the target artery with a blood pressure cuff.[90] Flow mediated vasodilation is mainly dependent on NO[94] and is a valid correlate of endothelial function but its application is technically challenging and requires extensive training and standardization.[90]

Using a specifically designed device (EndoPAT, Itamar Medical) finger plethysmography measures pulsatile arterial volume changes in the fingertips using pneumatic probes.[95] Reactive hyperemia is achieved in the same way as FMD with a blood pressure cuff. The outcome measure, the reactive hyperemia index (RHI) is calculated using the ratio of the amplitude of the pulse wave during reactive hyperemia over the baseline, corrected using measurements of the contralateral arm. (Figure 1.6)




Reactive hyperemia index **RHI** = $\frac{A/B}{C/D}$ x correction factor

**Figure 1.6 Example of EndoPAT test and calculation of reactive hyperemia index (RHI)**

Indeed, a main advantage of the system is that the contralateral arm serves as control that can be used to correct for any systemic drift in vascular tone during the test. This index is validated marker for endothelial function but reflects changes in flow, as well as in digital microvessel dilatation and is only partly dependent on NO.[96] In two large cross-sectional studies (the Framingham cohort and the Gutenberg Heart Study)[97,98] digital vascular dysfunction was associated with traditional and metabolic cardiovascular risk factors but not or only modestly with FMD, likely measuring different aspects of vascular biology.[90]

Similarly, assessment of coronary microvascular function relies on the quantification of blood flow through the coronary circulation at rest and during maximal vasodilation. Maximal vasodilation can be achieved by adenosine, dypiridamole or regadenoson, acting directly on vascular smooth muscle cells (**endothelium-independent** microvascular function). The use of acetylcholine

explores **endothelium-dependent** microvascular function specifically (Figure 1.7). Coronary flow reserve (CFR) is used to describe the increase in flow from basal perfusion to maximal vasodilation in one given coronary artery. In the same way, myocardial flow reserve (MFR), defined as the ratio of myocardial blood flow (MBF) at peak stress to MBF at rest, represents the vasodilatory reserve of the entire coronary circulation. Since resistance is primarily determined by the microvasculature, CFR and MFR are indirect measure of the coronary microvascular function. Coronary or myocardial blood flow can be assessed using different imaging techniques (echocardiography, CMR, PET scan).

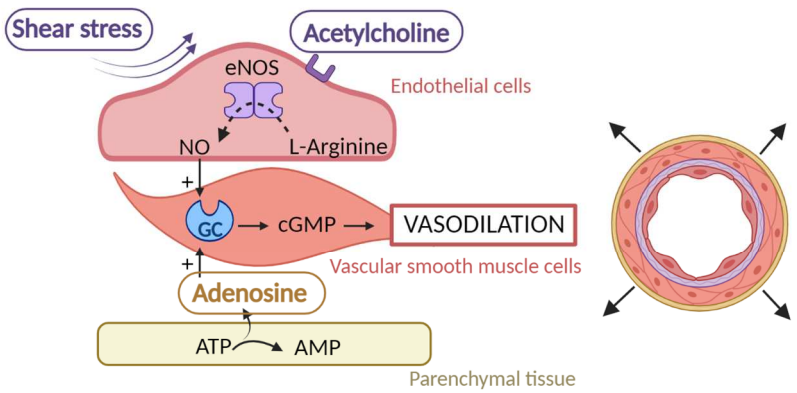



**Figure 1.7 Endothelium-dependent and Endothelium-independent vasodilation.**
Stimulation of the endothelial cells by acetylcholine (ACh) or shear stress results in endothelium dependent formation and release of nitric oxide (NO). Substances such as adenosine elicit vasodilation by direct action on vascular smooth muscle.

**Evidence of endothelial dysfunction in HFpEF**

Using non-invasive methods, **endothelial dysfunction** in HFpEF was first identified in the **peripheral systemic vasculature** (Table 1.2), with evidence of impaired endothelium-mediated vasodilation.[13,23,99,100] The largest study by Akiyama et al.[23] compared 321 HFpEF patients with age-, gender-, hypertension, and diabetes mellitus-matched subjects using EndoPAT. Reactive hyperemia index (RHI) was significantly lower in patients with HFpEF and a RHI below the median predicted adverse events. Endothelial function was correlated with markers of oxidative stress and diastolic function. RHI below normal was associated with reduced exercise capacity and more severe symptoms, suggesting a role of endothelial dysfunction in exertional intolerance.[101] Results of studies assessing flow mediated dilation (FMD) were conflicting. Some concluded that HFpEF patients had no further decline in FMD beyond that due to age alone, and that FMD did not significantly contribute to reduced exercise capacity[102,103] while other found a significantly lower FMD in patients with HFpEF than in controls.[104,105] Interestingly Farrero et al.[104] showed a significant inverse correlation between FMD and pulmonary vascular resistance, measured invasively in 20 patients. Peripheral endothelial dysfunction may be associated with impaired pulmonary endothelial function and could contribute to pulmonary hypertension.

Advocating in favour of a causal role for **coronary microvascular dysfunction (CMD)**(Table 1.3), Taqueti et al.[106] found that CMD (defined as CFR <2) was associated with diastolic dysfunction (defined as E/e'>15) and with incident risk of HFpEF in a population of hypertensive patients. Coronary flow reserve (CFR) was altered in HFpEF compared with controls and hypertensive subjects in both a CMR and a Rb-82 PET study.[107,108] Interestingly, in both studies, coronary blood flow at rest was higher in HFpEF than in controls. This may reflect increased resting

metabolic demand, whereas reserve myocardial capacity for pharmacological stress is decreased. Finally, Shah et al.[109], showed that CFR was correlated with renal function, peripheral endothelial function (endoPAT RHI), NTproBNP levels, RV function and LV strain. Across studies, prevalence of CMD in HFpEF patients reached 75%.

All aforementioned studies used adenosine or dipyridamole to induce hyperemia, probing essentially endothelium-independent microvascular function (Figure 1.7). Studies evaluating endothelium-dependent CMD with acetylcholine are rare. Tschöpe et al.[110] showed some degree of endothelium-dependent CMD in asymptomatic patients with diastolic dysfunction, even before the onset of HF. Yang et al.[111] evaluated both endothelium-dependent and independent coronary microvascular function in HFpEF. Intriguingly, they found only a modest correlation ($r=0.27$, $p=0.001$) between the two types of CMD. Prevalence of both were similar (about 30% each) but only 10% of patients showed combined endothelium dependent and independent dysfunction.

Altogether, these data indicate that peripheral endothelium-dependent vasodilation and coronary microvascular function are altered in a significant proportion of patients with HFpEF (40-75%) and are associated with multiple indices of abnormal cardiac function. Interestingly, studies in HFrEF showed no relationship between coronary and peripheral endothelial function[112,113], hence different mechanisms may be implied depending on the vascular bed. Furthermore, besides functional alteration, there is evidence of capillary rarefaction, contributing to altered oxygen delivery, both in peripheral beds[24] and in the heart.[11] By impairing myocardial blood supply, coronary microvascular dysfunction may promote cardiomyocyte injury and increased interstitial fibrosis, leading to an alteration in cardiac structure and function.

| **Table 1.2 Peripheral endothelial dysfunction in patients with HFpEF** | | | | | |
|---|---|---|---|---|---|
| Reference | Technique | Outcome variable | HFpEF (N) | Control group (N) | Main findings |
| Borlaug et al., 2010[13] | RHI (PAT) | Ln (PAT ratio 60 – 120 sec)<br><br>ED: RHI <2.0 | 21 | 19 hypertensive (HT)<br>10 healthy matched for age and gender | RHI lower in HFpEF vs healthy (0.85±0.42 vs 1.33±0.34),<br>but not in HFpEF vs HT (0.85±0.42 vs 0.92±0.38)<br>Prevalence ED 42% |
| Akiyama et al., 2012[23] | RHI (PAT) | Ln (PAT ratio 90–150 sec)<br><br>ED: RHI <1.63 | 321 | 173 matched for age, gender, hypertension and diabetes | RHI lower in HFpEF vs controls<br>(0.53±0.20 vs 0.64±0.20, p<0.001)<br>RHI associated with CV events (per 0.1) HR 0.72 (0.61 – 0.85), p<0.001 |
| Matsue et al., 2012[100] | RHI (PAT) | Ln (PAT ratio 90–150 sec)<br>ED: RHI <1.63 | 159 | / | RHI associated with the composite endpoint of death or hospitalization for HF<br>(per 0.1) HR 0.59 (0.43–0.81), p<0.001 |
| Yamamoto et al., 2015[101] | RHI (PAT) | Not reported | 64 | 64 matched for age, gender, hypertension, diabetes, and coronary artery disease | RHI lower in HFpEF (1.70 [1.55;1.88] vs 2.01 [1.64;2.42], p <0.001)<br>BH4/BH2 ratio decreased in HFpEF (3.21 ± 2.05 vs. 2.05 ± 1.62, p < 0.001).<br>Correlation of BH4/BH2 ratio with RHI (R=0.23, p=0.009) and with E/e' (-0.26, p=0.003) |

| | | | | | |
|---|---|---|---|---|---|
| Haykowsky et al.,2013[103] | FMD | % dilation brachial artery | 66 | 16 young<br>31 matched for age and gender | FMD lower in HFpEF vs young but comparable to old healthy (4.00±0.38%, p=0.86) |
| Farrero et al. 2014[104] | FMD | % dilatation brachial artery<br>*+ sublingual nitroglycerin (endothelium indep)* | 28 | 42 hypertensive matched for age | FMD lower in HFpEF vs controls (1.95 [−0.81–4.92] vs 5.02[3.90–10.12] %, p=0.002), no difference in shear rate.<br>FMD inversely correlated to pulmonary vascular resistance and mean PAP (r= -0.623, p=0.006 and r=-0.503, p=0.033) |
| Kishimoto et al.,2017[105] | FMD | % dilatation brachial artery<br>*+sublingual nitroglycerin* | 41 | 165 unmatched | FMD lower in HFpEF (2.9 ± 2.1 vs 4.6 ± 2.7%),<br>Nitroglycerine-induced vasodilation lower in HFpEF (9.3±4.1% versus 12.9± 4.9%) |
| Lee et al.,2016[105] | FMD | % dilatation brachial artery | 24 | 24 matched for age and gender | FMD lower in HFpEF (3.06±0.68 vs 5.06±0.53), but no difference when corrected for shear rate. (shear rate = 8 Vmean/arterial diameter) |

| **Table 1.3 Coronary microvascular dysfunction in patients with HFpEF** | | | | | |
|---|---|---|---|---|---|
| Reference | Technique | Outcome variable | HFpEF (N) | Control group | Main findings |
| Shah et al., 2018[109] | Echo-doppler Adenosine (EI) | CMD = CFR <2.5 | 202 | / | CMD prevalence in HFpEF = 75%<br>CFR is associated with smoking and AF<br>CFR is correlated to renal function (R=0.34, p=0.002), NTproBNP (R=-0.27, p<0.001), RHI (R=0.21, p=0.004) and TAPSE (R=0.26, p<0.001) |
| Taqueti et al., 2017[114] | Rb-82 PET Dipyridamole (EI) | CMD = CFR <2.0 | 201 without HFpEF (undergoing evaluation for suspected coronary artery disease) | | CMD is an independent risk factor for incident HFpEF (HR 2.47 [1.09;5.62], p=0.03)<br>Impaired CFR associated with E/e' septal >15 |
| Srivaratharajah et al., 2016[108] | Rb-82 PET Dipyridamole (EI) | CMD = CFR < 2.0 | 78 | 186 hypertensive, 112 controls, unmatched | CMD prevalence in HFpEF = 40%<br>CFR lower in HFpEF vs hypertensive and controls |
| Kato et al., 2016[107] | CMR Adenosine (EI) | CMD = CFR < 2.5 | 25 | 13 hypertensive LVH<br>18 controls, unmatched | CMD prevalence in HFpEF = 76%;<br>CFR lower in HFpEF vs hypertensive LVH and controls<br>CFR correlated with BNP levels (p<0.001) |
| Yang et al., 2019[111] | Invasive angiography<br>Acetylcholine (ED)<br><br>Adenosine (EI) | <br>CMD= increase in CBF ≤0%<br><br>CMD = CFR ≤ 2.5 | 162 | / | <u>Prevalence of CMD:</u><br>ED CMD : 29%, EI CMD: 33%, Combined: 10%<br><u>Prediction of mortality</u><br>ED CMD: HR 2.81 [0.94;8.34], p=0.06<br>EI CMD: adjusted HR 3.56, [1.14;11.12], p=0.03<br><u>Association with clinical characteristics</u><br>EI CMD associated with E/e' |
| EI: endothelium independent, ED: endothelium dependent, CMD: coronary microvascular dysfunction, CFR: coronary flow reserve, AF: atrial fibrillation, RHI: reactive hyperemia index, TAPSE: tricuspid annular plane systolic excursion, PAP: pulmonary artery pressure, HR: hazard ratio, LVH: left ventricular hypertrophy, CMR: cardiac magnetic resonance, PET: positron emission tomography, BH4: tetrahydrobiopterin, BH2: dihydrobiopterin | | | | | |

### 1.2.5 Nitric oxide imbalance

The role of nitric oxide (NO) dysregulation in the development of HFpEF was recently extended beyond the endothelial regulation of vascular tone following exploration of NO synthases (NOS) expression in animal models of HFpEF.[41,43,44] Endogenous NO is produced during the transformation of L-Arginine to L-Citrulline by NOS (Figure 1.9).[115] The three main isoforms of NOS are most commonly referred to as neuronal (nNOS), inducible (iNOS), and endothelial (eNOS), reflecting their initially identified location and condition of expression (Figure 1.8). nNOS and eNOS are constitutive, cytosolic, and Ca2+/calmodulin dependent, and release NO in small amount for short time periods, in response to receptor or physical stimulation (shear stress).[40] iNOS is Ca2+ independent, is induced after activation of macrophages, endothelial cells, and a number of other cells by endotoxin and pro-inflammatory cytokines, and once expressed, synthesizes NO for long periods of time and at higher concentrations.[116] Tetrahydrobiopterin (BH4) is a required cofactor for the stabilization of the NOS dimer and proper activity ('coupled' NOS activity); otherwise, electrons are transferred directly to O2, and superoxide anions are produced instead of NO ('uncoupled' NOS – activity), resulting in oxidative stress.[117] NOS activity also depends on the presence of nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen.

As previously outlined, in healthy conditions, the primary target of NO in the cardiovascular system is the enzyme soluble guanylyl cyclase (sGC). It's activation in the circulation culminates in a reduction of vascular smooth muscle tone (vasodilation) and in optimized blood flow.[118] In cardiomyocytes, it leads to activated protein kinase G (PKG), which plays an essential role by inhibiting inflammation, hypertrophy, and fibrosis. PKG also mediates the phosphorylation of titin, promoting the reduction in titin-based passive tension and thereby decreasing myocardial stiffness.[119-121]

Besides these beneficial effects, NO can also be deleterious [122,123] (Figure 1.8). NO generated at high concentration upon the expression of iNOS in conditions of oxidative stress reacts with the superoxide anion radical (O2−), producing peroxynitrite, (ONOO-).[124] This is further amplified by the uncoupling of eNOS since during inflammation, the tetrahydrobiopterin BH4 is not recycled from BH2. Peroxynitrite is toxic by direct oxidative mechanisms and can react with a number of biological molecules though nitration / nitrosation, altering their function.[125]

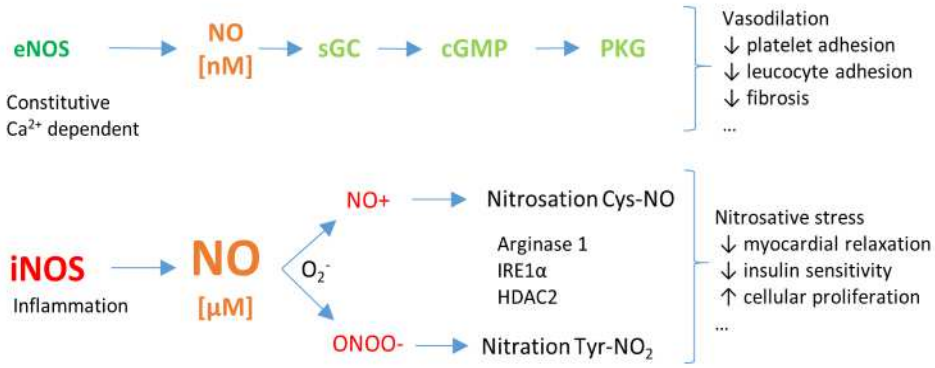



**Figure 1.8 Duality of nitric oxide signalling**. Endothelial nitric oxide synthase (eNOS) is constitutive and releases NO in response to receptor or physical stimulation. In healthy conditions, NO targets the soluble guanylate cyclase (sGC) and culminates in vasodilation and in the inhibition of inflammation, hypertrophy, and fibrosis in cardiomyocytes. Inducible NOS (iNOS) is induced in inflammatory conditions and synthesizes NO for long periods at high concentration. In conditions of oxidative stress, NO reacts with the superoxide anion radical (O2−), producing nitrosonium (NO+) and peroxynitrite, (ONOO-) with subsequent nitration / nitrosation of target proteins.
$Ca^{2+}$: Calcium, cGMP: cyclic guanosine monophosphate, PKG: protein kinase G, IRE1α: inositol-requiring protein 1 α, HDAC2: histone deacetylase 2

The elegant study by Schiattarella et al.[41] was the first to explore the contribution of nitrosative stress to the HFpEF phenotype using a 'two-hit' mouse model, consisting of mice exposed to a high-fat diet and treated with L-NAME, mimicking the coincidence of metabolic stress (obesity and metabolic syndrome) and mechanical stress (hypertension). They demonstrated elevated plasma levels of

proinflammatory cytokines, such as interleukin-1β (IL1β), TNFα, and interleukin 6 (IL6), and resulting over-activation of iNOS in cardiomyocytes. NO produced in high concentration lead to S-nitrosation of the endonuclease inositol-requiring protein 1α (IRE1α), culminating in defective XBP1 splicing and reduced unfolded protein response (UPR). The UPR is a regulatory system that protects the endoplasmic reticulum from an overload of improperly folded proteins. To explore causality, Schiattarella et al. genetically suppressed iNOS and overexpressed XBP1s in affected mice. Each intervention ameliorated the HFpEF-like phenotype: the treated mice had lower left ventricular filling pressures, lower lung weight and could run a greater distance than control mice.

Recently, Yoon et al.[44] also demonstrated overexpression of nNOs in cardiomyocytes and its contribution in the development of diastolic dysfunction through S-nitrosation of histone deacetylase 2. Although the animal models they used were rather models of left ventricular hypertrophy (SAUNA - SAlty drinking water, unilateral Nephrectomy, Aldosterone and mild transverse aortic constriction mice) and do not recapitulate the metabolic component of HFpEF, their data adds to Schiattarella's findings incriminating nitrosative stress and protein S-nitrosation as important drivers of HFpEF.

Furthermore, Dhot and colleagues[43] showed that overexpression of the β3-adrenoreceptor in the endothelium of transgenic rats lead to increased iNOs and nNOS expression, while in contrast, eNOS levels were decreased. This imbalance in the NO pathways was associated with age-related diastolic dysfunction (increase in E/A ratio and LA dilation developing in 45 weeks old rats). They also observed a slight but significant increase in collagen deposition. This data suggests that endothelial-localized alteration in NO signalling can lead to cardiac structural and functional alteration.

To resume, in cardiomyocytes, reduced eNOS activity decreases NO available to activate the soluble guanylate cyclase (sGC), leading to decreased cGMP production, PKG activity and subsequently titin phosphorylation. Meanwhile, enhanced iNOS and nNOS activity lead to the amplification of oxidative stress through the formation peroxynitrite, and to S-nitrosation of proteins with deleterious consequences on diastolic function. In endothelial cells, similar alteration in NOS activity (decreased eNOS and increased iNOs and nNOS) contributes to diastolic dysfunction and collagen deposition.

These data emerge from animal studies, with evident limitations. However, obtaining tissue material from living subjects requires invasive procedures that are difficult to perform in practice, especially in this fragile population. Hence, available data is limited to the analysis of circulating NO metabolites that indirectly and imperfectly reflect the complexity of NO homeostasis. A recent study by Piatek et al.[126] investigated L-arginine (L-Arg), homoarginine (hArg), and asymmetric and symmetric dimethylarginine (ADMA and SDMA) as markers of NO metabolism (Figure 1.9). They could not identify statistically different concentrations of these metabolites between patients with definitive HFpEF and patients at risk. Hage et al.[88] on the other hand, reported higher SDMA and ADMA levels in patients with HFpEF compared with healthy controls. A possible explanation is that abnormalities in the NO metabolism are associated with comorbidities and occur early in the development of the disease. Interestingly, ADMA and hsCRP were correlated ($R=0.30$)[126], consistently with the proposed concept of a systemic proinflammatory state responsible for the impairment in the NO pathway.

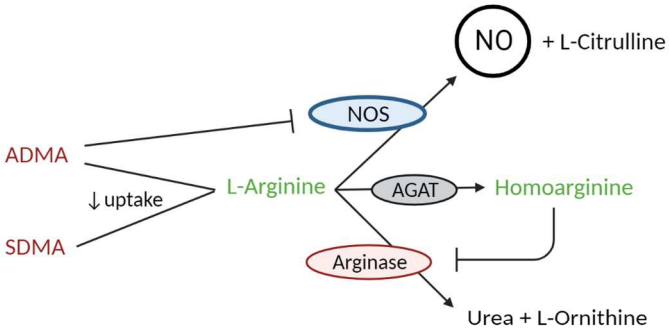



**Figure 1.9 Synthesis and interactions of methylated arginines and nitric oxide (NO).**
Symmetric dimethylarginine (SDMA) and asymmetric dimethylarginine (ADMA) are formed within post-translational modifications of protein-bound arginine residues. ADMA is a direct inhibitor of nitric oxide synthases (NOS). NOS synthesize nitric oxide (NO) from L-arginine. ADMA and SDMA can cause a reduced L-arginine uptake through a competitive transport and thereby reduce substrate availability for NO synthesis. Homoarginine is formed from L-arginine and lysine by the L-arginine:glycine amidinotransferase (AGAT). Homoarginine can inhibit the enzyme arginase and thereby augment L-arginine pools.
*Adapted from Piatek, K. et al. Nitric oxide metabolites: associations with cardiovascular biomarkers and clinical parameters in patients with HFpEF. ESC heart failure 2022.*

## 1.3 HFpEF, the translational perspective

### 1.3.1 Completed clinical trials

**NO-cGMP axis**

In contrast to the numerous advances in HFrEF, HFpEF remains a therapeutic challenge. Established HF drugs targeting the renin-angiotensin-aldosterone system have failed to improve prognosis leading to more specific approaches targeting the NO–cGMP–PKG-axis.[127] Therapeutic targets are represented in Figure 1.10 and results of clinical trials are outlined in Table 1.4.

**Nitrite**

For years, attempts were made to restore intracellular cyclic guanosine monophosphate (cGMP) signalling directly via nitrate and nitrite administration, using isosorbide mononitrate in the NEAT-HFpEF trial[128], and inhaled inorganic nitrite in the INDIE-HFpEF trial[129]. Both studies failed to improve exercise capacity, and the former even demonstrated a tendency to reduce the total physical activity level.

**PDE-5 inhibitors**

Phosphodiesterase-5 (PDE-5) metabolizes cGMP, and may thus limit the beneficial effect of nitric oxide. Hence PDE-5 inhibitors could have a favourable effect on cardiac structural and functional remodelling and enhance vascular, neuroendocrine, and renal function. The PDE-5 inhibitor Sildenafil indeed improved exercise capacity and hemodynamics in patients with idiopathic pulmonary arterial hypertension.[130] This benefit was confirmed in a small single center study of patients with HFpEF and severe pulmonary vascular disease.[131] However, in the larger, multicenter RELAX trial[132], sildenafil use did not improve exercise capacity in patients with typical HFpEF and showed no significant benefit over placebo in a

range of secondary endpoints regarding left ventricular remodelling, diastolic function parameters and quality of life scores. A substudy of this trial showed beneficial effects in the systemic and pulmonary vasculature but deleterious effects on left ventricular function.[133] Overall, PDE5-inhibitors could be beneficial in a subset of patients with precapillary or combined pre- and post-capillary pulmonary hypertension[134] but not in all-comers HFpEF. Moreover, therapeutic sildenafil levels were associated with minimal increases in plasma cGMP, hence the effect of PDE-5 inhibitors might be limited by insufficient endogenous production of cGMP by soluble guanylate cyclase (sGC). Hence, direct, NO-independent sGC stimulators were developed.

**sGC activator**

The sGC activator Riociguat failed to improve mean pulmonary artery pressure in patients with HFpEF and pulmonary hypertension although it had a favourable effect on stroke volume and right ventricular end diastolic area.[135] Trials with Vericiguat yielded conflicting results. Post hoc analysis of SOCRATES-PRESERVED[136] showed benefit in quality of life in 68 patients, but this was not reproduced in VITALITY, a larger trial designed for this specific endpoint.[137]

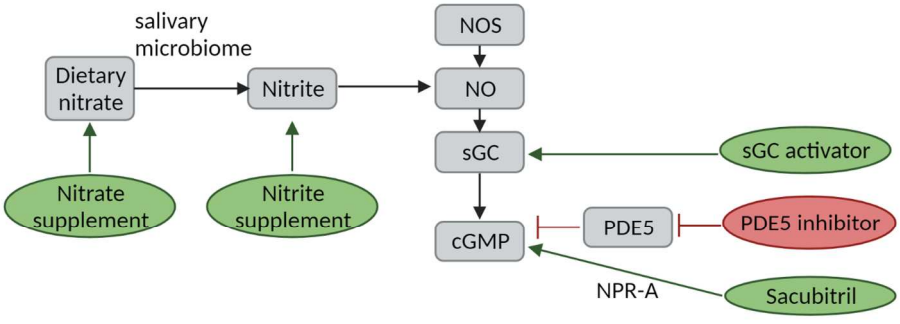



**Figure 1.10 Therapies targeting the NO-cGMP axis.**
sGC: soluble guanylate cyclase, cGMP: cyclic guanosine monophosphate, PKG: protein kinase G; NOS: nitric oxide synthase, PDE: phosphodiesterase, NPR-A: natriuretic peptide receptor-A

**Table 1.4 Clinical trials targeting the NO-sGC-cGMP-PKG axis**

| Trial | Year | Treatment protocol | HFpEF (n) | Outcome |
|---|---|---|---|---|
| Nitrite or nitrate supplementation | | | | |
| NCT01932606[138] | 2015 | Acute infusion sodium nitrite | 28 | Improved pulmonary artery pressure and cardiac output at rest and during exercise |
| NCT02262078[139] | 2016 | Acute inhalation sodium nitrite | 26 | Reduction of pressure and pulmonary artery pressure at rest and during exercise |
| NEAT-HFPEF[128] | 2015 | Isosorbide mononitrate vs placebo | 110 | **Decrease** in daily activity level (P=0.02) |
| INDIE-HFpEF[129] | 2018 | Inhaled nitrite for 1 month | 105 | No difference in peak VO2 (P = 0.27) |
| INABLE training NCT02713126 | | Oral sodium nitrite capsules and cardiac rehabilitation | | No published results Endpoint: Change in peak VO2 |
| KNO3CK OUT HFPEF NCT02840799 | | Potassium nitrate (KNO3) capsules 6 weeks | | No published results Endpoint: Change in peak VO2 |
| Phosphodiesterase type 5 inhibitors | | | | |
| NCT01156636[131] | 2011 | Sildenafil vs placebo 12 months | 44 PH-HFpEF | Improved pulmonary artery pressure at 6 and 12 months (Δ-42.0±13.0%) |
| RELAX[132] | 2013 | Sildenafil | 216 | Decrease in arterial pressure, no improvement in exercise capacity |

| | | | | |
|---|---|---|---|---|
| NCT01726049[140] | 2015 | Sildenafil vs placebo 12 weeks | 52 PH-HFpEF | No improvement in pulmonary pressures, cardiac output, and exercise capacity |
| NCT01201257849[134] | 2020 | Sildenafil vs placebo 6 months | 50 PH-HFpEF (precapillary PH) | Improvement in exercise capacity, pulmonary pressures, and right ventricular function |
| Soluble guanylate cyclase activators or stimulators | | | | |
| DILATE-1[135] | 2014 | Single dose Riociguat | 20 PH-HFpEF | No significant effect on pulmonary pressures |
| DYNAMIC[141] | 2022 | Riociguat 26 weeks | 118 PH-HFpEF | Increase cardiac output and decrease pulmonary pressures but no effect on symptoms |
| SOCRATES - Preserved[136] | 2017 | Vericiguat | 477 | Unchanged NTpro-BNP and atrial volume but improvements in quality of life |
| VITALITY-HFpEF[137] | 2020 | Vericiguat | 789 | No improvement in KCCQ score, nor in 6 minutes walking distance |
| CAPACITY-HFpEF[142] | 2020 | Praliciguat 12 weeks | 196 | No improvement in peak VO2 nor in quality of life. More dizziness, hypotension and headaches |
| Sacubitril (NP degradation inhibitor)/valsartan* | | | | |
| PARAGON-HF[35] | 2019 | Sacubitril Valsartan | 4822 | No reduction in hospitalizations for HF and death from CV causes<br>Possible benefit in patients with lower ejection fraction (<57%) and in women |

Natriuretic peptides (NPs) augment the intracellular level of cGMP via natriuretic peptide receptor-A*

Peak VO2: maximal oxygen consumption during cardiopulmonary exercise testing; KCCQ: Kansas city cardiomyopathy questionnaire assessing quality of life in patients with heart failure; PH: pulmonary hypertension; CV: cardiovascular; HF: heart failure

**Anti-inflammatory strategies**

Despite current understanding that inflammation is a key player in the development of HFpEF, only a few trials targeted inflammation in that population.[143] Pharmacological strategies that specifically target the NLRP3 inflammasome has been investigated in several cardiovascular diseases but poorly in HFpEF.[76] Only the D-HART pilot study and DHART-2 trial[144], both with the IL-1β antagonist anakinra were investigated. Despite a reduction in CRP and in NT-proBNP, no improvement of exercise capacity was observed. This might be due to the limited power of the phase I study and predominant obesity of participants in D-HART2 that may have confounded exercise tolerance.

**SGLT2 inhibitors**

As briefly introduced previously, sodium glucose cotransporters-2 (SGLT2) inhibitors is the first class of treatment with demonstrated favourable effect on adverse events in HFpEF. Empalglifozin led to a 21% lower relative risk of cardiovascular death or hospitalization for heart failure in EMPEROR-Preserved[33], benefit confirmed with Dapaglifozin in the lately published DELIVER trial.[34]

SGLT2 are major transport proteins responsible for reabsorption of glucose in the kidneys. Medication inhibiting those protein (SGLT2 inhibitors) were initially developed for the treatment of diabetes mellitus but rapidly proved beneficial on cardiovascular outcome.[145] Different mechanisms of action have been elucidated. First, an evident diuretic effect with consequent decrease of congestion. SGLT2 inhibitors have been shown to rapidly lower pulmonary pressures, improving symptoms and exercise capacity. Secondly, SGLT2 inhibitors may reduce systemic inflammation and oxidative stress, improving endothelial function both in the

myocardium and skeletal muscles. In an animal study, the interaction between endothelial cells and cardiomyocyte was altered by the mediator of inflammation TNF-α and restored by empagliflozin.[146] Empagliflozin significantly acted on inflammation and endothelial function by suppressing increased levels of TNF- α, IL-6, and adhesion molecules ICAM-1 and VCAM-1 in human and murine HFpEF myocardium.[81,147] As a consequence, SGLT2 inhibitors exert anti-fibrotic properties reducing myofibroblasts and macrophages infiltration and have been demonstrated to reduce LV mass and to improve diastolic function.[148] It has also been postulated that SGLT2i exerted beneficial effect through improved myocardial metabolism. As glucose availability is reduced upon treatment with SGLT2i, lipolysis and ketogenesis have shown to be increased, leading to a shift from carbohydrate usage to lipid usage and to the reduction of visceral and subcutaneous adipose tissues. Overall, evidence points towards multifactorial effects associated with reduced systemic and myocardial inflammation and oxidative stress.

### 1.3.2 Patient phenotyping for targeted therapeutics

In light of evidence reviewed in previous chapters, inflammation, oxidative stress and endothelial dysfunction are present in a significant proportion of patients with HFpEF. However, doubt remains whether endothelial dysfunction causes diastolic dysfunction. Diastolic dysfunction could also be initiated by cardiomyocyte impairment which subsequently induces ED or the 2 phenomenon could occur concomitantly.[149] An argument in favour of diastolic dysfunction initiated ahead of endothelial dysfunction emerged from a study by Waddingham et al.[150] where non-obese diabetic rats developed diastolic dysfunction associated with inflammation and oxidative stress while endothelium dependent vasodilation was not altered. In any case, the disappointing results of trials targeting NO-sGC-cGMP questioned the

causal role of this pathway in the development of the disease. Furthermore, the fact that decreased NO bioavailability in endothelial cells may be responsible for decreased titin phosphorylation has not been firmly demonstrated. Endothelial dysfunction could also be causative in some but not all patients. Indeed the difficulty when it comes to HFpEF is the great multifactoriality and heterogeneity of the disease. Compared with HFrEF, primarily due to myocardial damage (eg ischemia, cardiomyopathies, and toxicity) and associated with significant neurohormonal activation, HFpEF is the end result of a wide variety of cardiovascular diseases and risk factors, and involves multiple cardiac and extra-cardiac abnormalities. One reason researchers struggle to unravel HFpEF pathophysiology is that there is no animal model recapitulating all features of the disease. Instead, several animal models exist, all of them representative of some but not all aspects of HFpEF.[79,151] This illustrates that diverse combinations of hits occurring in different orders can lead to similar consequences on diastolic function and exercise capacity.

This heterogeneity is also observable among patients and is a challenge for clinicians. Each patient displays a unique combination of risk factors, comorbidities and end organ failure associated with HFpEF. This makes the syndrome resistant to a “one size-fits-all” approach and complicates its management. Consequently, attempts have been made to divide patients into clusters with distinct clinical features (“phenogroups”), representing distinct pathophysiological mechanisms that can be targeted for therapeutic purposes.

Shah et al.[152] initially used a form of machine learning unbiased clustering in 397 patients with HFpEF, which they termed “phenomapping”. This method confirmed HFpEF heterogeneity and identified 3 separate clusters of patients exhibiting differences in clinical characteristics, biomarkers, cardiac structure/function,

pathophysiology, and outcomes. Since then, multiple studies used similar methods to identify clusters of patients.[152-156] Depending on the included population and the data available for clustering, studies identified different subgroups. However, some phenotypes share similar characteristics and prognosis across different cohorts. Principal phenogroups are reviewed by Galli et al.[156] (Figure 1.11). They identify five common phenogroups, three of them representing different pathophysiological pathways: the natriuretic peptide deficiency syndrome (1), the obesity/cardiometabolic phenotype (2) and the atrial myopathy phenotype (3). The last two clusters are rather related to the evolution of the disease and encompass patients in end stage heart failure, where kidney failure (4) and / or pulmonary hypertension and right ventricular dysfunction (5) prevails. Patients from different clusters might respond differently to treatments, and clinical trials distinguishing those subgroups could be useful in the future. Hence, identifying biomarker profiles and / or echocardiographic characteristics that are discriminant between groups is of great clinical interest and represents the focus of future research in the field of HFpEF.

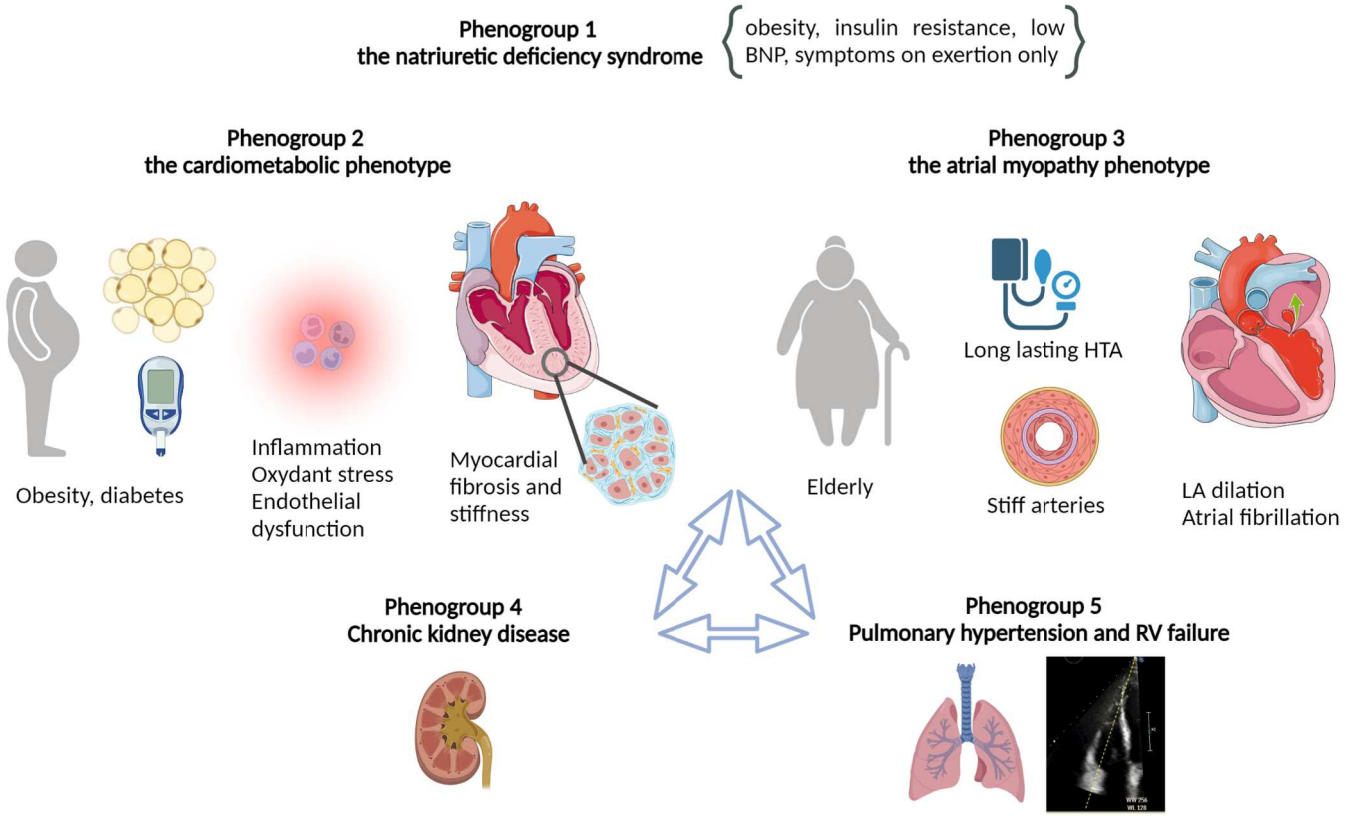



**Figure 1.11 Five common phenogroups of HFpEF**
The natriuretic peptide deficiency syndrome (1) the obesity/cardiometabolic phenotype (2) elderly with atrial myopathy and stiff arteries (3) advanced kidney failure (4) and pulmonary hypertension with right ventricular dysfunction (5) *Adapted from Galli, E., et al. (2021). "Phenomapping Heart Failure with Preserved Ejection Fraction Using Machine Learning Cluster Analysis: Prognostic and Therapeutic Implications." Heart Fail Clin 17(3): 499-518*

# 2. AIMS OF THE THESIS

In light of the available evidence, it is clear that HFpEF is a systemic, multifactorial pathology. Pathophysiological mechanisms and phenotypic presentations are diverse (1). Comorbidity-driven inflammation, oxidative stress and endothelial dysfunction play an important role, at least in a significant proportion of patients (2). Strategies are needed to differentiate those patients for targeted therapeutics (3). Disruption of nitric oxide homeostasis is implicated in the development of HFpEF in preclinical studies but data from human studies are scarce. In practice, human tissue is difficult to obtain, especially in this fragile population and nitric oxide is an unstable gas, hard to quantify in vivo (4).

Hence, the aims of this thesis were (1) to characterize patients with HFpEF in our real life setting, (2) to differentiate profiles of patients based on metabolic comorbidities (diabetes mellitus and obesity) and assess their prognosis and (3) to evaluate whether myeloperoxidase levels could discriminate subgroups of patients according to their level of inflammation and endothelial dysfunction. Finally, (4) based on the available body of evidence at the start of the study, we hypothesized circulating nitric oxide would be decreased in HFpEF and aimed to demonstrate this with the measurement of nitrosylated hemoglobin.

# 3. METHODS AND RESULTS

## 3.1 Heart failure with preserved ejection fraction in Belgium: characteristics and outcome of a real-life cohort

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**ABSTRACT**

BACKGROUND: Due to aging of the population and the increase of cardiovascular risk factors, heart failure and preserved ejection fraction (HFpEF) is a rising health issue. Few data exist on the phenotype of HFpEF patients in Belgium and on their prognosis.

OBJECTIVES: We describe clinical characteristics and outcomes of Belgian HFpEF patients.

METHODS: We prospectively enrolled 183 HFpEF patients. They underwent clinical examination, comprehensive biological analysis and echocardiography, and were followed for a combined outcome of all-cause mortality and first HF hospitalization.

RESULTS: Belgian patients with HFpEF were old (78±8 years), predominantly females (62%) with multiple comorbidities. Ninety five percent were hypertensive, 38% diabetic and 69% overweight. History of atrial fibrillation was present in 63% of population, chronic kidney disease in 60 % and anemia in 58%. Over 30±9 months, 55 (31%) patients died, 87 (49%) were hospitalized and 111 (63%) reached the combined outcome. In multivariate Cox analysis, low body mass index (BMI), NYHA class III and IV, diabetes, poor renal function and loop diuretic intake were independent predictors of the combined outcome ($p < 0.05$). BMI and renal function were also independent predictors of mortality, as were low hemoglobin, high E/e' and poor right ventricular function.

CONCLUSION: Belgian patients with HFpEF are elderly patients with a high burden of comorbidities. Their prognosis is poor with high rates of hospitalization and mortality. Although obesity is a risk factor for developing HFpEF, low BMI is the strongest independent predictor of mortality in those patients.

## INTRODUCTION

Heart failure with preserved ejection fraction (HFpEF) is characterized by signs and symptoms of heart failure, including peripheral oedema, dyspnea and exercise intolerance, in the absence of a reduced left ventricular ejection fraction (LVEF > 50%).[157] The fundamental pathophysiological perturbation leading to HFpEF remains incompletely understood but traditionally it has been attributed to hypertensive left ventricular remodelling.[10] Systemic microvascular endothelial inflammation and dysfunction related to coexisting comorbidities has been proposed as an additional mechanism leading to myocardial inflammation and fibrosis.[37]

Although it is not a rare condition (HF affecting 1-3% of the adult population, half of whom have a preserved ejection fraction[25]) there are currently no treatment consistently improving prognosis for patients with HFpEF. Yet, prognosis is as grim as in heart failure and reduced ejection fraction (HFrEF) with a 5-year mortality rate after hospitalization for acute HF around 75%, which is worse than most cancers.[28] Guidelines currently advise to treat symptoms with diuretics, and to control comorbidities such as hypertension and diabetes tightly.[26,158]

Due to aging of the population and increasing presence of cardiovascular risk factors[25,159] the prevalence of HFpEF will rise in the coming decades. This epidemic proportion together with the lack of treatment makes HFpEF one of the greatest unmet need in 21st century cardiology.[30]

In Belgium, cardiovascular disease is the leading cause of death among women and the second leading cause of death among men after cancer. In 2014, 30 260 people died from cardiovascular diseases (accounting for 31% of all deaths among women and 27% of all deaths among men).[159]

Few data exist on the phenotype of HFpEF patients in Belgium and on their prognosis. Hence, we sought to describe clinical characteristics and outcome of a Belgian cohort, and compare them to patients enrolled in PARAGON-HF[160-162] (most recent clinical trial in HFpEF comparing efficacy and safety of Sacubitril Valsartan versus Valsartan) and to a recent Asian registry.[163]

**METHODS**

*Study population*

Between December 2015 and June 2017, consecutive patients with HFpEF were prospectively evaluated for inclusion in the study. The following criteria had to be fulfilled for study inclusion (Table 1): New York Heart Association (NYHA) functional class ≥II, typical signs of HF, NT-proBNP > 350 pg/ml and/or an hospitalization for HF in the previous 12 months, left ventricular ejection fraction ≥50%, and relevant structural heart disease (left ventricular (LV) hypertrophy/left atrial (LA) enlargement) and/or diastolic dysfunction by echocardiography[164]. The exclusion criteria were severe valvular disease, infiltrative or hypertrophic cardiomyopathy, acute coronary syndrome in the previous 30 days, chronic obstructive pulmonary disease GOLD 3 or 4, congenital heart disease, pericardial disease, atrial fibrillation with a ventricular response >140 bpm, and severe anemia (hemoglobin <8 g/dl). A total of 183 patients satisfied the inclusion criteria. Patients underwent blood sampling and complete transthoracic echocardiography. The local ethics committee approved the study, and all patients gave written informed consent before study enrolment (Clinical trial NCT03197350). The investigation conforms to the principles outlined in Declaration of Helsinki.

**Table 1. Summary of inclusion and exclusion criteria for admission in our Belgian cohort, in Paragon-HF and in Asian-HF.**

| | Belgian cohort | PARAGON – HF[10-12] | ASIAN – HF[13] |
|---|---|---|---|
| Inclusion criteria | Age > 50 years<br>NYHA functional class II-IV,<br>Clinical signs of HF,<br>NT-proBNP >350pg/ml and/or an hospitalization for HF < 12 months,<br>LVEF ≥50%,<br>LVH or LA enlargement and/or diastolic dysfunction | Age > 50 years<br>NYHA functional class II to IV<br>Diuretic therapy for 30 days before screening,<br>High NT-proBNP<br><br>LVEF > 45 %<br><br>LVH or LA enlargement[1] | Age >18 years<br><br>Symptomatic HF (at least one episode of decompensated HF in the previous months )<br><br><br>LVEF ≥50% |
| Exclusion criteria | Severe valvular disease,<br>Alternative diagnosis<br>Severe uncontrolled HTA | Prior LVEF <40%<br>Alternative diagnosis<br>Systolic blood pressure <110 or > 180mmHg<br>AF limited to 33%<br>eGFR < 30mL/min/1.73m2<br>History of malignancy < 5 years<br>Intolerance of Sacubitril Valsartan (run in) | Severe valvular disease<br>Life-threatening co-morbidity with a life expectancy <1year<br><br>Unable or unwilling to give consent |

NYHA: New York Heart Association; HF: heart failure; LVEF: left ventricular ejection fraction; LVH: left ventricular hypertrophy (septal or posterior wall thickness > 1.1 cm); LA enlargement: left auricular enlargement (width > 3.8 cm, length > 5.0 cm, area > 20 cm2, volume > 55 ml, or volume index >29 ml/m2); HTA: hypertension; AF: atrial fibrillation; eGFR: estimated glomerular filtration rate. Alternative diagnosis including chronic obstructive pulmonary disease (COPD) stage GOLD III – IV, severe anemia (Hb<8g/dl), pericardial disease, congenital heart disease, hypertrophic / infiltrative cardiomyopathy, acute coronary syndrome within 30 days

*Clinical data*

Patients were interrogated about symptoms, medical history and treatment and were thoroughly examined. Other information was retrieved from medical files and from review of hospital records.

*Echocardiography*

Standardized complete transthoracic echocardiography (TTE) exams were acquired according to established guidelines using iE33 ultrasound systems (Philips Medical Systems, Andover, Massachusetts) equipped with a 3.5/1.75-MHz phased-array transducer and stored on a XCELERA 2.1 PACS server (Philips Medical Systems, Andover, Massachusetts).

*Follow up*

Patients were prospectively followed by ambulatory visits and phone calls at 6-months intervals. Clinical and survival status was obtained by follow up visits and by phone contact with the patients, their relatives, or their physician if necessary. The primary endpoint was a composite of all-cause mortality or hospitalization for HF, whichever came first. Hospitalization was defined as patients diagnosed with heart failure and requiring IV diuretics, either treated in the emergency room or admitted to the hospital. The secondary endpoint was overall mortality.

*Statistical analysis*

Statistical analyses were performed using SPSS version 25 (SPSS Corp., Somers, New York). All tests were 2-sided and p-value <0.05 was considered statistically significant. Continuous variables were expressed as mean ± 1 standard deviation (SD) and categorical variables as count and proportion. To determine predictors of the primary and secondary endpoints, univariate Cox proportional hazards models were used. Hazard ratios (HR) were expressed as mean and 95% confidence interval (CI). All clinical and biological parameters were proposed for inclusion in the univariate model. Then, a backward multivariate Cox regression including all significant ($p < 0.05$) univariate correlates of survival was used to determine independent predictors of prognosis. Differences among groups according to body

mass index (BMI) were examined using "p for trend" analyses (by ANOVA or Chi-square test for linear association, when appropriate). Kaplan Meier curves were used to illustrate survival, and event-free survival of HFpEF patients. The log-rank test was used to compare survival among different groups.

## RESULTS

*Baseline characteristics*

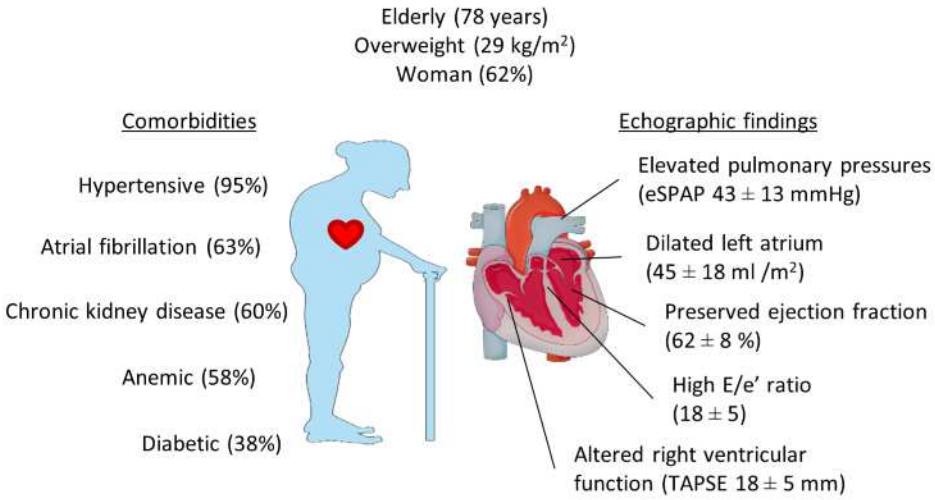
Baseline characteristics of patients are illustrated in Figure 1 and summarized in Table 2. Our cohort is composed of relatively old patients (78 ± 8 years), predominantly females (62%) with high burden of cardiovascular risk factors. Nearly all patients were hypertensive (95%), the prevalence of diabetes was of 38%, and the mean BMI was of 29±6 kg/m², with more than two thirds of the population being at least overweight (33% overweight and 36% obese). History of atrial fibrillation was the most common comorbidity, present in 63% of the study population, closely followed by chronic kidney disease (CKD) with 60% of the population having an estimated glomerular filtration rate (eGFR) lower than 60 ml/min/1.73m$^2$. Anemia, defined as a hemoglobin level <12g/dl in women and <13g/dl in men was present in 58% of the population.

The Minnesota Living with Heart Failure Questionnaire (MLHFQ) was collected for 73 patients. The mean MLHFQ score was 36.21±19.54. Patients were evenly distributed among groups representing good, moderate or poor quality of life. Indeed, 24 (33%) patients had MLHFQ score < 24, 26 (35%) had a score between 24 and 45, and 23 (32%) had a score higher than 45.

Regarding medication, the majority of patients took diuretics and/or angiotensin converting enzyme (ACE) inhibitors. Due to the high frequency of atrial fibrillation, anticoagulant and beta-blockers were also frequently prescribed.

Imaging parameters confirmed a preserved ejection fraction (62±8%) and showed signs of LV diastolic dysfunction with high LA volume index (45±18 mL/$m^2$) and high E/e' ratio (19±8). They also had high pulmonary pressures, with a mean estimated systolic pulmonary artery pressure (eSPAP) of 43±13 mmHg) and a poor right ventricular function (TAPSE 18±5 mm).

**Figure 1. Clinical and echographic characteristics of our HFpEF population**




Adapted from Servier Medical Art online image bank https://smart.servier.com/smart_image/heart-4/ and /shape-16/

**Table 2. Baseline characteristics of HFpEF patients**

| | **Belgian cohort (N=183)** | **PARAGON – HF[10-11] (N=4822)** | **ASIAN – HF[13] (N = 1204)** |
|---|---|---|---|
| **Age, y** | **78±8** | **73±8** | **68±12** |
| **Female sex** | **62%** | **52%** | **50%** |
| **Body mass index, kg/m$^2$** | **29±6** | **30±5** | **27±6** |
| NYHA class II | 47% | 72% | 59% |
| III | 33% | 27% | 21% |
| **IV** | **14%** | **0.6%** | **3%** |
| Heart rate, beats per min | 73±14 | 70±12 | 76±16 |
| Systolic blood pressure, mm Hg | 138±21 | 136±15 | 132±22 |
| Diastolic blood pressure, mm Hg | 74±13 | 77±11 | 73±13 |
| Medical History | | | |
| **Prior heart failure hospitalization** | **68%** | **48%** | **57%** |
| Coronary artery disease | 32% | 43% | 29% |
| **Atrial fibrillation/atrial flutter** | **46%** | **32%** | **24%** |
| **History of AF** | **63%** | **52%** | **29%** |
| Left bundle branch block | 12% | 7% | 3% |
| Hypertension | 95% | 96% | 71% |
| Diabetes mellitus | 38% | 43% | 45% |
| Stroke | 14% | 10% | 8% |
| COPD | 10% | 14% | 9% |
| Biology | | | |
| Glomerular filtration rate **<45** | **38%** | **18%** | **50%** |
| (ml/min) ≥45,<60 | 22% | 30% | **(<60)** |
| ≥60 | 40% | 53% | 50% |
| **NT-proBNP, pg/mL** | **1927 (1032 -3726)** | **855 (863-908)** | **1448 (528 – 3290)** |
| Hemoglobin, g/dL | 12±2 | | 12±2 |
| Medication | | | |
| Diuretic | 68% | 96% | 64% |
| Mineralocorticoid receptor antagonists | 17% | 24% | 22% |
| ACE inhibitors | 68% | 85% | 66% |
| β-blockers | 64% | 75% | 68% |
| Anticoagulant | 58% | 27% | |
| Statin lipid-lowering medication | 42% | 62% | |
| Echocardiographic parameters | | | |

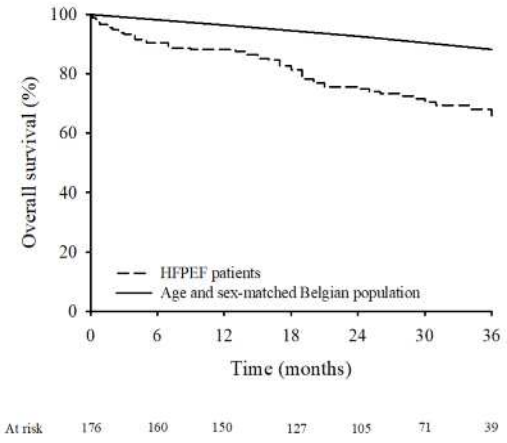
| | | | |
|---|---|---|---|
| LV ejection fraction, % | 62±8 | 58±8 | 60±5 |
| LA volume index, ml/m$^2$ | 45±18 | 39±16 | 34±10 |
| Septal E/e' ratio | 19±8 | 17±7 | 16±4 |
| TAPSE, mm | 18±5 | 18±4 | |
| eSPAP, mmHg | 43±13 | 34±10 | |
| Outcome | | | |
| Event rate (hospitalization for heart failure or death withing 1 year follow up) | 33% | 14% | 12% |

Continuous variables are expressed as mean ± SD and categorical variables as proportion
NYHA: New York Heart Association; AF: atrial fibrillation; COPD: chronic obstructive pulmonary disease; ACE: angiotensin converting enzyme, LV: left ventricle; LA: left atrium; TAPSE: tricuspid annular plane systolic excursion; eSPAP: estimated systolic pulmonary artery pressures.

*Outcome*

The follow up was completed for 177 (97%) patients for a mean duration of 30±9 months. Over this period of time, 55 (31%) patients died, 87 (49%) were hospitalized and 111 (63%) reached the combined outcome of overall death or HF hospitalization, whichever came first. Kaplan Meier curves of survival (Figure 2) illustrate the prognosis of HFpEF patients compared to age and sex matched Belgian population. Amongst the deaths, 30 (55%) were from cardiovascular origin, 18 (33%) were from other causes, and the cause was unknown for 7 (12%) patients.

**Figure 2. Kaplan Meier curves of survival in HFpEF patients and age and sex matched Belgian population.**



In univariate Cox regression analysis (Table 3 and 4), low BMI, high NT-proBNP, low hemoglobin, low eGFR, high E/e', low TAPSE and high eSPAP were significant predictors of both the combined outcome and overall mortality. The severity of symptoms (NYHA functional class III and IV), the presence of diabetes and loop diuretic intake were only predictors of the combined outcome.

Low BMI, NYHA functional class III and IV, diabetes, low eGFR and loop diuretic medication were independent predictors of the combined outcome in multivariate Cox regression (Table 3). Low BMI and low eGFR were also an independent predictor of mortality, as were low hemoglobin, high E/e' and low TAPSE (Table 4).

**Table 3. Univariate and Multivariate Cox Regression analysis for prediction of the primary endpoint (composite of all cause deaths and HF hospitalization)**

| | **Univariate** | | | **Multivariate** | | |
|---|---|---|---|---|---|---|
| | **HR** | **95 % CI** | **P** | **HR** | **95 % CI** | **P** |
| Age | 1.01 | 0.99 – 1.04 | 0.35 | | | |
| Female sex | 1.37 | 0.97 – 2.03 | 0.12 | | | |
| **BMI** | **0.97** | **0.94 – 0.99** | **0.044** | 0.95 | 0.92 – 0.99 | **0.004** |
| **NYHA class III – IV** | **1.62** | **1.11 – 2.36** | **0.012** | 1.96 | 1.33 – 2.88 | **0.001** |
| | | | | | | |
| Atrial Fibrillation | 1.43 | 0.97 – 2.12 | 0.069 | | | |
| Ischemic | 1.08 | 0.73 – 1.60 | 0.70 | | | |
| **Diabetes** | **1.65** | **1.13 – 2.40** | **0.009** | 1.85 | 1.24 – 2.77 | **0.003** |
| COPD | 1.67 | 0.96 – 2.88 | 0.067 | | | |
| | | | | | | |
| **NT-proBNP** | **1.30** | **1.09 – 1.56** | **0.004** | - | - | - |
| **eGFR** | **0.98** | **0.97 – 0.99** | **< 0.001** | 0.98 | 0.97 – 0.99 | **< 0.001** |
| **Hemoglobin** | **0.84** | **0.76 – 0.93** | **0.001** | - | - | - |
| | | | | | | |
| **Loop diuretics** | **1.86** | **1.19 – 2.91** | **0.006** | 1.99 | 1.26 – 3.15 | **0.003** |
| Anticoagulant | 1.44 | 0.97 – 2.13 | 0.068 | | | |
| | | | | | | |
| LA volume index | 1.01 | 1.00 – 1.02 | 0.063 | | | |
| **E/e'** | **1.03** | **1.01 – 1.05** | **0.001** | - | - | - |
| **TAPSE** | **0.96** | **0.93 – 0.99** | **0.019** | - | - | - |
| **eSPAP** | **1.02** | **1.01 – 1.03** | **0.008** | - | - | - |

**Table 4. Univariate and Multivariate Cox Regression analysis for prediction of the secondary endpoint (overall mortality)**

| | Univariate | | | Multivariate | | |
|---|---|---|---|---|---|---|
| | **HR** | **95 % CI** | **P** | **HR** | **95 % CI** | **P** |
| Age | 1.02 | 0.99 – 1.06 | 0.19 | | | |
| Female sex | 1.05 | 0.61 – 1.81 | 0.87 | | | |
| **BMI** | **0.92** | **0.88 – 0.97** | **0.001** | 0.92 | 0.88 – 0.97 | **0.001** |
| **NYHA class III – IV** | 1.23 | 0.72 – 2.10 | 0.45 | - | - | - |
| | | | | | | |
| History of Atrial Fibrillation | 0.90 | 0.52 – 1.54 | 0.70 | | | |
| Ischemic cardiomyopathy | 1.01 | 0.58 – 1.76 | 0.98 | | | |
| **Diabetes** | 1.58 | 0.93 – 2.68 | 0.092 | | | |
| COPD | 0.93 | 0.37 – 2.33 | 0.87 | | | |
| | | | | | | |
| **NT-proBNP** | **1.43** | **1.11 – 1.83** | **0.005** | **-** | **-** | **-** |
| **eGFR** | **0.98** | **0.96 – 0.99** | **0.002** | 0.99 | 0.97 – 1.00 | **0.050** |
| **Hemoglobin** | **0.78** | **0.68 – 0.90** | **0.001** | 0.84 | 0.72 – 0.97 | **0.019** |
| | | | | | | |
| **Loop diuretics** | 1.76 | 0.91 – 3.42 | 0.094 | | | |
| Anticoagulant | 1.28 | 0.74 – 2.21 | 0.39 | | | |
| | | | | | | |
| LA volume index | 1.01 | 0.99 – 1.03 | 0.081 | | | |
| **E/e'** | **1.04** | **1.01 – 1.07** | **0.005** | 1.03 | 1.00 – 1.06 | **0.048** |
| **TAPSE** | **0.96** | **0.89 – 0.99** | **0.031** | 0.94 | 0.87 – 0.99 | **0.030** |
| **eSPAP** | **1.03** | **1.01 – 1.05** | **0.005** | **-** | **-** | **-** |

BMI: body mass index, NYHA: New York Heart Association; COPD: chronic obstructive pulmonary disease; eGFR: estimated glomerular filtration rate, LA: left atrium; TAPSE: tricuspid annular plane systolic excursion; eSPAP: estimated systolic pulmonary artery pressures.
HR: hazard ration; CI: confidence interval

*BMI and mortality*

To explore the association between BMI and mortality, the cohort was divided among 4 groups according to BMI (< 25, 25 – 30, 30 – 35, or > 35 kg/m$^2$) (table 5). Across groups of increasing BMI, patients were younger (p-for trend < 0.001) and suffered more often from diabetes (p for trend = 0.001). Although trend analysis

was not significant (p=0.087) there was an important proportion of females (80%) in the morbidly obese group (BMI > 35 kg/m$^2$). Other biological and echocardiographic parameters did not differ significantly across groups.

**Table 5. Baseline Characteristics according to body mass index (BMI)**

| | BMI <25 N = 55 (30%) | BMI 25 – 30 N = 63 (34%) | BMI 30-35 N = 30 (16%) | BMI > 35 N = 35 (19%) | P for trend |
|---|---|---|---|---|---|
| **Age, years** | **80 ± 8** | **81 ± 7** | **75 ± 9** | **73 ± 9** | **<0.001** |
| **Female sex** | **34 (62%)** | **33 (52%)** | **18 (60%)** | **28 (80%)** | **0.087** |
| NYHA class III – IV | 29 (53%) | 28 (44%) | 16 (53%) | 14 (40%) | 0.38 |
| CAD | 15 (27%) | 24 (38%) | 9 (30%) | 12 (34%) | 0.64 |
| History of AF | 35 (64%) | 43 (68%) | 14 (47%) | 23(66%) | 0.64 |
| Hypertension | 50 (93%) | 60 (95%) | 28 (93%) | 34 (97%) | 0.45 |
| **Diabetes** | **14 (25%)** | **21 (33%)** | **16 (53%)** | **19 (54%)** | **0.001** |
| eGFR, mL/min | 54 ± 23 | 57 ± 27 | 56 ± 20 | 52 ± 20 | 0.72 |
| NT-proBNP, pg/mL | 2207 [1332–5079] | 1141 [1927–3714] | 1924 [699–3451] | 1148 [484-2425] | 0.20 |
| Hemoglobin, g/dL | 11 ± 2 | 12 ± 2 | 12 ± 2 | 12 ± 2 | 0.064 |
| Septal E/e' ratio | 18 ± 8 | 20 ± 9 | 21 ± 9 | 18 ± 6 | 0.48 |
| TAPSE, mm | 18 ± 5 | 18 ± 6 | 19 ± 6 | 19 ± 4 | 0.48 |
| eSPAP, mmHg | 43 ± 13 | 44 ± 13 | 45 ± 12 | 42 ± 12 | 0.64 |

Continuous variables are expressed as mean ± SD and categorical variables as count and proportion. P-values are derived from p-for-trend analysis, by ANOVA or Chi-square test for linear association when appropriate.
BMI: body mass index; NYHA: New York Heart Association; CAD: coronary artery disease; AF: atrial fibrillation, eGFR: estimated glomerular filtration rate, TAPSE: tricuspid annular plane systolic excursion; eSPAP: estimated systolic pulmonary artery pressures.

Figure 3 shows how the one-year mortality rate decreases across groups of increasing BMI. As already shown, lower BMI was significantly associated with mortality in univariate Cox regression analysis (unadjusted HR 0.92 [0.88 – 0.97], p<0.001). This stayed true after adjustment for age, sex and diabetic status (adjusted HR 0.91 [0.86 – 0.95], p<0.001). Figure 4 shows the Kaplan Meier curve of survival of HFpEF patients according to BMI groups, adjusted for age, sex and diabetic status.

Finally, this association between BMI and mortality was also significant when accounting only for cardiovascular deaths. The unadjusted hazard ratio of BMI for the prediction of cardiovascular deaths was 0.94 [0.88 – 0.99], p=0.046 and 0.93 [0.86 – 0.99], p = 0.034 after adjustment for age, sex and diabetic status.

**Figure 3. One-year mortality rate among increasing body mass index in patients with HFpEF.**

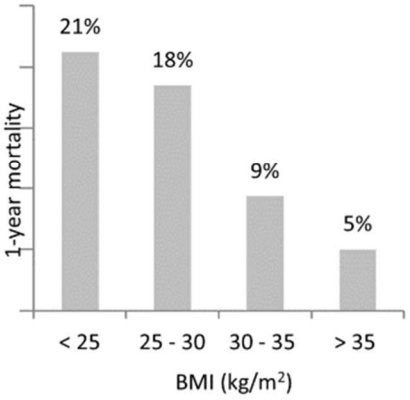
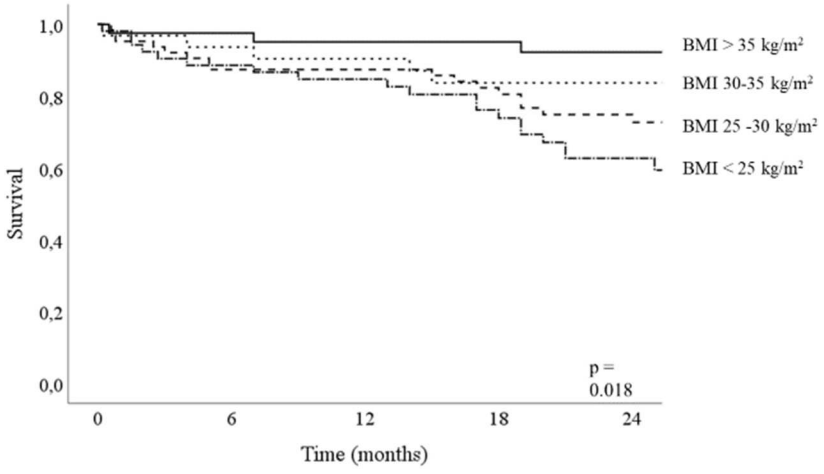



**Figure 4. Kaplan Meier curves of survival in HFpEF patients acording to body mass index groups, adjusted for age, sex and diabetic status.**




| | | | | | |
|---|---|---|---|---|---|
| BMI > 35 | 34 | 33 | 30 | 27 | 25 |
| BMI 30-35 | 28 | 26 | 25 | 20 | 19 |
| BMI 25 -30 | 62 | 54 | 53 | 45 | 34 |
| BMI < 25 | 53 | 47 | 42 | 34 | 24 |

**DISCUSSION**

This study describes baseline characteristics and outcome of Belgian patients with HFpEF. Patients were relatively old (78±8 years) and carried a high comorbidity burden. The prevalence of anemia and CKD was particularly important (58% and 60% respectively). Atrial fibrillation was the most frequent cardiac comorbidity among Belgian HFpEF patients (63%), which is consistent with prior data.[15,165,166] A recent review reported that between 22 and 53 % of HFpEF patients suffered from anemia, 26 to 52 % from CKD, 33 – 43 % from diabetes mellitus and that 33 – 51 % were obese[39]. Prevalence of those comorbidities in HFpEF patients is very high compared to the general population of Belgium. Indeed, based on the Health Interview Surveys of 2013 (self-reported information on approximately 10.000 inhabitants), the prevalence of diabetes was 5.3% while 16.5% of the population acknowledged an elevated blood pressure. The mean self-reported BMI was estimated at 25.4 kg/m², with an obesity rate of 14%.[159,167] As it has already been hypothesized[2,30,37], the very high prevalence of comorbidities among HFpEF patients may suggest they play a pathophysiological role. More importantly, it underlines the importance of close cardiovascular monitoring of patients at risk to prevent HF before the onset of symptoms, and open the way for preventive strategies.

In comparison with one of the largest contemporary trial involving HFpEF patients (PARAGON-HF)[160], some differences are to be highlighted (Table 2). The patients in our real-life cohort appeared to be more advanced heart failure patients. They were more symptomatic (14 % of NYHA functional class IV VS 0.6 %), with higher levels of NT-proBNP, lower hemoglobin and lower renal function. Furthermore, the PARAGON study limited the prevalence of AF to 30% although it seems to be more prevalent among HFpEF patients in real life. These differences are mainly explained

by the design of the study (Table 1), which excluded frailer patients with renal insufficiency or intolerance to Sacubitril-Valsartan during a “run in” period. These considerations remind us that study populations are not always a reliable reflection of patients encountered in daily practice.

The Belgian cohort was also compared to a real-life registry in Asia[163] (Table 2). Interestingly, we found that the Asian cohort was composed of younger and leaner patients, with a higher prevalence of diabetes. Hypertension and atrial fibrillation, however were less frequent among Asian patients. The prevalence of CKD, the levels of NT-proBNP and the medication use were similar in both groups. Differences between Asian and Belgian patients may support current theories that HFpEF is a global term encompassing different phenotypes with distinct pathophysiological pathways, which might be one of the key reasons why clinical trials have failed to find effective treatment.[42,168] A recent study[169] using data from large contemporary trials (CHARM-preserved, I-PRESERVE, and TOPCAT) suggests a potential dichotomization of HFpEF phenotypes, with young, diabetic and obese HFpEF on one hand, versus elderly women with a higher comorbidity load, in particular atrial fibrillation, on the other hand. The latter seems to be the predominant phenotype in our study.

With regards to outcome, HFpEF has a strong impact on both quality of life and life expectancy. Two thirds of the patients reported an altered quality of life as a result of heart failure symptoms (MLHFQ > 24) and the 1-year mortality rate was found to be 11.9%, which is consistent with data from other Western cohorts[170]. After a mean follow up of two years, more than two third of the patients had reached the combined outcome and one third was dead. Kaplan Meier curves of survival (Figure 2) illustrate the poor prognosis of HFpEF patients compared to age and sex matched Belgian population. The ratio of CV- versus non CV deaths was similar to previous

data[171,172], with CV deaths accounting for 55% of the mortality. As expected[173,174], low hemoglobin and low renal function were associated with an increase in mortality. As were signs of diastolic dysfunction (high E/e') and of right ventricular dysfunction (low TAPSE).

It is interesting to note that although obesity is one of the main risk factor for developing HFpEF[42,50], patients with low BMI had the worse prognosis. In fact, low BMI was the strongest independent predictor of mortality (Table 4). This has already been referred to as "the obesity paradox"[175,176]. In 2010, Kapoor et al.[177] demonstrated a U-shaped relationship between BMI and mortality in HFpEF, with better survival for groups with BMI up to 45kg/m$^2$. Since then, this entity has been controversial. Some authors believe that the obesity paradox is the result of incorrect statistical analysis with lack of adjustment for confounding factors and collider stratification bias[178,179]. Indeed, the protective effect of BMI is only observable in patients with chronic disease and cannot be generalized. This can be explained by the catabolic state associated with chronic disease, resulting in fat mass, as well as lean mass loss (i.e. cachexia), which carries a devastating prognosis in heart failure, especially in the elderly[180,181]. Furthermore, the utility of BMI to assess obesity has been criticized for its inability to differentiate between fat, muscle and skeletal weight and individuals with similar BMI may have very different metabolic profiles[181]. It can also be argued that this effect is due to weight loss secondary to concomitant diseases associated with poor prognosis such as cancer or chronic inflammatory disease. However, in our cohort of elderly HFpEF patients, we found a protective effect of higher BMI, even after adjustment for traditionally described confounding factors, and this was also true for cardiovascular mortality.

Certainly, describing the obesity paradox is not a promotion of overweight and obesity. Obesity clearly increases cardiovascular risk among the general population

and controlling this risk factor before the onset of heart failure may lead to decreased morbidity and mortality. However, one of the main purposes for reporting the obesity paradox is to emphasize that physicians should be more concerned about the poor prognosis in their leaner or underweight patients with HFpEF.

*Limitations*

Several limitations should be addressed when interpreting the results of this study. First, this is a single center study. All patients were recruited from the cardiology service of the Cliniques Universitaires Saint Luc in Brussels, at ambulatory visits or during hospitalization for heart failure. Hence, the cohort might not reflect the overall Belgian population, nor the population of HFpEF patients seen by general practitioners. There is also a potential bias linked to willingness of patients to participate the study. Although data were collected prospectively, the association between BMI and mortality were derived from retrospective analyses. As such, the obesity paradox is subject to collider stratification bias and our data do not allow to generalize this finding beyond HFpEF patients. Also, other data parameters, such as biomarkers of nutritional status and invasive hemodynamics, that may have improved risk adjustment, were unavailable.

**CONCLUSION**

Our study illustrates that HFpEF patients in Belgium are elderly, with high burden of comorbidities. It emphasizes the poor prognosis for HFpEF patients and the need to pursue research aimed at better understanding the development of the disease to discover therapeutic targets. Finally, our data shows that in HFpEF, low BMI is associated with an increase in mortality and clinicians should be concerned about the poor prognosis in their leaner or underweight HF patients.

## 3.2 Diabetic phenotype and prognosis of patients with heart failure and preserved ejection fraction in a real life cohort

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**ABSTRACT**

BACKGROUND: Heart failure with preserved ejection fraction (HFpEF) is a heterogeneous syndrome, with several underlying etiologic and pathophysiologic factors. The presence of diabetes might identify an important phenotype, with implications for therapeutic strategies. While diabetes is associated with worse prognosis in HFpEF, the prognostic impact of glycemic control is yet unknown. Hence, we investigated phenotypic differences between diabetic and non-diabetic HFpEF patients (pts), and the prognostic impact of glycated hemoglobin (HbA1C).

METHODS*:* We prospectively enrolled 183 pts with HFpEF (78±9 yrs, 38% men), including 70 (38%) diabetics (type 2 diabetes only). They underwent 2D echocardiography (n=183), cardiac magnetic resonance (CMR) (n=150), and were followed for a combined outcome of all-cause mortality and first HF hospitalization. The prognostic impact of diabetes and glycemic control were determined with Cox proportional hazard models, and illustrated by adjusted Kaplan Meier curves.

RESULTS: Diabetic HFpEF pts were younger (76±9 vs 80±8 yrs, p=0.002), more obese (BMI 31±6 vs 27±6 kg/$m^2$, p=0.001) and suffered more frequently from sleep apnea (18% vs 7%, p=0.032). Atrial fibrillation, however, was more frequent in non-diabetic pts (69 % vs 53 %, p=0.028). Although no echocardiographic difference could be detected, CMR analysis revealed a trend towards higher LV mass (66±18 vs 71±14 g/$m^2$, p=0.07) and higher levels of fibrosis (53% vs 36% of patients had ECV by T1 mapping > 33%, p=0.05) in diabetic patients.
Over 25±12 months, 111 HFpEF pts (63%) reached the combined outcome (24 deaths and 87 HF hospitalizations). Diabetes was a significant predictor of mortality and hospitalization for heart failure (HR: 1.72 [1.1 – 2.6], p = 0.011, adjusted for age, BMI, NYHA class and renal function). In diabetic patients, lower levels of

glycated hemoglobin (HbA1C <7%) were associated with worse prognosis (HR: 2.07 [1.1 – 4.0], p=0.028 adjusted for age, BMI, hemoglobin and NT-proBNP levels).

CONCLUSION: Our study highlights phenotypic features characterizing diabetic patients with HFpEF. Notably, they are younger and more obese than their non-diabetic counterpart, but suffer less from atrial fibrillation. Although diabetes is a predictor of poor outcome in HFpEF, intensive glycemic control (HbA1C < 7%) in diabetic patients is associated with worse prognosis.

**BACKGROUND**

Heart failure with preserved ejection fraction (HFpEF) is increasingly being recognized as an umbrella term describing a heterogeneous group of clinical and pathophysiological phenotypes. HFpEF is a diagnostic challenge, especially since important features are mainly apparent on exercise and require dynamic testing [182]. Furthermore, the phenotypic heterogeneity among patients is a key reason for current lack of treatment improving outcome. Indeed most recent clinical trial using sacubitril-valsartan in HFpEF had disappointing results [35], although it could decrease the rate of hospitalisation in specific subgroups [183]. All eyes are now turned towards ongoing studies with sodium-glucose cotransporter-2 inhibitors (SGLT-2i) [184]. Type 2 diabetes (T2D) is one of the most frequent comorbidity associated with HFpEF (prevalence varying from 33 to 43 %)[39], but there are still numerous uncertainties surrounding the mechanisms by which these two conditions interact. There is a need to understand the clinical characteristics of patients with HFpEF and diabetes in order to guide therapeutic decision making, highlight potential phenotype-specific targets, and aid in the development of risk stratification tools. Sub studies of large clinical trials (RELAX-HF[63], I-PRESERVE[64], CHARM[65] and TOPCAT[66]) comparing diabetic and nondiabetic patients showed that HFpEF patients with T2D were younger, more obese, displayed greater structural echocardiographic abnormalities (higher left ventricular mass) and had a worse prognosis than patients without T2D. Those studies were clinical trials with restrictive inclusion criteria and might not reflect HFpEF patients encountered in daily practice. The same differences in clinical characteristics were found in a large American registry (GWTG-HF registry)[185] but imaging parameters were not available for analyzes. Previous studies [186,187] showed a U-shaped association between HbA1C and prognosis in heart failure patients. Those studies either were conducted among patients with HFrEF alone, or did not make a distinction between patients according

to ejection fraction. Glycemic variability was found to be associated with diastolic dysfunction and with poor outcome in HFpEF [188,189], but data remain limited. Accordingly, we aimed to investigate phenotypic differences between diabetic and nondiabetic patients with HFpEF in a prospective, real life cohort. The prognostic impact of glycemic control assessed by HbA1C was also evaluated in this population.

**METHODS**

*Study population*

Patients with HFpEF encountered in our division of cardiology between December 2015 and June 2017 (in hospital and at ambulatory visits) were prospectively screened for inclusion in the study. Inclusion and exclusion criteria were reported in previous publications.[190] Briefly, the following criteria had to be fulfilled: New York Heart Association (NYHA) functional class ≥II, typical signs of HF, NT-proBNP > 350 pg/ml and/or an hospitalization for HF in the previous 12 months, left ventricular ejection fraction ≥50%, and relevant structural heart disease (left ventricular (LV) hypertrophy/left atrial (LA) enlargement) and/or diastolic dysfunction by echocardiography[164]. The exclusion criteria were: history of reduced ejection fraction (LVEF < 50%), severe valvular disease, infiltrative or hypertrophic cardiomyopathy, acute coronary syndrome in the previous 30 days, severe chronic obstructive pulmonary disease, congenital heart disease, pericardial disease, atrial fibrillation (AF) with a ventricular response >140 bpm, and severe anemia (hemoglobin <8 g/dl). A total of 183 patients satisfied the inclusion criteria. Patients underwent blood sampling and complete transthoracic echocardiography and cardiac magnetic resonance (CMR) in the absence of following contra-indications: pacemaker, claustrophobia or estimated glomerular filtration rate (eGFR) < 30mL/min/1.73m$^2$ (N=151). The local ethics committee approved the study, and all

patients gave written informed consent before study enrolment (Clinical trial NCT03197350). The investigation conforms to the principles outlined in Declaration of Helsinki.

*Clinical data*

Patients were interrogated about symptoms, medical history and treatment and were thoroughly examined. Other information, including diagnosis and treatment of diabetes were retrieved from medical files and from review of hospital records.

*Echocardiography*

Standardized complete transthoracic echocardiography (TTE) exams were acquired according to established guidelines using iE33 ultrasound systems (Philips Medical Systems, Andover, Massachusetts) equipped with a 3.5/1.75-MHz phased-array transducer and stored on a XCELERA 2.1 PACS server (Philips Medical Systems, Andover, Massachusetts).

*Cardiac magnetic resonance*

CMR was performed using a 3 Tesla system (Ingenia, Philips Medical Systems, Best, The Netherlands). The different sequences have been previously described.[191] Pre- and post-contrast MOLLI images were processed using the open-source software MRmap v1.4 under IDL. Pre- and post-myocardial T1 times were measured in six regions of interest in the myocardium (anterior, anterolateral, inferolateral, inferior, inferoseptal, anteroseptal). We calculated the average T1 time of the six different regions of interest. Areas of ischemic focal fibrosis identified by late gadolinium enhancement (LGE) were excluded from the analysis. Extracellular volume (ECV) was then computed according to the formula[192]. A cut off of ECV > 33% was used to define significant diffuse myocardial fibrosis [191].

*Follow up*

Patients were prospectively followed by ambulatory visits and phone calls at 6-months intervals. Clinical and survival status was obtained by follow up visits and by phone contact with the patients, their relatives, or their physician if necessary. The primary endpoint was a composite of all-cause mortality or hospitalization for HF, whichever came first. Hospitalization was defined as patients diagnosed with heart failure and requiring intravenous diuretics, either treated in the emergency room or admitted to the hospital.

*Statistical analysis*

Statistical analyses were performed using SPSS version 25 (SPSS Corp., Somers, New York). All tests were 2-sided and p-value <0.05 was considered statistically significant. Continuous variables were expressed as mean ± 1 standard deviation (SD) and categorical variables as count and proportion. Differences of characteristics between groups were examined using independent sample t-test or Chi square test when appropriate. Uni- and multivariate Cox regression analyzes were used to determine the prognostic impact of diabetes and HbA1C. Diabetic patients enrolled in the study who completed the follow up (67/70, 96%) and with at least one HbA1c measurement in the three months previous to inclusion were used for analyzes about the prognostic impact of glycemic control (62/70, 89%). Adjusted Kaplan Meier curves were used to illustrate event-free survival of HFpEF patients. The log-rank test was used to compare survival among different groups.

## RESULTS

*Characteristics and outcome of diabetic versus nondiabetic HFpEF patients (Table 1)*

The total population was constituted of 183 HFpEF patients (78±9 years, 62% women), including 70 (38%) diabetics. Diabetic HFpEF patients were younger (76±9 vs 80±8 yrs, p=0.002) and more obese (body mass index (BMI) 31±6 vs 27±6 kg/m$^2$, p=0.001). They suffered more frequently from chronic coronary artery disease (47% vs 24%, p=0.001) and obstructive sleep apnea (18% vs 7%, p=0.032). Atrial fibrillation, however, was more frequent in nondiabetic patients (69 % vs 53 %, p=0.028). Although no echocardiographic difference could be detected between the two groups, CMR analysis revealed a trend towards higher LV mass in the diabetic population (66±18 vs 71±14 g/m$^2$, p=0.07). Interestingly, more diabetic patients (53% vs 36%, p=0.05) had high levels of myocardial fibrosis (defined as ECV by T1 mapping > 33%)[191]. The main differences between diabetic and nondiabetic patients are summarized in Figure 1.

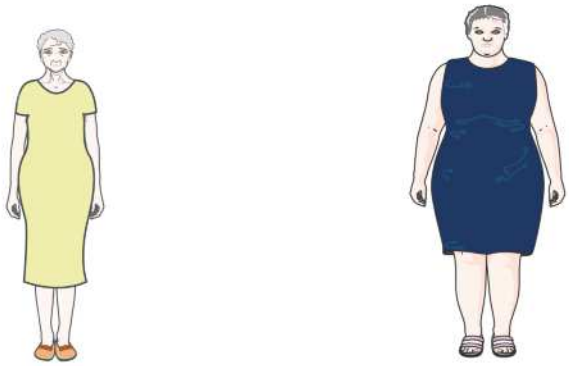
**Table 1. Clinical, echocardiographic and CMR characteristics of diabetic versus nondiabetic HFpEF patients.**

| | Nondiabetic<br>N = 113 (62%) | Diabetic<br>N = 70 (38%) | P-value |
|---|---|---|---|
| Age (years) | 80 ± 8 | 76 ± 9 | 0.002 |
| Female (n, %) | 71 (63%) | 42 (60%) | 0.70 |
| Body mass index (kg/m$^2$) | 27 ± 6 | 31 ± 6 | 0.001 |
| NYHA III – IV (n, %) | 60 (53%) | 27 (39%) | 0.056 |
| Hospitalized for HF at inclusion (n,%) | 73 (65%) | 43 (61%) | 0.53 |
| Atrial fibrillation (n, %)<br>History<br>At inclusion | <br>78 (69%)<br>57 (50%) | <br>37 (53%)<br>26 (37%) | <br>0.028<br>0.079 |
| Ischemic cardiomyopathy (n, %) | 27 (24%) | 33 (47%) | 0.001 |
| Smoking (n, %) | 50 (45%) | 27 (39%) | 0.42 |
| Hypertension (n, %) | 105 (93%) | 67 (97%) | 0.23 |
| Hypercholesterolemia (n, %) | 66 (59%) | 49 (70%) | 0.13 |

| | | | |
|---|---|---|---|
| Sleep apneas (n, %) | 8 (7%) | 12 (18%) | 0.032 |
| COPD (n, %) | 12 (11%) | 7 (10%) | 0.88 |
| Medication | | | |
| Loopdiuretics (n, %) | 73 (65%) | 51 (73%) | 0.25 |
| MRA (n, %) | 19 (17%) | 13 (19%) | 0.76 |
| Beta blockers (n, %) | 77 (68%) | 41 (59%) | 0.19 |
| ACE inhibitors/ARB (n, %) | 78 (69%) | 46 (66%) | 0.64 |
| Biology | | | |
| eGFR (ml/min/1.73m$^2$) | 58 ± 22 | 50 ± 24 | 0.026 |
| Hemoglobin (g/dL) | 12 ± 2 | 11 ± 2 | 0.041 |
| NT-proBNP (pg/mL) | 1937[1040–3775] | 1745 [955–3710] | 0.56 |
| Troponin (pg/mL) | 22 [13 – 37] | 31 [17 – 42] | 0.034 |
| Echocardiography | | | |
| Indexed LA volume (mL/m$^2$) | 46 ± 19 | 45 ± 16 | 0.67 |
| LV ejection fraction (%) | 62 ± 7 | 61 ± 8 | 0.35 |
| E wave velocity (mm/s) | 91 ± 32 | 97 ± 26 | 0.23 |
| Septal E/e' | 19 ± 9 | 20 ± 7 | 0.17 |
| TAPSE (mm) | 19 ± 5 | 18 ± 5 | 0.40 |
| eSPAP (mmHg) | 43 ± 11 | 45 ± 15 | 0.27 |
| CMR | N=94 | N=57 | |
| CMR indexed LA Volume (mL/m$^2$) | 70 ± 31 | 62 ± 25 | 0.12 |
| CMR indexed LV EDV (mL/m$^2$) | 72 ± 18 | 74 ± 17 | 0.37 |
| CMR LV ejection fraction (%) | 62 ± 8 | 62 ± 9 | 0.62 |
| CMR indexed LV mass (g/m$^2$) | 66 ± 18 | 71 ± 14 | 0.07 |
| CMR RV ejection fraction (%) | 56 ± 8 | 58 ± 8 | 0.41 |
| CMR indexed RV EDV (mL/m$^2$) | 79 ± 25 | 83 ± 27 | 0.36 |
| ECV > 33 % | 34 (36%) | 28 (53%) | 0.05 |

Continuous variables are expressed as mean ± 1 standard deviation (SD) and categorical variables as count and proportion. P-values are derived from independent sample t-test or Chi square test when appropriate.
NYHA: New York Heart Association; COPD: chronic obstructive pulmonary disease; GFR: glomerular filtration rate estimated by CKD-epi; MRA: mineralocorticoid receptor antagonist; ACE: angiotensin-converting enzyme; ARB: angiotensin II receptor blockers LV: left ventricle; LA: left atrium; TAPSE: tricuspid annular plane systolic excursion; eSPAP: estimated systolic pulmonary artery pressures; CMR: cardiac magnetic resonance; EDV: end diastolic volume; RV: right ventricle; ECV: extracellular volume estimated by T1 mapping.

**Figure 1. Characteristics of diabetic versus nondiabetic HFpEF patients**

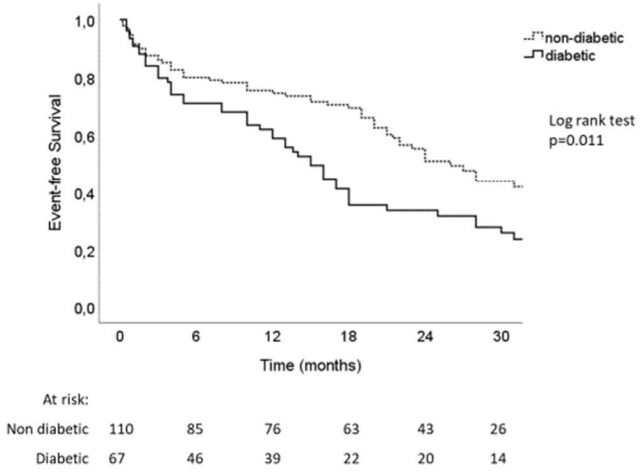


| Nondiabetic | Diabetic |
|---|---|
| Older | Younger |
| Leaner | More obese |
| More patients in NYHA class III - IV | High prevalence of ischemic cardiomyopathy and sleep apneas |
| High prevalence of atrial fibrillation | Higher LV mass and more myocardial fibrosis |

The follow up was completed for 177 (97%) patients, including 67 diabetics (96%) over a mean duration of 30±9 months. Over this period of time, 27/67 (40%) diabetic patients died, and 52/67 (78%) reached the combined outcome, versus 28/110 (25%) deaths and 59/110 (54%) combined outcome in the nondiabetic group. As such, T2D was associated with worse prognosis in univariate Cox regression (HR 1.65 [1.1 - 2.4], p = 0.009). Although it shortly missed statistical significance for mortality alone, the association between diabetes and single outcomes taken separately went in the same direction (for all-cause mortality HR 1.58 [0.9 – 2.7], p=0.092 and for hospitalization HR 1.64 [1.1 - 2.5], p=0.022). After adjustment for age, body mass index, NYHA functional class and glomerular filtration rate, diabetes remained a significant predictor of mortality and

hospitalization for heart failure (HR: 1.72 [1.1 – 2.6], p = 0.011) as shown by the adjusted Kaplan Meier curves (Figure 2).

**Figure 2. Kaplan Meier curves of event-free survival in diabetic versus non-diabetic HFpEF patients.** Adjustments were made for age, body mass index, NYHA functional class and glomerular filtration rate




*Characteristics and outcome of diabetic HFpEF patients according to glycemic control (Table 2)*

Overall, the diabetic patients in our population had well controlled diabetes with median HbA1C of 7.1 [6.1 – 7.8] %. Almost half (32/65, 49%) were treated with insulin, alone or in combination with Metformin. Details of hypoglycemic treatments can be found in Figure 3. Note that no patient was taking sodium-glucose cotransporter-2 inhibitors (SGLT-2i) as, in Belgium, they were reimbursed according to strict criteria at the time of inclusion. The subgroup of diabetic patients were compared among each other according to glycemic control (HbA1C <7% versus >7%, Table 2). Patients with HbA1C <7% were leaner, with a mean BMI of 29 ± 6 versus 32 ± 7 kg/m$^2$ (p=0.048). They had slightly lower hemoglobin levels and showed a tendency, although not statistically significant, toward higher NT-proBNP

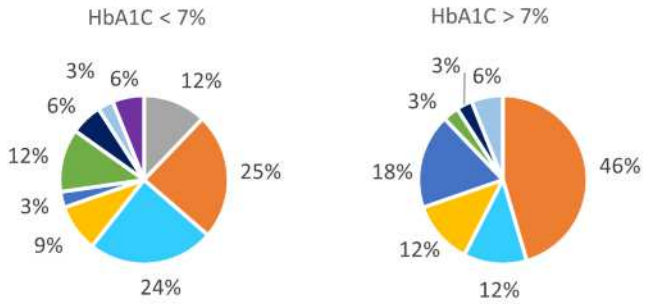
levels. The two groups were homogenous regarding age, sex and comorbidities, and had similar renal functions. Patients with HbA1C > 7% were more often treated with insulin.

**Table 2. Clinical characteristics of diabetic HFpEF patients according to glycemic control.**

| | Diabetic<br>N = 65 | HbA1C < 7%<br>N = 32 | HbA1C > 7%<br>N = 33 | P-value |
|---|---|---|---|---|
| Age (years) | 76 ± 9 | 76 ± 8 | 75 ± 10 | 0.79 |
| Duration of diabetes (yrs) | 19.3 ± 8 | 19.2 ± 9 | 19.4 ± 8 | 0.96 |
| Female (n, %) | 42 (60%) | 20 (62%) | 20 (61%) | 0.88 |
| Body mass index (kg/m$^2$) | 31 ± 6 | 29 ± 6 | 32 ± 7 | 0.048 |
| NYHA III – IV (n, %) | 27 (39%) | 14 (44%) | 12 (36%) | 0.54 |
| Hospitalized for HF at inclusion (n,%) | 41 (63%) | 21 (66%) | 20 (61%) | 0.55 |
| Atrial fibrillation (n, %) | 37 (53%) | 18 (56%) | 16 (48%) | 0.53 |
| Ischemic cardiomyopathy (n, %) | 33 (47%) | 13 (41%) | 18 (55%) | 0.26 |
| Smoking (n, %) | 27 (39%) | 10 (31%) | 14 (42%) | 0.35 |
| Hypertension (n, %) | 67 (97%) | 32 (100%) | 31 (94%) | 0.49 |
| Hypercholesterolemia (n, %) | 49 (70%) | 22 (69%) | 24 (73%) | 0.72 |
| Sleep apneas (n, %) | 12 (18%) | 4 (13%) | 8 (27%) | 0.16 |
| COPD (n, %) | 7 (10%) | 3 (9%) | 3 (9%) | 0.97 |
| Biology | | | | |
| HbA1C (%) | 7.1 [6.1 – 7.8] | 6.1 [5.8 – 6.5] | 7.7 [7.2 – 8.4] | <0.001 |
| eGFR (ml/min/1.73m$^2$) | 50 ± 24 | 49 ± 27 | 48 ± 18 | 0.78 |
| Hemoglobin (g/dL) | 11 ± 2 | 11 ± 2 | 12 ± 2 | 0.046 |
| NT-proBNP (pg/mL) | 1745<br>[955 – 3710] | 2373<br>[1148 – 5264] | 1464<br>[506 – 3696] | 0.086 |
| Antidiabetic treatment | | | | |
| Insulin (n, %) | 32 (46%) | 11 (34%) | 21 (64%) | 0.018 |

| | | | | |
|---|---|---|---|---|
| Metformin (n, %) | 31 (44%) | 15 (47%) | 13 (39%) | 0.54 |
| Sulfonylureas (n, %) | 16 (23%) | 9 (28%) | 5 (15%) | 0.20 |
| Gliptins (n, %) | 8 (11%) | 4 (13%) | 4 (12%) | 0.96 |
| Continuous variables are expressed as mean ± 1 standard deviation (SD) and categorical variables as count and proportion. P-values are derived from independent sample t-test or Chi square test when appropriate.<br>HbA1C: glycated hemoglobin; NYHA: New York Heart Association; COPD: chronic obstructive pulmonary disease; GFR: glomerular filtration rate estimated by CKD-epi. | | | | |

**Figure 3. Hypoglycemic treatment of diabetic patients**

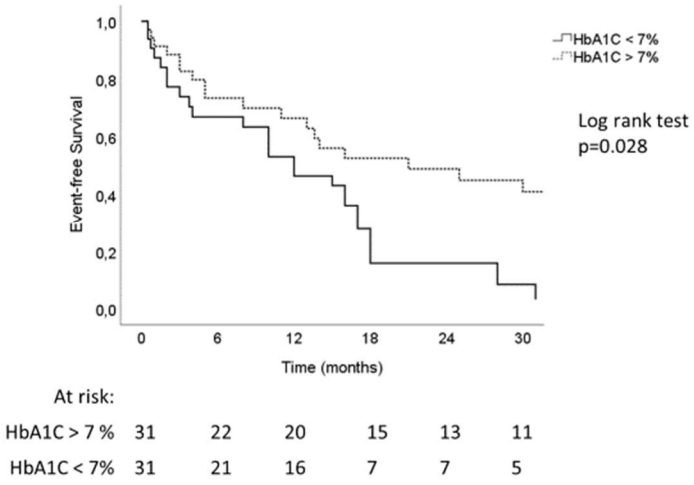



| | All Diabetics | HbA1C < 7% N = 32 | HbA1C > 7% N = 33 |
|---|---|---|---|
| Lifestyle | 5 (7%) | 4 (13%) | 0 (0%) |
| Monotherapy | 45 (64%) | 19 (60%) | 23 (70%) |
| Insulin | 23 | 8 | 15 |
| Metformin | 14 | 8 | 4 |
| Sulfonylurea | 8 | 3 | 4 |
| Bitherapy | 18 (26%) | 7 (22%) | 10 (30%) |
| Insulin + Metformin | 7 | 1 | 6 |
| Sulfonylurea + Gliptines | 5 | 4 | 1 |
| Metformin + Sulfonylurea | 3 | 2 | 1 |
| Metformin + Gliptines | 3 | 1 | 2 |
| Tritherapy | 2 (3%) | 2 (6%) | 0 (0%) |
| Insulin + Metformin +Sulfonylurea | | | |

Out of 65 diabetic patients with HbA1C data, 62 (95%) completed the follow up. In two years, 15/31 (48%) diabetic patients with HbA1C <7% died and 28/31 (90%) reached the combined outcome, versus 10/31 (32%) deaths in patients with HbA1C >7% and 20/31 (65%) combined outcome. Lower levels of glycated hemoglobin

were associated with worse prognosis (HR 2.07 [1.1 – 3.8], p=0.016). Although it shortly missed statistical significance for hospitalization alone, the association between diabetes and single outcomes taken separately went in the same direction (for all-cause mortality HR 2.36 [1.1 – 5.5], p=0.047 and for hospitalization HR 1.86 [0.96 – 3.6], p=0.064). After adjustment for age, body mass index, hemoglobin levels and NT-proBNP levels, HbA1C <7% remained a significant predictor of mortality and hospitalization for heart failure (HR: 2.07 [1.1 – 4.0], p=0.028). This can be seen in Figure 4, showing the adjusted Kaplan Meier curves of event-free survival among diabetic HFpEF patients according to HbA1C levels.

**Figure 4. Kaplan Meier curves of event-free survival in diabetic HFpEF patients according to HbA1C levels**. Adjustments were made for age, body mass index, hemoglobin and NTproBNP levels




**DISCUSSION**

The main findings of this study can be summarized as follows: 1. Diabetic patients with HFpEF show specific characteristics, including higher body mass index, lower

prevalence of atrial fibrillation, lower hemoglobin levels and worse renal function. No echocardiographic difference could be detected, but CMR showed a trend towards higher LV mass and more myocardial fibrosis (ECV > 33%). 2. Diabetes is associated with an excess of adverse events (hospitalization for HF and mortality) in HFpEF. 3. Lower levels of HbA1C levels are associated with worse prognosis in diabetic patients with HFpEF.

*Characteristics and outcome of diabetic versus nondiabetic HFpEF patients*

Regarding clinical characteristics, HFpEF patients with diabetes were younger and more obese than nondiabetic patients. This is consistent with sub studies from large clinical trials [63-65]. A large study examining age-related characteristics in HFpEF also observed that younger patients were more than twice as likely to be obese, and that the prevalence of diabetes ranged from 37% in the younger group versus 18% in the oldest group.[169] Although the reason for this difference is not completely elucidated, it might reflect that different pathophysiological pathways can lead to the development of HFpEF. The combination of diabetes and obesity, both conditions associated with a release of proinflammatory cytokines and decreased nitric oxide availability, could lead to the development of HFpEF at a younger age through myocardial remodelling and fibrosis.[60] Supporting this, diabetic patients also exhibited a trend towards higher LV masses and higher levels of myocardial fibrosis than their nondiabetic counterparts, consistently with previous studies.[64,67,68] This can contribute to the worse prognosis conferred by diabetes, as we previously showed that extracellular matrix expansion (higher ECV by CMR) was associated with adverse events in HFpEF.[191]

Atrial fibrillation, on the other hand, was more prevalent in the nondiabetic group. This is consistent with previously published literature [193-195]. AF and HFpEF often coexist and it is still unclear whether one affection leads sequentially to the other.

More likely, the two disorders share a common mechanistic substrate, which causes AF and HFpEF [16,166] and develop in parallel. A recent meta-analysis underlined that AF was associated with poor prognosis in HFpEF, although it is unclear whether AF is only a marker of more severe heart failure, or a cause of mortality in itself.[196] Atrial fibrillation is also an age-related marker, hence, it is not surprising that the prevalence of AF is higher in the older nondiabetic group. Studies have also suggested differences in cardiac remodelling, with diabetic patients showing smaller LA volumes, which might contribute to this phenomenon.[193] However, the presence of AF was retrieved from medical files, patients' interrogation, and a standard electrocardiogram at inclusion, but no long term rhythm monitoring was performed. As such, the prevalence of AF and other arrhythmias could have been underestimated in both groups.

The event rate in our study was high compared to clinical trials (16.1 / 100 persons-year overall mortality in the diabetic group versus 6.8-8.8 in pooled data from I-Preserve, Charm-Preserved and TOPCAT[193]), but similar to a large community based study (15.2/100 persons-year [197]). Compared to clinical trials, our population is almost 10 years older (76 vs 69 years) had higher NT-proBNP levels (1745 vs 430 – 581 pg/mL), lower hemoglobin (11 vs 12.9 – 13.5 g/dL) and worse renal function (50 vs 62.7 – 71.4 mL/min/1.73m²), all parameters associated with adverse events. The association between diabetic status and prognosis (hospitalization for HF and mortality) is consistent with the existing literature [63,64,66,185,193]. There are numerous pathophysiologic processes in diabetes that are thought to alter the myocardium resulting in less effective relaxation and contraction, including oxidative stress, inflammation and disorders in calcium transport, as well as alterations in substrate metabolism, and mitochondrial dysfunction [56,58]. Furthermore, extra-cardiac effects of diabetes such as decreased arterial compliance, renal angiopathy, and autonomic dysfunction can also accelerate the progression of HFpEF [56]. In

particular, hyperglycemia causes up-regulation of the sodium-glucose cotransporter-2 (SGLT-2) leading to increased proximal renal sodium absorption, volume expansion, and decreased responsiveness to diuretics [60-62]. A better understanding of the interplay between diabetes and HF is crucial for the development of new therapies. This has recently been emphasized by the promising results of studies using SGLT-2 inhibitors in diabetic patients with HF[145,198]. The results of ongoing randomized controlled trials using SGLT-2i in HFpEF [60,184] are eagerly awaited. Nevertheless, a retrospective study showed less impressive effects of SGLT2i on cardiac remodeling in HFpEF compared to HFrEF, tempering enthusiasm for this class of treatment.[198]

*Characteristics and outcome of diabetic HFpEF patients according to glycemic control.*

While the presence of diabetes conferred a worse prognosis to our HFpEF patients, tight glycemic control did not seem to reverse this association. On the contrary, patients with best controlled diabetes (HbA1C <7%) were more at risk for adverse event (hospitalization for heart failure and all-cause mortality). Previous studies [186,187,199,200] showed a U-shaped association between HbA1C and prognosis in heart failure patients, with the lowest risk in the group of patients with HbA1C between 6.5 and 7.5%. However, those studies were either conducted among patients with HFrEF alone, or did not make a distinction between patients according to ejection fraction, while the interplay between diabetes and outcome seems to differ in those populations. In the CHARM trial, the relative risk conferred by diabetes was significantly greater in patients with preserved ejection fraction (EF) than in those with low EF [65] and a recent study highlighted that the presence of T2D was associated with a reduction of exercise capacity (lower peak VO2) in the LVEF <40% and LVEF 40-49%, but not in the LVEF >50% subgroup [201].

Data about glycemic control and outcome in HFpEF are scarce. A study by Gu et al.[188] did not find baseline HbA1C to be an independent predictor of outcome, but they analyzed it in the overall population of HF with T2D, and not only in HFpEF. Glycemic variability, however, was associated with outcome in the HFpEF subgroup [188] and was associated with signs of diastolic dysfunction in patients without HF[189]. Finally, the GAMIC cohort, a large population-based propensity-matched study of patients with HF [197] observed an increased mortality and morbidity (hospitalizations and visits) in patients who developed diabetes, particularly in those with a mean HbA1c higher than 7.0%.

How can we explain that, in our population, patients with higher HbA1C levels seem "protected" and suffer from less adverse events, while recent research emphasized the direct role of glucotoxicity on cardiomyocytes in the development of diabetic cardiomyopathy [56,59,202]? Firstly, glucotoxicity plays a part in the pathophysiology of the disease but its role in the evolution of symptoms and outcomes is yet unknown. Heart failure in diabetic patients occurs in a broad context of metabolic disorders including lipotoxicity, glucotoxicity and insulin resistance and resulting in impaired mitochondrial oxidative capacity and increased reactive oxygen species (ROS) production and surely, hyperglycemia is not the only mechanism involved. This is supported by the fact that, before the SGLT-2 inhibitor era, no study could demonstrate a favourable effect of glucose lowering therapies on events related to heart failure [203]. Conversely, some glucose-lowering therapies, including peroxisome proliferator-activated receptor (PPAR) agonists even increased the risk of heart failure in individuals with type 2 diabetes. Note that those drugs were seldom taken by patients in our cohort (Figure 3) and cannot solely be responsible for the difference in event-free survival.

For years it has been assumed that insulin resistance observed in diseases characterized by nutrient excess (ie T2D and obesity), was fundamental to the pathogenesis of these diseases. As stated above, insulin resistance into the heart has been considered to favour myocardial contractile dysfunction and to be involved in the pathophysiology of diabetic cardiomyopathy. However, an alternative view, which recently gained researchers' interest, is that adaptations occurring in metabolic diseases can be viewed as protective in nature, and that insulin resistance could act as a defence mechanism to prevent or delay pathological intracellular substrate accumulation when substrate uptake exceeds energy demand [204-207]. Fundamental to this hypothesis is that, although these metabolic alterations are deleterious in the long term for complications associated with obesity and diabetes, they provide immediate protection against cell death in response to excess nutrients. Supporting this, it has been shown that cardiac contractile function was preserved, or even improved, in hearts subjected to metabolic and haemodynamic stress when myocardial insulin resistance was induced in response to elevated glucose levels or upon high-fat diet [208,209]. Conversely, excessive insulin signaling exacerbates systolic dysfunction when the heart is subjected to pressure overload [210]. In light of this, the discrepancy between our study and the results of the GAMIC cohort [197] might be explained by the difference in disease duration. The GAMIC cohort excluded patients with a previous diagnosis of diabetes, while the mean duration of diabetes in our population was 19 ± 8 years. Possibly our results do not apply to new onset diabetes, as the adaptation to excess nutrients have not yet taken place.

In this context of old patients with long standing diabetes, the utility of therapeutically targeting glycemia in those patients, particularly through insulin sensitization, is questionable as it may result in exposure of cells and tissues to additional nutrients that will further challenge their survival. This could explain why

PPAR agonists, important insulin sensitizers favouring nutrient uptake and storage, have been associated with adverse cardiovascular outcomes in T2D patients. On the other hand, treatment reducing nutrient overload might be beneficial in this context and should be preferred. Metformin, for example, which has shown beneficial effect on mortality in HF patients [211], although often referred to as insulin sensitizer, has its main glucose-lowering effect via reducing hepatic glucose production. Similarly, SGLT-2 inhibitors lower blood glucose by promoting glycosuria.

This cardio protective effect of insulin resistance could be involved in the better prognosis observed in heart failure patients with higher BMIs, referred to as the "obesity paradox" [177,190]. Although we did not measure insulin resistance per se, we can hypothesize that the group with HbA1C > 7% is more insulin resistant as they are more obese and show higher glycemia levels though intensively treated.

Furthermore, hyperglycemia was shown to be involved in irreversible epigenetic changes, known as "glycemic memory", and HbA1c at time of the study cannot reflect the whole history of diabetes [212,213]. Similarly, intermittent hyperglycemia, rather than chronic elevation of blood glucose, with a lesser repercussion on HbA1C levels, exacerbates the production of reactive oxygen species, impairs endothelial function and induces cytokines release and contributes to pejorative evolution [214]. Finally, hypoglycemia could also be involved in the progression of cardiovascular diseases and mortality through sympatho-adrenal response [215].

In short, together with existing literature, this study underlines that other mechanisms besides glucotoxicity must be involved in the development and worsening of heart failure in diabetic patients, and that the effect of intensive glycemic control on cardiovascular associated morbidity is not fully understood.

Current guidelines recommend that the appropriate target for HbA1C should be individualized based on overall health and life expectancy. As such it is generally accepted that the glycemic goal should be somewhat higher (HbA1C ≤8%) in frail older adults with medical and functional comorbidities [216,217]. Patients with HFpEF generally match this description (mean age of 78 years and high comorbidity burden in our population). However, these recommendations are based on consensus and there are virtually no trials that have examined glycemic control and complications focusing on the older patient, and even less on older patients with HFpEF. Hence, an important issue that is still unsolved is the optimal target level of HbA1c in that population. Given published data, glycemic variability should be avoided once the optimal target is reached [188]. Our study is a retrospective analysis of a relatively small population and does not allow answering this question. Furthermore, very few patients in our population had severely uncontrolled diabetes. However, this study generates the hypothesis that low levels of HbA1C are associated with more adverse events in and that physicians should not be too stringent about glycemic control in HFpEF patients with long standing diabetes. In addition, it underlines the need for future studies: fundamental studies to unravel the interaction between diabetes, insulin resistance and heart failure, and clinical studies designed to determine the optimal HbA1C target in HFpEF.

*Limitations*

This study was conducted in a single center with a relatively small number of patients. Although data were collected prospectively, the association between HbA1C and mortality were derived from retrospective analyses. As such, this observation is subject to collider stratification bias and our data do not allow generalizing this finding beyond HFpEF patients. The diagnosis of diabetes was reported by investigators and did not require systematic documentation using

standardized diagnostic criteria. Its prevalence is, therefore, likely to have been underestimated. Also, unmeasured confounders, such as biomarkers of nutritional status, invasive hemodynamics, and duration of heart disease, that may have improved risk adjustment, were unavailable.

**CONCLUSION**

Together with previous data, this study suggests a potential differentiation of HFpEF phenotypes, with young obese and diabetic HFpEF on one hand, versus elderly HFpEF with atrial fibrillation on the other. This might reflect distinct pathophysiological pathways that perhaps should be targeted more specifically in future clinical trials. Furthermore, these results strengthen evidence on the prognostic significance of diabetes in HFpEF. It underlines that patients with HFpEF and diabetes are at high risk of hospitalization for HF and should benefit of closer monitoring and intensive treatment of comorbidities and congestion. Finally, it shows that a stringent glycemic control has a negative impact on prognosis. This opens the way for future research to better understand the interplay between diabetes and heart failure, and to determine an optimal HbA1C target in this specific population.

## 3.3 Association of Plasma Myeloperoxidase with Inflammation and Diabetic status in HFpEF.

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**ABSTRACT**

BACKGROUND: Inflammation and oxidative stress are thought to play an important role in the pathophysiology of HFpEF through the development of endothelial dysfunction. Myeloperoxidase (MPO) functions as a link between oxidative stress and inflammation and is an interesting therapeutic target. The objective of this observational cohort study was to compare MPO levels between HFpEF and old controls, to define clinical characteristics associated with high levels of MPO and to assess the relation between MPO levels and vascular function.

METHODS : Patients with HFpEF (N=55) and controls > 60 years (N=18) were prospectively included. All subjects underwent complete echocardiography and blood analysis. MPO levels were dosed by ELISA assay. Effective arterial elastance (Ea) and peripheral arterial tonometry (EndoPAT reactive hyperemia index RHI and augmentation index AIx) were used to assess vascular function. Characteristics between groups defined by the median of MPO were compared using independent samples t-test or chi square test.

RESULTS : Patients with HFpEF (80 ± 8.7 years, 65 % female) had higher levels of MPO compared to controls (75 ± 5.0 years, 72% female) (34.7 ng/mL [22.7 ; 44.0] versus 22.6 [18.2 ; 32.0], p=0.026). MPO levels were correlated with markers of inflammation; C-reactive protein (Pearson's R=0.46, p=0.001) and neutrophile to lymphocyte ratio (R=0.36, p=0.031) and with signs of left ventricular (LV) remodelling and elevated filling pressures, namely NT-proBNP levels (R=0.32, p=0.019), decreased LV ejection fraction (LVEF, R=-0.36, p=0.008) and E/e' ratio (R=0.35, p=0.011). HFpEF patients with levels of MPO above the median were more often men (48% vs 21%, p=0.037) and suffered more often from diabetes (48% vs 18%, p=0.017). Intriguingly, they had lower indices of vascular stiffness (augmentation index (11.1 [0.1 ; 30.] vs 19.9 [10.5 ; 33.4], p=0.018 and arterial

elastance Ea (2.06 ± 0.676 vs 2.43 ± 0.721, p=0.065) and there was no difference in endothelial function (1.82 [1.34 ; 2.30] versus 1.66 [1.32 ; 1.95], p=0.55).

CONCLUSIONS: HFpEF patients have higher levels of MPO than controls, reflecting leukocyte activation and oxidative stress. Among patients, high levels of MPO are associated with male sex, diabetic status, subtle left ventricular dysfunction and pronounced diastolic dysfunction. The association between oxidative stress and vascular stiffness, on the other hand could not be demonstrated.

**KEYWORDS**

Heart failure with preserved ejection fraction, myeloperoxidase, oxidative stress, inflammation, diabetes, vascular stiffness

## 1. INTRODUCTION

Heart failure with preserved ejection fraction (HFpEF) is characterized by signs and symptoms of heart failure, including peripheral oedema, dyspnea and exercise intolerance, in the absence of a reduced left ventricular ejection fraction (LVEF ≥ 50%).[157] Current understanding of molecular mechanisms underlying HFpEF[37] relates coexisting comorbidities to myocardial remodelling and dysfunction, through a systemic pro inflammatory state. Non-cardiac co-morbidities such as diabetes, obesity, hypertension, and chronic kidney disease are common in HFpEF and have the ability to induce systemic inflammation. During inflammation, microvascular endothelial cells produce reactive oxygen species (ROS), which limits nitric oxide (NO) bioavailability leading to oxidative stress and endothelial dysfunction.

Oxidative stress and inflammation are closely interconnected. Transcription factors that regulate the expression of pro inflammatory cytokines are activated under oxidative stress conditions and in turn, induce the generation of ROS, thus creating a vicious cycle of oxidation and inflammation.[84] Myeloperoxidase (MPO), a leukocyte-derived enzyme, functions as a link between oxidative stress and inflammation. During inflammation, MPO is released and uses $H_2O_2$ as a substrate to produce hypochlorous acid, a powerful pro-oxidant and pro inflammatory molecule.

Studies suggest that plasma MPO levels are elevated in patients with HF compared to controls and that increasing levels of MPO are associated with restrictive diastolic stage, right ventricular systolic dysfunction and tricuspid regurgitation in HFrEF.[87] Furthermore, MPO may be involved in the pathophysiology of atrial fibrillation through atrial accumulation of MPO and consequent increase in fibrosis.[86] These results imply that MPO may be important also for the development

of HFpEF where diastolic dysfunction, atrial fibrillation and fibrosis are major components. Indeed, a recent study showed that HFpEF patients displayed higher plasma concentration of MPO compared to healthy controls.[88] Furthermore, since oxidative stress and microvascular endothelial dysfunction are suggested as fundamental parts of the pathophysiology and development of HFpEF, MPO inhibition appears as an interesting therapeutic approach and a clinical trial investigating MPO inhibitor "AZD4831" (ENDEAVOR NCT04986202 and NCT03611153) is currently ongoing. Heterogeneity among patients with HFpEF has been singled out to explain the difficulty to find treatments improving prognosis in this population. Hence, identifying characteristics associated with high levels of MPO could be interesting to target subgroups of patients most likely to benefit from treatment with MPO inhibitors.

In this context, the objective of our study was to reinforce data about MPO elevation in HFpEF, to assess the relation between MPO levels and clinical parameters including vascular function and to determine patient characteristics associated with high levels of MPO.

## 2. METHODS

### *2.1 Population*

Patients with HFpEF encountered in our division of cardiology between May 2019 and May 2021 were prospectively screened for inclusion in the study. HFpEF was diagnosed according to the 2016 guidelines of the European society of cardiology. [26] Briefly, patients had to be symptomatic (New York Heart Association (NYHA) functional class ≥II or hospitalization for HF in the previous 12 months), have a left ventricular (LV) ejection fraction ≥ 50%, show echocardiographic signs of elevated

filling pressures (LV hypertrophy, left atrial (LA) enlargement, elevated E/e' ratio or elevated pulmonary pressures) and elevated NT-proBNP (> 220 pg/ml in sinus rhythm, >660 pg/mL in atrial fibrillation (AF)). The exclusion criteria were: history of reduced ejection fraction (LVEF < 50%), severe valvular disease, infiltrative or hypertrophic cardiomyopathy, acute coronary syndrome in the previous 30 days, severe chronic obstructive pulmonary disease, congenital heart disease, pericardial disease, AF with a ventricular response >140 bpm, and severe anemia (hemoglobin <8 g/dl). A total of 55 patients satisfied the inclusion criteria. Patients underwent blood sampling and complete transthoracic echocardiography. All except 10 also underwent endothelial function measurement by endoPAT (6 patients had finger deformities or injuries preventing the probes use and, there was a technical problem with the device on the day of the study for 4 patients). To constitute a control group of similar age and sex, asymptomatic volunteers aged between 60 and 90 years were screened by advertisement in the local community. They all underwent a full clinical exam, blood sampling, ECG, echocardiography and endoPAT. Exclusion criteria were any evidence of heart disease as indicated by clinical history, physical exam and echocardiography. Eighteen subjects satisfied the inclusion criteria. The local ethics committee approved the study, and all subjects gave written informed consent before study enrolment (Clinical trial NCT03197350). The investigation conforms to the principles outlined in Declaration of Helsinki. Patients and controls were interrogated about symptoms, medical history and treatment and were thoroughly examined. Other information was retrieved from medical files and from review of hospital records.

*2.2 Echocardiography*

Standardized complete transthoracic echocardiography (TTE) exams were acquired according to established guidelines [218] using iE33 ultrasound systems

(Philips Medical Systems, Andover, Massachusetts) equipped with a 3.5/1.75-MHz phased-array transducer and stored on a XCELERA 2.1 PACS server (Philips Medical Systems, Andover, Massachusetts). Annular e' velocity, average E/e' ratio, LA volume index and peak TR velocities were measured to evaluate LV diastolic function.[164]

*2.3 Blood sampling*

Blood samples were collected from the cubital vein. Samples were immediately centrifuged and aliquots of plasma and serum were stored in microcentrifuge tubes at -80˚C until analysis. Plasma MPO concentration was determined by an enzyme-linked immunosorbent assay (ELISA) method (#DMYE00B, R&D Systems) according to the manufacturer's instructions.

*2.4 Vascular function: effective arterial elastance, reactive hyperemia index and augmentation index*

Effective arterial elastance (Ea) was calculated as described in the literature[45]: end-systolic pressure divided by stroke volume. End-systolic pressure was estimated as systolic pressure times 0.9, as previously validated [219]. Digital hyperemia response was measured at finger (index) tips using an EndoPat2000 device (Itamar Medical, Israël) (Fig. 1). Briefly, pulse wave amplitude (PWA) changes were assessed as beat-to-beat plethysmographic signals in the index finger by high-sensitive pneumatic probes (EndoPAT, Itamar). The signals were measured at basal state during 5 minutes from each fingertip. Then brachial blood flow was interrupted for 5 minutes by inflation of a sphyngomanometer cuff placed on one proximal forearm, and signals were recorded during occlusion (5 minutes) and after restoration of blood flow (5 minutes). Data were digitized and computed automatically by EndoPat2000 software; the reactive hyperemia index (RHI) was defined as the ratio

of mean post-deflation signal (in the 90 to 120-second post-deflation interval) to baseline signal in hyperemic finger normalized by the same ratio in the contra-lateral finger and multiplied by a baseline correction factor (K=0.523976*log(mean baseline amplitude)-0.2). Arterial stiffness was approximated by the augmentation index (AI), which is calculated through software identification of the systolic peak (P1) and reflected wave (P2) inflection points and then using the formula AI = (P1 − P2)/P1 × 100, averaged over multiple valid pulses collected during the baseline period. It is then normalized to heart rate of 75bpm (referred to as AIx in the manuscript). Lower AI values (including negative results) reflect better arterial elasticity. This method has been shown to correlate well with other methods of AI derivation.[220]

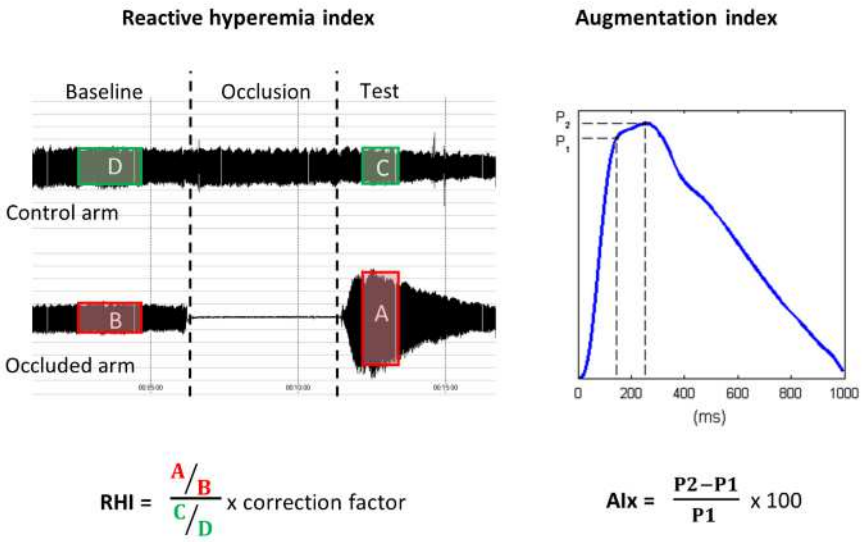



**Figure 1.** Calculation of the reactive hyperemia index (RHI) and the augmentation index (Aix) by the EndoPAT2000 software (Itamar Medical)

*2.5 Statistical analysis*

Statistical analyses were performed using SPSS version 25 (SPSS Corp., Somers, New York). All tests were 2-sided and p-value <0.05 was considered statistically significant. The sample size of the control group was determined to reach a power of 80%, $\alpha = 0.05$, with an expected difference of 15% of plasma MPO levels between patients and controls[88] (minimum n = 15). Continuous variables are expressed as mean ± standard deviation (SD) or median [P25 ; P75] if not normally distributed. Non normal biomarkers (NT-proBNP, Troponin, CRP, MPO) were log-transformed to achieve normality. Categorical variables are expressed as count and proportion. Receiver operating characteristic (ROC) curves were established and the area under the ROC curves (AUC) were calculated to establish the diagnostic value of MPO levels compared to NT-proBNP levels. Correlation between variables was assessed using Pearson coefficient of correlation (R). Differences of characteristics between groups were examined using independent sample t-test, Mann Whitney U test, Chi-square test or Fisher exact test when appropriate. Multivariate logistic regression was used to evaluate the association between MPO levels and diabetic status after correction for age and sex.

## 3. RESULTS

*3.1 MPO levels in HFpEF compared to controls*

The characteristics of all 55 patients with HFpEF are presented in Table 1. Patients were 80 ± 8.7 years old, mostly women (65%) and about one third was suffering from advanced heart failure (36% NYHA class III or IV). One third (33%) of the patients had diabetes. While ejection fraction was preserved, patients displayed functional and morphological signs of diastolic dysfunction including increased E/e'

ratio and dilated left atrium. The 18 healthy controls (75 ± 5.0 years) were 72% women (Supplementary Table 1).

Besides expected differences in NT-proBNP levels and echocardiographic parameters, patients with HFpEF had higher levels of CRP (3.1 mg/L [1.2 ; 8.4] vs 1.2 mg/L [1.0 ; 1.75], p=0.001), uric acid (7.3 ± 2.66 vs 5.2 ± 1.01, p<0.001) and MPO (34.7 ng/mL [22.7 ; 44.0] vs 22.6 [18.2 ; 32.0], p=0.026) reflecting higher degree of inflammation and oxidative stress (Fig. 2). However, there were no significant differences in vascular function. In controls, the reactive hyperemia index was 1.80 [1.42 ; 2.55] and the augmentation index 17.7 [4.6 ; 36.9] versus 1.67 [1.33 ; 2.02] and 17.81 [2.64 ; 31.24] in patients (respectively p=0.26 and 0.70). Effective arterial elastance was also not different (1.99 ± 0.570 vs 2.24 ± 0.716, p=0.21).

The AUC of the ROC curves for myeloperoxidase was 0.72 (0.59 ; 0.84) p=0.006 indicating moderate diagnostic value for HFpEF. Expectedly, NT-proBNP levels had a very good diagnostic value of 0.94 (0.89 ; 1.00) p<0.001 (Supplemental Figure 1.)

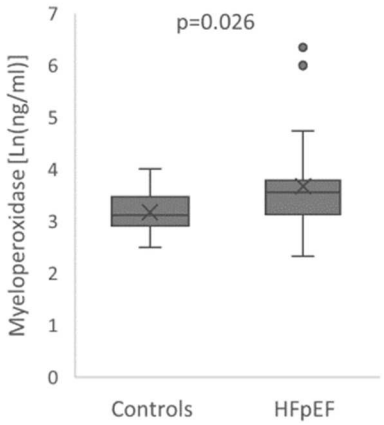



**Figure 2. Boxplot of myeloperoxidase levels in heart failure and preserved ejection fraction patients and controls.**

Center line: median; box limits: upper and lower quartiles; whiskers: 1.5x interquartile range; cross: mean; points: outliers.

*3.2 Correlation between MPO levels and patients' characteristics*

Among HFpEF patients, MPO levels were correlated with markers of inflammation; CRP (R=0.46, p=0.001) and neutrophile to lymphocyte ratio (R=0.36, p=0.031) and with signs of LV remodelling and elevated filling pressures, namely NT-proBNP levels (R=0.32, p=0.019), decreased LV ejection fraction (LVEF, R= - 0.36, p=0.008) and E/e' ratio (R=0.35, p=0.011) (Fig. 3). There was no correlation with age (R=0.12, p=0.41), body mass index (R=0.09, p=0.54), nor renal function (glomerular filtration rate estimated by Chronic Kidney Disease Epidemiology Collaboration CKD-EPI equation) (R=-0.13, p=0.34)[221].

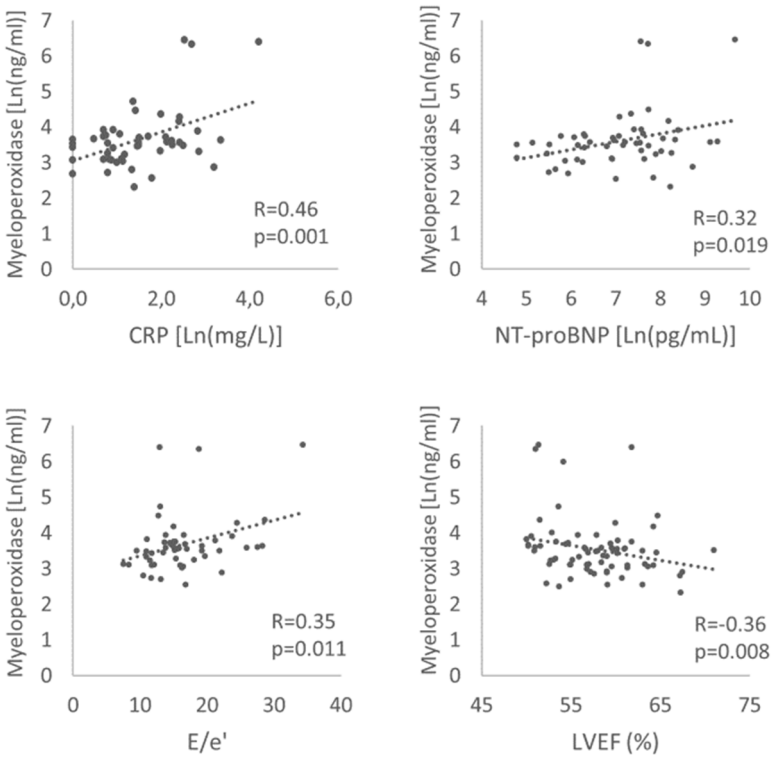
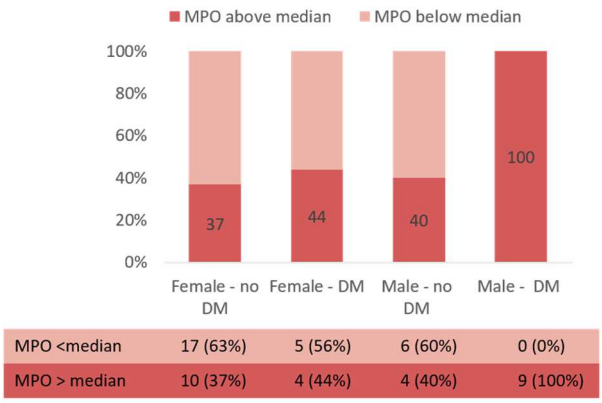



**Figure. 3** Correlations between myeloperoxidase and C-reactive protein (CRP), NT-proBNP, E/e' ratio, left ventricular ejection fraction (LVEF) in heart failure and preserved ejection fraction patients.

*3.3 Characteristics associated with high MPO levels*

Patients with MPO levels above the median consistently had higher levels of CRP and NT-proBNP levels. They also showed lower LVEF (55.8 ± 4.71% vs 59.5 ± 4.89%, p=0.007) and higher E/e' ratio (18.2 ± 6.40 vs 14.4 ± 3.96, p=0.012). Patients with MPO levels above the median suffered more often from diabetes (48 vs 18%, p=0.017) and were more often males (48 vs 21%, p = 0.037) than patients with MPO levels below the median (Table 1). In multivariable logistic regression, diabetic status remained predictive of high levels of myeloperoxidase after adjustment for age and sex (OR= 4.7, 95%CI 1.15-19.19, p=0.031). Figure 4 illustrates the proportion of patients with MPO levels above or below median according to sex and diabetic status. Interestingly, all men suffering from diabetes (9, 100%) had MPO levels above the median, while in women (both with or without diabetes) and in men without diabetes the proportion was similar, around 40%.




| | | | | |
|---|---|---|---|---|
| MPO <median | 17 (63%) | 5 (56%) | 6 (60%) | 0 (0%) |
| MPO > median | 10 (37%) | 4 (44%) | 4 (40%) | 9 (100%) |

**Figure. 4.** Proportion of patients with MPO levels above or below median according to sex and diabetic status.

Intriguingly, patients with higher levels of MPO showed lower augmentation index (11.1 [0.1 ; 30.] versus 19.9 [10.5 ; 33.4], p=0.018) and a trend towards lower effective arterial elastance (2.06 ± 0.676 vs 2.43 ± 0.721, p=0.065) indicating less vascular stiffness. Endothelial function did not differ between groups (1.82 [1.34 ; 2.30] versus 1.66 [1.32 ; 1.95], p=0.55).

**Table 1. Characteristics of HFpEF patients stratified by levels of myeloperoxidase.**

| | All patients (N=55) | MPO below median N=28 | MPO above median N=27 | P-value |
|---|---|---|---|---|
| Age (years) | 80 ± 8.7 | 79 ± 9.7 | 80 ± 7.9 | 0.72 |
| Female (n, %) | 36 (65%) | 22 (79%) | 14 (52%) | 0.037 |
| Body mass index (kg/m$^2$) | 28.2 ± 4.97 | 28.3 ± 5.94 | 28.3 ± 3.84 | 0.99 |
| Systolic blood pressure (mmHg) | 134 ± 20 | 138 ± 17 | 129 ± 22 | 0.099 |
| Diastolic blood pressure (mmHg) | 74 ± 14 | 75 ± 14 | 72 ± 15 | 0.45 |
| Heart rate at inclusion (bpm) | 72 ± 13 | 74 ± 12 | 70 ± 13 | 0.26 |
| NYHA III – IV (n, %) | 20 (36%) | 10 (36%) | 10 (37%) | 0.92 |
| Diabetes (n,%) | 18 (33%) | 5 (18%°) | 13 (48%) | 0.017 |
| Atrial fibrillation (n, %)<br>Paroxysmal (n,%)<br>Permanent (n,%) | 42 (76%)<br>10 (18%)<br>32 (58%) | 23 (85%)<br>6 (21%)<br>17 (61%) | 19 (68%)<br>6 (22%)<br>13 (48%) | 0.13 |
| Ischemic cardiomyopathy (n, %) | 21 (38%) | 8 (29%) | 13 (48%) | 0.14 |
| Smoking (n, %) | 18 (18%) | 9 (32%) | 9 (33%) | 0.93 |
| Hypertension (n, %) | 52 (95%) | 26 (93%) | 26 (93%) | 1 |
| Hypercholesterolemia (n, %) | 39 (71%) | 17 (61%) | 22 (81%) | 0.09 |
| Sleep apneas (n, %) | 6 (11%) | 3 (11%) | 3 (11%) | 1 |
| COPD (n, %) | 6 (11%) | 4 (14%) | 2 (7%) | 0.67 |
| Medication | | | | |
| Loopdiuretics (n, %) | 42 (76%) | 19 (68%) | 23 (85%) | 0.13 |
| MRA (n, %) | 18 (33%) | 11 (39%) | 7 (26%) | 0.19 |
| Beta blockers (n, %) | 34 (62%) | 21 (75%) | 22 (81%) | 0.56 |
| ACE inhibitors/ARB (n, %) | 43 (78%) | 16 (57%) | 18 (67%) | 0.47 |
| Statins (n, %) | 35 (64%) | 15 (54%) | 20 (74%) | 0.11 |
| Biology | | | | |
| eGFR (ml/min/1.73m$^2$) | 49.4 ± 18.26 | 51.4 ± 16.13 | 47.4 ± 20.35 | 0.42 |
| Hemoglobin (g/dL) | 12.0 ± 1.75 | 12.3 ± 1.52 | 11.7 ± 1.94 | 0.19 |
| NT-proBNP (pg/mL) | 1302 [498 ; 2435] | 1015 [361 ; 2251] | 1668 [824 ; 3386] | 0.044 |
| Troponin (pg/mL] | 21 [11 ; 40] | 16 [10 ; 40] | 32 [16 ; 41] | 0.47 |
| CRP (mg/L) | 3.1 [1.2 ; 8.4] | 2.1 [1.2 ; 4.2] | 4.7 [1.4 ; 10.2] | 0.045 |
| Myeloperoxidase (ng/ml) | 34.7 [22.7 ; 44.0] | 23.9 [18.4 ; 32.0] | 44.0 [37.8 ; 78.5] | By design |
| Uric acid (mg/dL) | 7.3 ± 2.66 | 6.6 ± 2.34 | 8.0 ± 2.84 | 0.06 |
| Neutrophiles | 4.3 ± 1.44 | 4.3 ± 1.37 | 4.2 ± 1.54 | 0.98 |
| Lymphocytes | 1.6 ± 0.66 | 1.7 ± 0.61 | 1.5 ± 0.72 | 0.45 |
| Monocytes | 0.68 ± 0.219 | 0.65 ± 0.179 | 0.71 ± 0.257 | 0.56 |
| Neutrophile to lymphocyte ratio | 3.2 ± 2.12 | 3.0 ± 1.9 | 3.3 ± 2.3 | 0.63 |
| Echocardiography | | | | |
| Indexed LA volume (mL/m$^2$) | 37.6 ± 11.42 | 36.1 ± 13.29 | 39.2 ± 9.17 | 0.32 |
| LV ejection fraction (%) | 57.7 ± 5.11 | 59.5 ± 4.89 | 55.8 ± 4.71 | 0.007 |
| E wave velocity (mm/s) | 108.5 ± 30.29 | 105.6 ± 23.9 | 111.4 ± 35.78 | 0.49 |

| | | | | |
|---|---|---|---|---|
| E/e' ratio | 16.3 ± 5.63 | 14.4 ± 3.96 | 18.2 ± 6.40 | 0.012 |
| TAPSE (mm) | 19.2 ± 6.59 | 19.9 ± 6.09 | 18.7 ± 7.17 | 0.53 |
| eSPAP (mmHg) | 50.7 ± 13.82 | 49.8 ± 13.32 | 51.7 ± 14.59 | 0.66 |
| Vascular function | | | | |
| Effective arterial elastance (mmHg/mL) | 2.24 ± 0.716 | 2.43 ± 0.721 | 2.06 ± 0.676 | 0.065 |
| EndoPAT<br>Reactive hyperemia index (RHI) | (n=45)<br>1.67 [1.33 ; 2.02] | (n=22)<br>1.66 [1.32 ; 1.95] | (n=23)<br>1.82 [1.34 ; 2.30] | 0.55 |
| Augmentation Index (AIx) | 17.81 [2.64 ; 31.24] | 19.9 [10.5 ; 33.4] | 11.1 [0.1 ; 30.7] | 0.018 |
| NYHA: New York heart association, COPD: chronic obstructive pulmonary disease, MRA: mineralocorticoid receptor antagonist, ACE: angiotensin-converting enzyme, ARB: angiotensin II receptor blocker, eGRF: estimated glomerular filtration rate, NT-proBNP: N-terminal of brain natriuretic peptide, CRP: C-reactive protein, LA: left atrium, LV: left ventricle, TAPSE: tricuspid annular plane systolic excursion, eSPAP: estimated systolic pulmonary artery pressure.<br>P values are for differences of characteristics between the groups MPO above median versus MPO below median and are derived from independent sample t-test, Mann Whitney U test, Chi-square test or Fisher exact test when appropriate. | | | | |

**DISCUSSION**

The findings of this study are as follows: patients with HFpEF have higher levels of MPO than controls, MPO levels in HFpEF are positively correlated with inflammation (CRP levels), diastolic dysfunction (E/e') and congestion (NT-proBNP) and negatively with left ventricular ejection fraction. Patients with MPO levels above the median suffer more often from diabetes, are more often males but tend to show less vascular stiffness (lower AIx) than patients with MPO levels below the median.

Several studies have shown a strong correlation between MPO and cardiovascular disease (CVD) including acute coronary syndrome, atherosclerosis, hypertension, and stroke [87,222]. Consistently, recent studies that target MPO in animal models of CVD have demonstrated favourable outcomes with regard to disease progression.[223] However, data in HFpEF are limited to the study by Hage and collegues[88]. Our study corroborates their finding that MPO is elevated in HFpEF

patients compared to controls and demonstrates that this applies also when the control group is older (74 ± 6 years) and with a proportion of women comparable to the HFpEF group (65 and 72% respectively). MPO levels showed moderate diagnostic value for HFpEF, less powerful in that regard than NT-proBNP levels (ROC curves Supplemental Figure 1.)

MPO-mediated oxidative stress may be one of the mechanistic link between comorbidities, inflammation and endothelial dysfunction at the source of HFpEF.[84] Comorbidities, namely obesity, diabetes, and ageing generate inflammation[80-83], during which MPO is released and uses $H_2O_2$ as a substrate to produce hypochlorous acid, a potent pro-oxidant and proinflammatory molecule. MPO levels in our study were indeed correlated with markers of inflammation (CRP and NLR) and signs of myocardial remodeling, namely NT-proBNP levels, decreasing LVEF (although within the normal range) and increasing E/e'. It is still to be determined whether MPO plays a causative role in the development of the disease or if it is merely a bystander of neutrophils activation. Indeed, recent studies directly incriminate activated neutrophils in aggravating diastolic dysfunction in mice subject to pressure overload[224], and in HFpEF patients.[225,226]

High MPO levels were associated with diabetic status. This is not surprising since diabetes is known to promote a systemic pro-inflammatory state [56,57]. Furthermore, MPO was shown to be predictive of insulin resistance in a population of obese patients [227]. Interestingly, the combination of male sex and diabetic status seem particularly associated with higher levels of MPO among patients with HFpEF. Indeed, all men suffering from diabetes had MPO levels above the median, while the proportion was limited to 40% in the other subgroups (Figure 4). This finding is consistent with the sex-specific proteomic profile of patients with HFpEF in the

PROMIS study [228], where they demonstrated that inflammation-related pathways predominated in men.

On the other hand, we found no association between vascular stiffness or endothelial function and MPO levels. Even more surprising, vascular stiffness seemed less important in the patients with higher MPO levels (lower AIx, lower Ea). The augmentation index (AIx) is calculated from pulse waveforms as the ratio of the difference between the early and late systolic peaks of the waveform relative to the early peak (Fig. 1) and represents the relative importance of the reflected wave.[229] Multiple small reflections travel back to the proximal aorta and merge into a "net" reflected wave whose magnitude and timing depend on vascular stiffness. In older subjects, systolic wave reflections mediate late systolic load, with an important impact on LV relaxation.[230,231] The augmentation index is not simply a measure of arterial stiffness and wave reflection, but was also shown to be elevated in conditions of increased LV contractility and may reflect overall ventricular-vascular coupling.[232] In HFpEF, high AIx was associated with abnormal LV diastolic responses to exercise, particularly in women, suggesting that arterial stiffness may contribute to the pathophysiology of HFpEF more commonly in women than in men.[233]The finding that patients with MPO levels above median do not display more endothelial dysfunction, nor vascular stiffness might be an indication that the sequence: comorbidities, inflammation, oxidative stress, endothelial dysfunction, myocardial remodelling is not straightforward. Rather, different mechanisms are probably involved in the development of myocardial remodeling and impaired vascular function, while both condition can ultimately lead to HFpEF. Recent data from phenomapping point towards the same direction. Indeed, although studies identify slightly different clusters depending on available variables,[152-156] two clusters seem to be commonly differentiated: one with older patients with stiff arteries, small highly contractile LVs and high rates of electrical remodelling (atrial

fibrillation) and the other with high rates of metabolic comorbidities, mainly diabetes, marked LV remodelling and advanced diastolic dysfunction. Inflammation and oxidative stress may play a more prominent role in the latter, hence the elevation of MPO (Fig. 4). Accordingly, there were more men and more patients suffering from diabetes in the group of patients with MPO levels above the median and they displayed lower (although ≥ 50%) LVEF and higher E/e'. These two subgroups might reflect two distinct pathophysiological mechanisms underlying HFpEF.

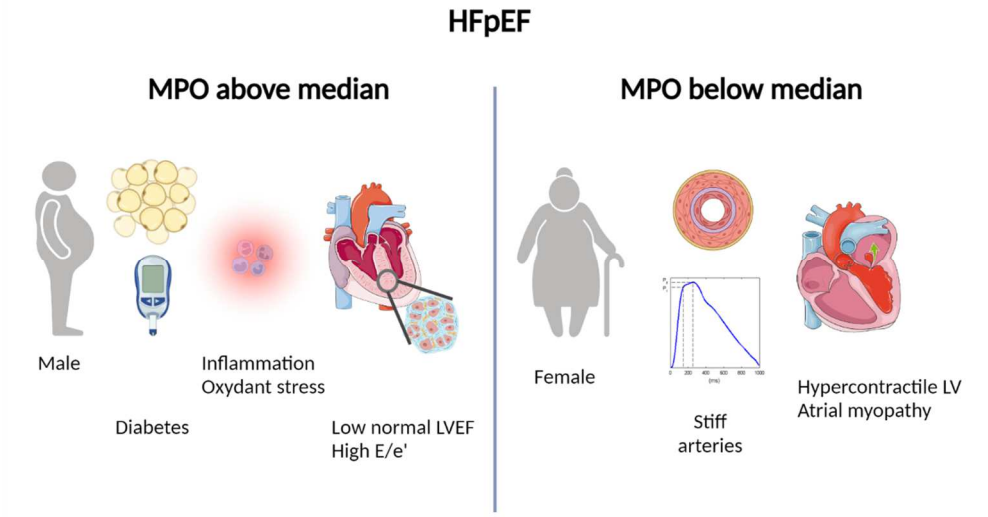



**Figure 5.** Illustration of patients' characteristics associated with levels of myeloperoxidase below or above the median. Patients with heart failure and preserved ejection fraction and myeloperoxidase above the median are more often men, suffer more often form diabetes, show subtle left ventricular dysfunction and pronounced diastolic dysfunction (high E/e') while patients with myeloperoxidase below the median are more often women with elevated vascular stiffness and high left ventricular ejection fraction.

## 4. Limitations

We acknowledge this single centre study has several limitations. Maybe the most important arising from the small sample size. Unfortunately, restrictions related to

the COVID pandemic interrupted the recruitment for several months. Furthermore, due to limitations of the EndoPAT technique, we could not obtain RHI and AIx for all patients. Despite our best effort to include controls of similar age and sex, both groups are not accurately matched for these characteristics. However, our groups are more alike than the only other published study demonstrating higher MPO levels in HFpEF[88]. Furthermore, the association between diabetic status and high MPO levels in HFpEF was not described before. In the context of the development of treatment with MPO inhibitor "AZD4831" (NCT03611153) it is interesting to note that not all patients might respond homogeneously. The results of our study suggest that patients with metabolic comorbidities, particularly diabetes, subtle LV dysfunction and evident diastolic dysfunction might benefit more from treatment targeting MPO while patients with predominant arterial stiffness (mostly females) and hyper contractile LV might be less responsive. Hence, while this study should be considered exploratory and hypothesis generating, it adds relevant information to existing literature. Future studies should aim at exploring the sex specific interplay between vascular inflammation and stiffness in this population, with special interest in features of metabolic stress such as obesity and diabetes.

## 5. Conclusion

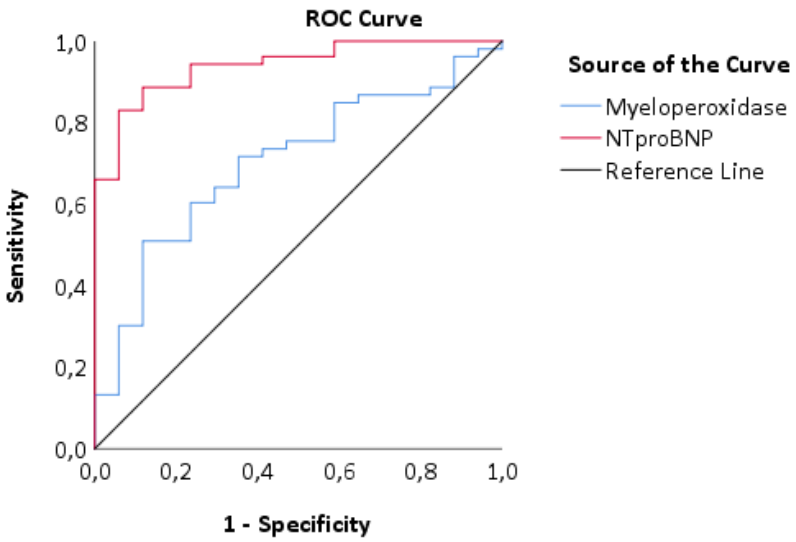
Myeloperoxidase levels are elevated in HFpEF compared to controls, reflecting leukocyte activation and oxidative stress. Patients with levels of MPO above the median are more often males and suffer more often from diabetes. MPO levels in HFpEF are positively correlated with diastolic dysfunction and congestion and negatively with left ventricular ejection fraction. The association between oxidative stress and vascular stiffness, on the other hand could not be demonstrated and deserves future attention.

## Supplementary material

**Supplementary Table 1.** Baseline characteristics of the control group.

| | Controls<br>N=18 | Patients<br>N=55 |
|---|---|---|
| Age (years) | 75 ± 5.0 | 80 ± 8.7 |
| Female (n, %) | 13 (72%) | 36 (65%) |
| Body mass index (kg/m$^2$) | 24 ± 2.8 | 28 ± 5.0 |
| Systolic blood pressure (mmHg) | 125 ± 15 | 134 ± 20 |
| Diastolic blood pressure (mmHg) | 77 ± 9 | 74 ± 14 |
| Heart rate at inclusion (bpm) | 63 ± 9 | 72 ± 13 |
| NYHA III – IV (n, %) | 0 (0%) | 20 (36%) |
| Diabetes (n,%) | 1 (6%) | 18 (33%) |
| Smoking (n, %) | 2 (11%) | 18 (18%) |
| Hypertension (n, %) | 13 (72%) | 52 (95%) |
| Hypercholesterolemia (n, %) | 10 (56%) | 39 (71%) |
| Sleep apneas (n, %) | 0 (0%) | 6 (11%) |
| COPD (n, %) | 0 (0%) | 6 (11%) |
| Loopdiuretics (n, %) | 0 (0%) | 42 (76%) |
| MRA (n, %) | 0 (0%) | 18 (33%) |
| Beta blockers (n, %) | 2 (11%) | 34 (62%) |
| ACE inhibitors/ARB (n, %) | 6 (33%) | 43 (78%) |
| Statins (n,%) | 1 (6%) | 35 (65%) |
| eGFR (ml/min/1.73m$^2$) | 70 ± 15.6 | 49 ± 18.3 |
| Hemoglobin (g/dL) | 13 ± 0.9 | 12 ± 1.8 |
| NT-proBNP (pg/mL) | 128 [90 ; 236] | 1302 [498 ; 2435] |
| Troponin (pg/mL) | 8 [5 ; 11] | 21 [11 ; 40] |
| CRP (mg/L) | 1.2 [1.0 ; 1.75] | 3.1 [1.2 ; 8.4] |
| Myeloperoxidase (ng/ml) | 22.6 [18.2 ; 32.0] | 34.7 [22.7 ; 44.0] |
| Uric acid (mg/dL) | 5.2 ± 1.01 | 7.3 ± 2.66 |
| Neutrophiles (10$^3$/µL) | 3.7 ± 1.29 | 4.3 ± 1.44 |
| Lymphocytes (10$^3$/µL) | 1.7 ± 0.51 | 1.6 ± 0.66 |
| Monocytes (10$^3$/µL) | 0.57 ± 0.222 | 0.68 ± 0.219 |
| Neutrophile to lymphocyte ratio | 2.4 ± 1.14 | 3.2 ± 2.12 |
| Indexed LA volume (mL/m$^2$) | 21.9 ± 9.40 | 37.6 ± 11.42 |
| LV ejection fraction (%) | 57.7 ± 3.86 | 57.7 ± 5.11 |
| E/e' ratio | 9.8 ± 2.64 | 16.3 ± 5.63 |
| Effective arterial elastance (mmHg/mL) | 1.99 ± 0.570 | 2.24 ± 0.716 |
| EndoPAT<br>Reactive hyperemia index (RHI) | 1.80 [1.42 ; 2.55] | 1.67 [1.33 ; 2.02] |
| Augmentation Index (AIx) | 17.7 [4.6 ; 36.9] | 17.81 [2.64 ; 31.24] |
| NYHA: New York heart association, COPD: chronic obstructive pulmonary disease, MRA: mineralocorticoid receptor antagonist, ACE: angiotensin-converting enzyme, ARB: angiotensin II receptor blocker, eGRF: estimated glomerular filtration rate, NT-proBNP: N-terminal of brain natriuretic peptide, CRP: C-reactive protein, LA: left atrium, LV: left ventricle | | |

**Supplementary Figure 1.** Receiver operating characteristic curves of plasma myeloperoxidase levels and NT-proBNP levels to diagnose HFpEF.




**Area under the curve**
Myeloperoxidase: 0.72 (0.59 ; 0.84) p=0.006
NT-proBNP: 0.94 (0.89 ; 1.00) p<0.001

## 3.4 Circulating nitric oxide in heart failure and preserved ejection fraction: too much of a good thing?

**Unpublished Data**

**SHORT COMMUNICATION (In Revision)**

**Abstract:** Endothelial dysfunction and decreased nitric oxide (NO) bioavailability are hypothesized to play a fundamental role in the pathophysiology of heart failure with preserved ejection fraction (HFpEF). On the other hand, pharmacologic attempts to restore circulating NO levels have had disappointing results and inducible NO synthase (iNOS) seems overexpressed in cardiomyocytes of patients with HFpEF. Hence, the state of NO homeostasis remains poorly understood. To address this question, we quantified circulating nitrosylated hemoglobin (HbNO) in controls and patients with HFpEF. Patients were prospectively recruited and underwent standard echocardiography and quantitative measurements of 5-coordinate α-HbNO in erythrocytes by electron paramagnetic resonance (EPR) spectroscopy. In a population of 40 HFpEF patients (80±9 years) and 16 controls (62±10 years), we found significantly higher levels of HbNO in patients (456 pmol/gHb (368.6 ; 765.5) versus 276 (214.9 ; 346.7), p=0.002). ). Among patients, HbNO levels were predicted by renal function (B= -10.4 (-18.1 ; -2.8), p=0.009 in uni- and multivariate linear regression. From these preliminary data, it seems circulating NO levels are not decreased but increased in HFpEF patients.

**Keywords:** nitric oxide, heart failure, preserved ejection fraction

## 1. Introduction

Current understanding of molecular mechanisms underlying heart failure with preserved ejection fraction (HFpEF) relates coexisting comorbidities to myocardial remodelling and diastolic dysfunction, through a systemic pro-inflammatory state and endothelial dysfunction. While this understanding of the HFpEF pathophysiology presupposes decreased nitric oxide (NO) bioavailability, Schiattarella et al.[41] demonstrated an activation of the inducible NO synthase (iNOS) that would instead lead to an increased production of NO. In their mouse

model of HFpEF, they showed that the activation of iNOS culminates in the accumulation of unfolded protein in the myocardium through the IRE1α–XBP1 axis, leading to increased myocardial rigidity. Hence, their findings suggest that metabolic inflammation and its master mediator, iNOS, are critical elements in the pathophysiology of HFpEF. Furthermore, studies that have attempted to restore circulating NO levels have had disappointing results. [234] Finally, evidence of endothelial dysfunction in HFpEF is derived from techniques measuring changes in flow or vessel diameter (such as flow mediated dilation of coronary flow reserve)[235], a rather indirect evaluation of NO-dependent endothelial function. Measurements of circulating nitrite/nitrate and nitrosated proteins have been used in human studies with some limitations and are affected by confounding factors limiting their interpretation.[236,237] Hence, the state of NO homeostasis in HFpEF patients remains mysterious.

Therefore, we attempted to quantify circulating NO in HFpEF patients compared to controls. Measurement of the bioavailable NO in the human circulation in vivo is a challenge due to low NO stability and various processes influencing NO reaction with potential targets. Commonly used correlates of circulating NO in vivo include total nitric oxide metabolites (NOx, nitrite / nitrate), but those are highly influenced by confounding factors and do not accurately reflect bioactive nitric oxide (no association with clinical vasodilation)[238]. Similarly, the measurements of nitrosylated proteins, cGMP and phosphor-VASP content in tissue biopsies, or stable isotopic methods have been used in human studies but are indirect measurements, relying on precursors and products of reactions involving NO [237]. 5-coordinate α-nitrosyl-hemoglobin (HbNO) was proposed as a relatively stable correlate of bioavailable NO, and an indicator of vascular oxidant stress, with clinical significance.[239-242] Electron Paramagnetic Resonance (EPR) spectroscopy, a method for quantitative detection of different paramagnetic compounds in

biological samples, has been proposed to quantify HbNO in human venous erythrocytes [243,244].

Previous work demonstrated that EPR measured HbNO from erythrocytes was linearly and highly significantly correlated with added nitric oxide and with endothelial function measured by tonometry during hyperemia (reactive hyperemia index, RHI)[239].

**2. Materials and Methods**

*Study population*

Patients with HFpEF encountered in our division of cardiology between May 2019 and March 2020 (in hospital, after decongestion and at ambulatory visits) were prospectively screened for inclusion in the study. HFpEF was diagnosed according to the HFA-PEFF diagnostic algorithm[31]. Briefly, patients had to be symptomatic (New York Heart Association (NYHA) functional class ≥II or hospitalization for HF in the previous 12 months), have a left ventricular ejection fraction over 50%, show echocardiographic signs of elevated filling pressures (left ventricular (LV) hypertrophy, left atrial (LA) enlargement, elevated E/e' ratio or elevated pulmonary pressures) and elevated NT-proBNP. The exclusion criteria were: history of reduced ejection fraction (LVEF < 50%), severe valvular disease, infiltrative or hypertrophic cardiomyopathy, acute coronary syndrome in the previous 30 days, severe chronic obstructive pulmonary disease, congenital heart disease, pericardial disease, atrial fibrillation (AF) with a ventricular response >140 bpm, and severe anemia (hemoglobin <8 g/dl). Patients taking nitrate derivatives were excluded from the analysis to avoid confounding effect on HbNO levels. Patients coming to the consultation for cardiovascular check up with no history of cardiac disease and normal echocardiography were used as controls. Patients and controls underwent blood sampling and complete transthoracic echocardiography. The local ethics

committee approved the study (NCT03197350), and all subjects gave written informed consent before study enrollment. The investigation conformed to the principles outlined in the Declaration of Helsinki.

*HbNO measurement*

Technique for HbNO measurement was previously described.[244] Briefly, blood collected for the HbNO analysis was mixed with antioxidant solution (sodium ascorbate and N-acetylcysteine, 5 mmol/L each, added into a closed vacutainer using a Micro-Fine™ syringe), centrifuged (10 min, 800xg, at room temperature), then retrieved from the bottom of the vacutainer tube, transferred into three 1 ml syringes and immediately frozen for low-temperature Electron Paramagnetic Resonance (EPR) spectroscopy measurements. The EPR spectra from the frozen erythrocyte samples were recorded on a Bruker X-band EPR spectrometer (EMX-micro) at 77 K using an EPR quartz finger Dewar filled with liquid nitrogen. The relative concentration of the heme-FeII nitrosyl-hemoglobin (T-form) was quantified from the intensity of the hyperfine components of the HbNO signal after subtraction of the overlapping EPR signal of protein free radicals. The absolute HbNO concentration was determined from the calibration curve generated using the HbNO complexes synthesized after incubation of erythrocytes with a NO-donor system in anaerobic condition.

*Statistical analysis*

Statistical analyses were performed using SPSS version 25 (SPSS Corp., Somers, New York). Tests were 2-sided and p-value <0.05 was considered statistically significant. HbNO levels were corrected for corpuscular concentration of hemoglobin (HbNO*100 / MCHC with MCHC = hemoglobin*100 / haematocrit). Data is presented as median (P25 ; P75). Mann-Whitney U test was used to compare patients and controls and Pearson's R was used to assess correlation. Uni- and

stepwise multivariate linear regression analysis were used to evaluate the effect of key variables on HbNO levels (age, sex, diabetic status, renal function and CRP).

**3. Results**

The baseline characteristics of the final study population including 40 HFpEF patients (80±9 years) and 16 controls (62±10 years) are described in table 1. HbNO levels were significantly higher in patients compared to controls ((456 pmol/gHb (368.6 ; 765.5) versus 276 (214.9 ; 346.7), Mann-Whitney U test p=0.002) (Figure 1). Male sex and lower renal function were associated with higher levels of HbNO in univariate linear regression (Table 2). In multivariate stepwise linear regression analysis, only renal function remained a significant predictor of HbNO levels. Figure 2 illustrates the correlation between renal function and HbNO levels

**Table 1**. Baseline characteristics of patients and controls

| | HFpEF | Controls |
|---|---|---|
| Age (years) | 80 ± 8.9 | 62 ± 10 |
| Female sex (n,%) | 33 (83) | 5 (33) |
| NYHA III-IV (n,%) | 14 (35) | 0 (0) |
| BMI (kg/m$^2$) | 28.3 ± 5.1 | 24.8 ± 2.6 |
| Ischemic heart disease (n,%) | 10 (25) | 0 (0) |
| Hypertension (n,%) | 37 (92) | 6 (40) |
| Diabete (n,%) | 11 (27) | 0 (0) |
| Loopdiuretics (n,%) | 29 (71) | 0 (0) |
| MRA (n,%) | 17 (41) | 0 (0) |
| Beta blockers (n,%) | 33 (80) | 5 (33) |
| ACE inhibitors (n,%) | 22 (54) | 4 (27) |
| Statins (n,%) | 23 (56) | 4 (27) |

| | | |
|---|---|---|
| eGFR (ml/min/1.73m$^2$) | 52 ± 17.5 | 81 ± 12.3 |
| Hemoglobin (g/dL) | 11.9 ± 1.6 | 14.0 ± 1.2 |
| hs-CRP (mg/L) | 3.2 [1.2 ; 9.4] | 1 [0.5 ; 2.0] |
| Troponin T (pg/mL) | 20 [13 ; 43] | 5.5 [0 ; 6] |
| NT-proBNP (pg/mL) | 1453 [332 ; 2706] | NA |
| LVEF (%) | 58 ± 5.3 | 58 ± 3.7 |
| LA volume indexed (ml/m$^{2)}$) | 40 ± 12.2 | 26 ± 9.3 |
| E/e' | 16.9 ± 5.5 | 8.6 ± 1.4 |
| RV-RA gradient (mmHg) | 35 ± 11.4 | 18 ± 4.3 |
| HbNO (pmol/gHb) | 456.0 [368.6 ; 765.5] | 276.2 [214.9 ; 346.7] |

NYHA: New York Heart Association functionnal class, BMI: body mass index, eGFR: estimated glomerular filtration rate by Cockcroft-Gault equation, hs-CRP: high sensitivity C reactive protein, NT-proBNP: N-terminal brain natriuretic peptide, LVEF: left ventricular ejection fraction, LA: left atrium, RV: right ventricle, RA: right atrium, HbNO: nitrosylated hemoglobin, RHI: reactive hyperemia index. P-values are derived from Mann-Whitney U test or Fisher exact test.

**Figure 1.** Boxplot of HbNO levels in controls and HFpEF patients.

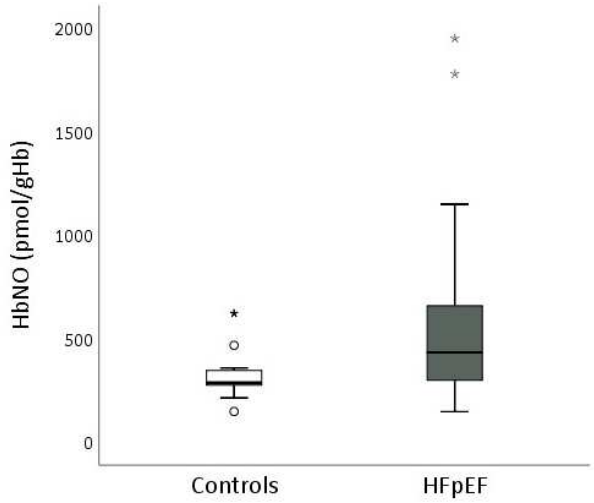
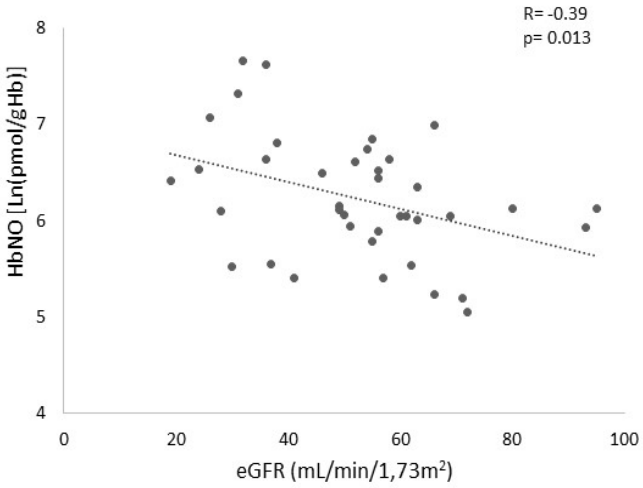


**Table 2.** Linear regression analysis for the prediction of HbNO levels.

| | Beta (95%CI) | p-value |
|---|---|---|
| Age (years) | 12.7 (-3.11 ; 28.50) | 0.11 |
| Female Sex | -424.0 (-776.5 ; -71.5) | 0.020 |
| Diabetes | 214.4 (-99.2 ; 528.1) | 0.18 |
| eGFR (ml/min/1.73m2) | -10.4 (-18.1 ; -2.8) | 0.009 |
| Hs-CRP (mg/L) | 10.2 (-2.5 ; 22.8) | 0.11 |

**Figure 2.** Correlation between HbNO levels and renal function in patients with HFpEF.



## 4. Discussion

The main finding of our study is that patients with HFpEF seem to have higher levels of circulating NO than control subjects and that those levels are correlated with renal function. Recent data from animal studies underlined the role of disrupted nitric oxide homeostasis in HFpEF. Reduced eNOS activity decreases NO available to activate the soluble guanylate cyclase, leading to decreased protein kinase G activity, titin hypophosphorylation and cardiomyocyte stiffness. Meanwhile, increased iNOS activity, leads to nitrosative stress and S-nitrosylation of key targets involved in diastolic function.[41,43,44] Hence, the activation of iNOS is a plausible explanation for the observed increase in HbNO among patients with HFpEF. Interestingly, a similar increase in HbNO was recently described among patients in septic shock[245], highlighting the possible role of inflammation-induced iNOS. However, in our population HbNO levels were not correlated with hs-CRP, a biomarker of inflammation. Inducible NOS is not the only possible source of NO. Zweier et al.[246] proposed that NO can be generated in condition of hypoxia in the heart by direct reduction of nitrite to NO. The obvious consequence of microvascular dysregulation observed in HFpEF[109] is the presence of areas of local intermittent ischemia that could contribute to NO formation. Furthermore, the enzyme-free reduction of nitrite to NO is enhanced in acidic condition, which might explain the moderate correlation between HbNO and renal function. This correlation is not a consequence of increased clearance since HbNO is not eliminated by the kidney. The formation and deformation of this complex depends on the conformational state of hemoglobin, hence on the oxygenation state of the environment  In venous blood, T-state deoxyhemoglobin favours the formation of HbNO (binding of NO to a heme-FeII α-chain, forming α-nitrosylhemoglobin). In the lungs, the presence of high oxygen concentration leads to the transition of hemoglobin to its R-state, where NO could be exchanged from the α-chain to the

β-chain forming S-nitrosohemoglobin, HbSNO or is converted to nitrate with formation of methemoglobin.[241,247]

*Limitations*

The authors acknowledge there are several limitations in this study. The study population was small, from a single center. The important difference in age and gender between the patients and the controls could have influenced the results. Other possible confounding factors such as the influence of dietary nitrate, medication intake and renal function were also not taken into account. Furthermore, the technique to measure HbNO is not free from pitfalls. Since circulating nitrite concentration varies with lifestyle and dietary intake, HbNO detected may not necessarily be a reflection of endothelial activity alone. Furthermore, HbNO formation from nitrite is complex, can occur via multiple interdependent routes and is influenced by oxygenation and redox status[248]. The digital subtraction of two EPR signals from composite spectra can be subject to interpretation and demands substantial experience. Thus, these data should be considered preliminary and hypothesis generating and should be confirmed in larger cohorts, following further exploration of sample processing and assay optimization.

However, the hypothesis that circulating NO levels are not decreased but increased in HFpEF patients, could be a game changer in the research for therapeutic targets. Our study adds data from a real life cohort to Schiattarella's fundamental findings and should encourage future research in this direction.

**ADDITIONAL DATA: BATCH 1**

**Comparison of patients with HbNO levels below or above the median (Table 1.)**

Patients with HbNO levels above the median had a tendency to be older and had a lower renal function. The prevalence of ischemic heart disease (42%) was higher in this group. There was no difference in body mass index, prevalence of diabetes mellitus or biomarker of inflammation (hs-CRP and NLR) between the two groups. Vascular function, measured by the reactive hyperemia index (RHI) and the augmentation index (AI75), was also not different.

**Table 3. Baseline characteristics of controls and HFpEF, and comparison of HFpEF patients according to HbNO levels below or above the medial**

| | Controls N=16 | HFpEF N=40 | HFpEF HbNO < med N=20 | HFpEF HbNO > med N = 20 | P-value* |
|---|---|---|---|---|---|
| Age | 62 ± 10.3 | 80 ± 8.9 | 77.7 ± 10.2 | 83.4 ± 6.5 | 0.082 |
| Female sex | 6 (38) | 33 (83) | 18 (90) | 14 (74) | 0.24 |
| NYHA III-IV | 0 (0) | 14 (35) | 6 (30) | 8 (40) | 0.52 |
| BMI (kg/m$^2$) | 25 ± 2.5 | 28.3 ± 5.1 | 28.4 ± 5.3 | 28.2 ± 53 | 0.96 |
| **Ischemic heart disease** | **0 (0)** | **10 (25)** | **2 (10)** | **8 (42)** | **0.032** |
| Hypertension | 7 (44) | 37 (90) | 18 (90) | 18 (90) | 1 |
| Diabetes | 0 (0) | 11 (27) | 5 (25) | 6 (30) | 0.73 |
| **eGFR (ml/min/1.73m$^2$)** | **79** ± 13.8 | **52 ± 17.5** | **58 ± 17.5** | **46 ± 16.1** | **0.033** |
| Hemoglobin | 14 1.21 | 12 ± 1.6 | 12 ± 1.4 | 11 ± 1.7 | 0.24 |
| hs-CRP (mg/L) | 1 [0.5 ; 2.5] | 3.2 [1.2 ; 9.4] | 3.9 [1.25 ; 12.0] | 2.2 [1.15 ; 7.12] | 0.44 |
| NLR | - | 3.4 [1.96; 4.50] | 3.4 [2.50 ; 4.25] | 2.8 [1.93 ; 5.12] | 0.55 |
| Troponin T (ng/L) | 6 [0.0 ; 6.0] | 20 [13 ; 43] | 17 [11.3 ; 31.0] | 31.5 [13.8 ; 61.0] | 0.30 |
| NT-proBNP (pg/mL) | - | 1453 [332 ; 2706] | 462 [235.4;3063] | 1734 [656.2; 2624.8] | 0.14 |

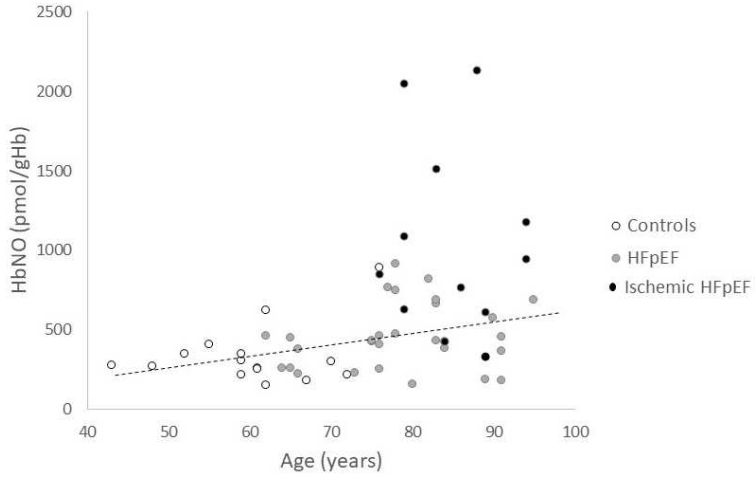
| | | | | | |
|---|---|---|---|---|---|
| Myeloperoxidase (ng/mL) | - | 40 [26.6 ; 64.6] | 43 [27.1 ; 113.4] | 36 [25.2 ; 60.4] | 0.66 |
| HbNO (pmol/gHb) | 286.2 [214.8;361.0] | 456.0 [368.6;765.5] | 375 [237.6;426.9] | 765 [635.4;1050.9] | By design |
| LVEF (%) | 58 3.7 | 58 ± 5.3 | 58 ± 6.2 | 58 ± 4.4 | 0.96 |
| LA volume (ml/m$^{2)}$) | 26 ± 9 | 40 ± 12 | 42 ± 13 | 38 ± 11 | 0.45 |
| E/e' | 8.6 ± 1.4 | 16.9 ± 5.5 | 16.5 ± 4.6 | 17.3 ± 6.4 | 0.97 |
| RV-RA gradient (mmHg) | 19 ± 4.0 | 35 ± 11.4 | 35 ± 10.8 | 35 ± 12.1 | 0.71 |
| RHI (n=25) | 1.78 [1.50 ; 2.95] | 1.52 [1.31 ; 1.90] | 1.45 [1.29 ; 1.88] | 1.67 [1.30 ; 2.44] | 0.37 |
| AI75 (n=30) | 3.61 [-4.15;14.59] | 17.9 [3.60 ; 29.23] | 19.9 [7.37 ; 30.14] | 12.1 [0.84 ; 26.08] | 0.37 |

NYHA: New York Heart Association functionnal class, BMI: body mass index, eGFR: estimated glomerular filtration rate by Cockcroft-Gault equation, hs-CRP: high sensitivity C reactive protein, NLR: neutrophile to lymphocyte ratio, NT-proBNP: N-terminal brain natriuretic peptide, LVEF: left ventricular ejection fraction, LA: left atrium, RV: right ventricle, RA: right atrium, HbNO: nitrosylated hemoglobin, RHI: reactive hyperemia index. AI75: Augmentation index corrected for heart rhythm. - : missing data. Normally distributed variables are presented as mean ± SD, other as median [P25;P75]. Binary variables are presented n (%).

*p-values are between HbNO below or above median, derived from Mann-Whitney U test or Fisher exact test.

## Effect of age

**Figure 3. Scatterplot of HbNO and age in controls and HFpEF patients.**




The difference of HbNO levels between HFpEF patients and controls could be due to confounding factors. Age in particular could play a role since patients were almost 20 years older than controls (80±9 years versus 62±10 years). Indeed, as illustrated in Figure 3 HbNO increases with age. However, some patients showed very high value of HbNO, not following the expected linear augmentation observed among controls (dotted line). Consistently, age and HbNO were correlated among the whole population (Spearman's rho (56) = 0.45, p=0.001) but not among patients alone (Spearman's rho (40) = 0.23, p=0.14). Hence we can hypothesize that the difference of HbNO is partly due to age, but not entirely. Interestingly, patients with the highest HbNO levels all had a history of ischemic cardiomyopathy (represented in black on Figure 3).

**HbNO in the coronary sinus**

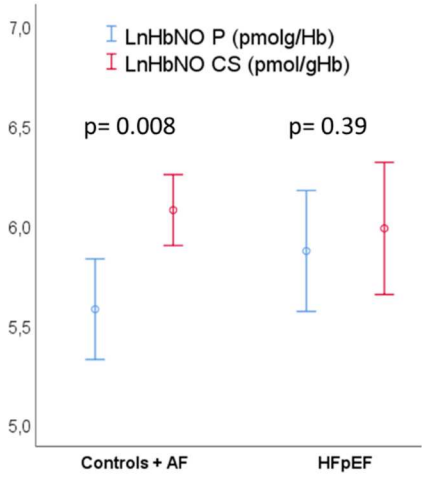
We also explored HbNO in samples from the coronary sinus in 10 subjects with atrial fibrillation without heart failure undergoing catheterization for isolation of pulmonary veins (IVP), and 10 HFpEF undergoing either IVP or right heart catheterization for measurement of pulmonary pressures.

| | Controls – AF (n=10) | HFpEF (n=10) |
|---|---|---|
| Age (years) | 60±8.5 | 76±7.7* |
| Female (n,%) | 2 (20%) | 10 (100%)* |
| BMI (kg/m$^2$) | 24.8±2.6 | 29.1±5.0 |
| Diabetes (n,%) | 0 (0%) | 2 (20%) |
| AF at sampling (n,%) | 1 (10%) | 3 (30%) |
| RHI | 2.2±0.9 | 1.4±0.5* |
| HbNO (pmol/gHb) | 268 [214;302] | 414 [244;454] |
| HbNO CS (pmol/gHb) | 454 [373;582] | 398 [280;561] |
| pvO2 (mmHg) | 40.7±8.4 | 40.0 ± 7.2 |
| pvO2 CS (mmHg) | 32.5±9.2 | 24.3±3.3* |
| SvO2 (%) | 73.8±11.6 | 71.8±11.5 |
| SvO2 CS (%) | 57.6±17.4 | 40 ± 9.0* |

BMI: body mass index, RHI: reactive hyperemia index, HbNO: nitrosylated hemoglobin, CS : coronary sinus, pvO2 : venous partial pressure in oxygen, Sv: venous saturation, *p<0.05

**Figure 4**

Peripheral (in blue) and coronary sinus (in red) HbNO in patients and controls in atrial fibrillation. Dots represents mean and bars 95% confidence interval. P-values were obtained using paired samples t-test.



Although not statistically significant in this small group, peripheral HbNO tended to be higher in HFpEF compared to controls. On the contrary, levels of HbNO in the coronary sinus of patients and controls were similar.

Paired analysis revealed higher HbNO levels in the coronary sinus of controls with AF compared to their own plasma levels (Figure 4). This was not the case in HFpEF, where coronary sinus and peripheral plasma levels of HbNO were similar. These results might illustrate that HFpEF is multisystemic, and not primarily a heart disease, in contrast to atrial fibrillation. This observation of differential NO levels in coronary and peripheral circulation in patients with AF is consistent with data from Han et al.[249] They showed that iNOs was overexpressed in the right atrium of patients in atrial fibrillation, with local increase in NOx levels, while plasma levels were decreased.

Finally, oxygen saturation and venous partial pressure of oxygen in the coronary sinus of HFpEF patients was lower than in the control subjects. This might be a consequence of enhanced oxygen extraction in HFpEF, secondary to coronary microvascular dysfunction and perfusion–demand imbalance.[109,133]

**ADDITIONAL DATA BATCH 2**

**Reproducibility**

Following those preliminary results, an important question was whether the elevation of HbNO we observed in HFpEF could be reproduced in a wider cohort, and, more importantly, if the difference was also observed if the control group was matched for age and gender.

The study population was extended to a total of 83 patients and 45 controls, including 22 controls in atrial fibrillation and 23 controls matched for age and sex (flowchart of the study population, Figure 5).

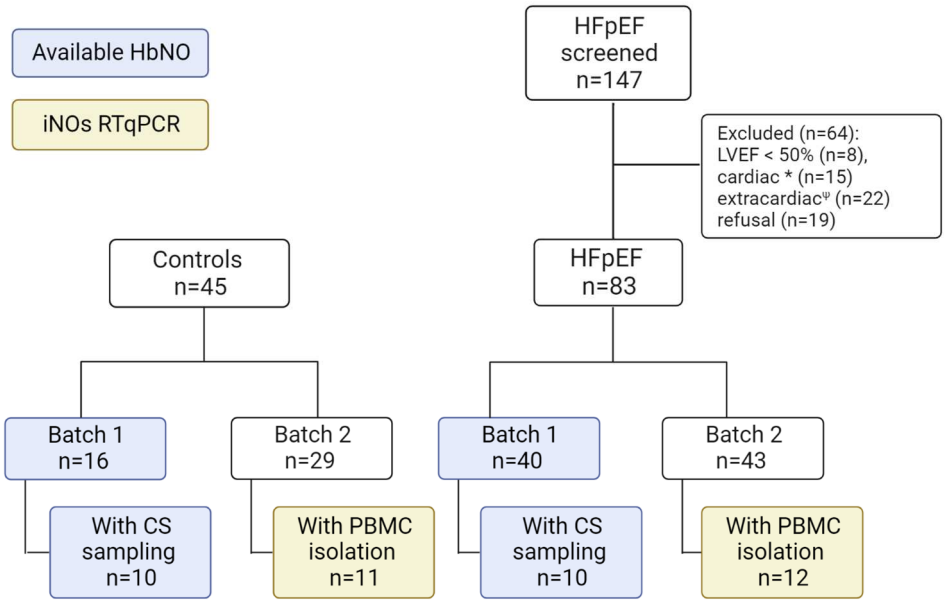



**Figure 5 Flowchart of included patients and controls in the HbNO study**
HbNO: nitrosylhemoglobin, iNOs: indufcible NO synthase, HFpEF: heart failure with preserved ejection fraction. LVEF: left ventricular ejection fraction, CS: coronary sinus, PBMC: peripheral blood mononuclear cells

Unfortunately, due to changes in the technique, the variability between measurements was too important to analyze data of both batches together and samples acquired in the second part of the study could not be used. Figure 6 shows the correlation and Bland Altman plots of samples read by two different operators. The validation of a new measurement technique is ongoing.

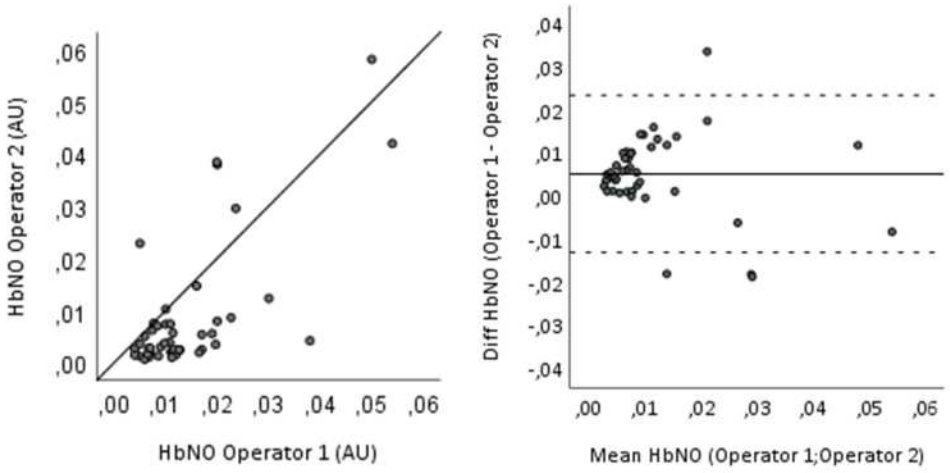



**Figure 6.** Correlation and Bland Altman plots showing poor reproducibility of HbNO measurements.

**Association with inflammation and iNOS**

In this second part of the study, we isolated peripheral mononuclear blood cells (PBMC) from 10 patients and 10 controls and quantified iNOS mRNA levels by RT-qPCR to explore the hypothesis that the elevation of HbNO was related to inflammation-induced iNOS. In this small population, we could not demonstrate significant overexpression of iNOS in the PBMCs of HFpEF patients (Figure 7).

| | CTL (n=11) | HFpEF (n=12) |
|---|---|---|
| Age (years) | 73 ± 1.8 | 76 ± 1.8 |
| Female (n,%) | 8 (73%) | 9 (75%) |
| BMI (kg/m$^2$) | 24 ± 3.0 | 29 ± 6 |
| Diabetes (n,%) | 0 (0%) | 5 (42%) |

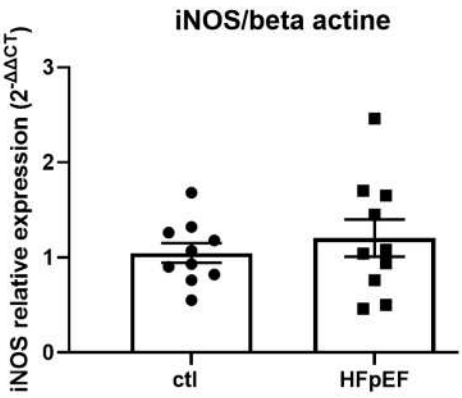



**Figure 7.** Inducible nitric oxide synthase (iNOS) mRNA levels in peripheral blood mononuclear cells (PBMCs) of patients with heart failure and preserved ejection fraction (HFpEF) and controls of similar age and gender.

**DISCUSSION**

Overall, this exploratory work with nitrosylated hemoglobin generated interesting results. In particular, levels of HbNO were increased in HFpEF patients compared to controls, hence circulating NO could be elevated and not reduced as previously postulated. We could not demonstrate that this increase was secondary to the overexpression of iNOs. Possible explanations include lack of statistical power in

the studied population (n=10). Furthermore, we quantified iNOS in circulating white blood cells while the overexpression of iNOS in HFpEF was initially demonstrated in cardiomyocytes.[41] However, since iNOs is primarily expressed in inflammatory cells, one might expect its activation in a context of systemic inflammation would have a repercussion in leucocytes. iNOs could also be more active but not overexpressed, which would not be detected by RT-qPCR. In the rat model of diastolic dysfunction by Dhot et al.[43] iNOs and nNOS protein levels were increased while the mRNA expression was not. This was associated with altered endothelial function (reduced vasorelaxation), counterintuitively related to an increase in NO.

Finally, the elevation of HbNO might not be secondary to increased iNOS activity. Multiple factors influence HbNO levels: NO production, affinity of NO for hemoglobin, and NO "elimination". Endogenous NO production is mainly driven by three NO synthases, hence the activity of eNOs and nNOS would also be interesting to quantify. Moreover, NO production is influenced by exogenous sources. Dietary nitrate from various types of green leafy vegetables is reduced to nitrite in the oral cavity by nitrate reductases of commensal bacteria. Circulating nitrite can in turn be reduced to NO in condition of hypoxia. The obvious consequence of microvascular dysregulation observed in HFpEF[109] is an imbalance between oxygen demand and supply, resulting areas of local intermittent ischemia that could contribute to this path of NO formation. In this context the observation that NO is particularly elevated is patients with history of ischemic cardiomyopathy is interesting. Furthermore, this enzyme-free reduction of nitrite to NO is enhanced in acidic condition, which might explain the moderate correlation between HbNO and renal function. This correlation is not a consequence of increased clearance since HbNO is not eliminated by the kidney. The formation and deformation of this complex depends on the conformational state of hemoglobin, hence on the

oxygenation state of the environment (Figure 8). In venous blood, T-state deoxyhemoglobin favours the formation of HbNO (binding of NO to an α-chain, forming α-nitrosylhemoglobin). In the lungs, the presence of high oxygen concentration leads to the transition of hemoglobin to its R-state, where NO is exchanged from the α-chain to the β-chain forming S-nitrosohemoglobin, HbSNO or is converted to nitrate with formation of methemoglobin.[241,247]

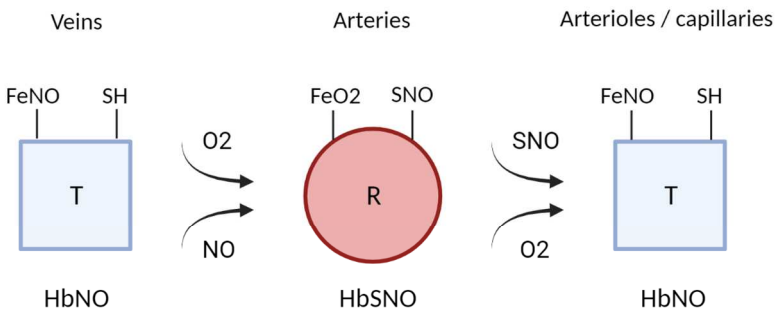



**Figure 8** In venous blood, T-state deoxyhemoglobin favours the formation of HbNO. In arterial blood, the presence of high oxygen concentration leads to the transition of hemoglobin to its R-state, where NO is exchanged from the α-chain to the β-chain forming S-nitrosohemoglobin, HbSNO or is converted to nitrate with formation of methemoglobin.

Our finding contrasts with a recent study by Chaar et al.[75] reporting a significant reduction of nitric oxide in patients with HFpEF. However, they quantified multi-species nitric oxide (nitric oxide and nitrite/nitrate) by enzyme-linked immunosorbent assay (ELISA). How HbNO levels and multi-species nitric oxide mix reflect bioavailable NO is unclear.

Limitations inherent to the measurement technique of HbNO prevented us from confirming these data and from drawing further conclusions. Electron paramagnetic resonance (EPR) is a difficult technique, with important operator dependency and requires training. Furthermore, HbNO is subject to many confounding factors related to the multiple possible sources of NO, the

compartmentalization of NO production and the variable affinity of NO for hemoglobin. Hence, HbNO might not be a suitable marker reflecting NO bioavailability, especially in a highly heterogeneous population as HFpEF.

# 4. CONCLUSION AND PERSPECTIVES

## 4.1 Final remarks and highlights

Many times over the past four years, I have been thinking of a quote from Oscar Wilde, which I find particularly relevant to HFpEF: “the truth is rarely pure and never simple”. Indeed, HFpEF is not a “pure” disease, caused by a single trigger. Instead, it is the result of multiple risk factors in combination with ageing, and the part played by each factor is probably unique for every patient.

With this work, we showed that patients we encounter in a tertiary centre in Brussels, Belgium are different from patients in Asia and from patients enrolled in clinical trials, illustrating this particularity. One striking dissimilarity was in the metabolic profile of patients. Accounting for this, we reported presentation and prognosis of patients according to their body mass index and diabetic status. Particularly in patients with metabolic comorbidities, it seems that inflammation and oxidative stress play an important role in the development of the disease. In accordance, we found that patients with high levels of myeloperoxidase were often diabetic and displayed more important alteration in cardiac structure and function. In other patients, we observed predominant vascular stiffness, maybe lying at the origin of HFpEF.

HFpEF is not a “simple” disease. During our research, we were often confronted with results we did not expect, in contradiction with our initial hypotheses. The first paradox we encountered is the well-known “obesity paradox”, described also in other types of heart failure. Much less described, a similar paradox arose among the diabetic patients. Patients with the best controlled diabetes were more at risk for hospitalisation for heart failure. Finally, starting the prospective study, we expected to find lower levels of HbNO in HFpEF patients, representing lower NO

bioavailability.[37] Instead, we observed an increase in HbNO levels. With the demonstration that iNOs was overexpressed in HFpEF cardiomyocytes, the article by Schiattarella and colleagues[41] came right on time to shed light on this finding.

These paradoxes remind us that we are still far from understanding all the intricate mechanisms at stake in HFpEF. Overall, this work raised at least as many questions it answered, but contributed to the existing knowledge over HFpEF.

## 4.2 Perspectives

### 4.2.1 Evolution in the definition of HFpEF

It is important to recognize that, even at the time of writing, the definition and classification of HF are in motion. Recently, a "universal" definition of heart failure was proposed to replace the traditional pathophysiologic definition, aiming to standardize its diagnosis across the world.[250] This "new" definition describes HF as a clinical syndrome including either symptoms or signs attributable to structural and/or functional cardiac abnormality and requires corroboration with either elevated natriuretic peptides or hemodynamic evidence of congestion. Hence the definition englobe all cases of symptomatic HF, regardless of ejection fraction. A revised version of the stages of heart failure was also included (A: at risk for HF, B: pre-HF, C: HF, D: advanced HF) underlining the continuum of risk and encouraging preventive approaches. Indeed, the most efficient attitude towards HF, and particularly HFpEF, is probably prevention. In this context, the patients with pre-heart failure (stage B), displaying echocardiographic features of diastolic dysfunction and / or elevated natriuretic peptide in the absence of symptoms represent a population of interest and should be included as intermediate group between controls and HF patients in future mechanistic studies. Given the growing consensus that in most cases, the origins of HFpEF are systemic and lie in the periphery, with cardiac injury as a secondary phenomenon, understanding of the transition from risk factors to HFpEF is our next challenge.

The classification of heart failure according to ejection fraction is also evolving.[250,251] Currently, accepted classification differentiate heart failure with reduced ejection fraction (HFrEF, EF <40%), mildly reduced EF (HFmrEF, EF 40-50%), preserved EF (HFpEF, EF >50%) and improved EF (HFimpEF). The strongest argument to use LVEF to categorize HF is that LVEF defines a group known to respond to therapy. The

growing body of evidence suggesting that standard therapy for HFrEF may be effective in patients at the lower end of the EF spectrum, formerly considered HFpEF (EF>40%) led to the introduction of HFmrEF . The recognition of this population enlarges the population who may potentially benefit from neurohormonal blockade; nonetheless, the cut-point of 50% is still debated, especially in women, the elderly, and in some racial/ethnic groups where the cut point is thought to be higher. In our work, we also observed that even among patients with EF>50%, ejection fraction could discriminate between phenogroups. Indeed, there was a substantial difference in EF between patients with high levels of myeloperoxidase (possibly the metabolic-inflammatory phenotype, LVEF 55.8± 4.71%) versus the others (59.5± 4.89%).

### 4.2.2 Evolution in the understanding of pathophysiological mechanisms

Pathophysiological research in the field of HFpEF still has a long way to go. Pieces of puzzle are added every day but we are far from seeing the complete picture. Here we present a few perspectives directly related to this thesis but this is not extensive.

The complex interactions between obesity, diabetes and heart failure is a fascinating topic. Recently visceral and epicardial adipose tissue (VAT and EAT) has come in the spotlight for its role in the development of HFpEF, especially in women and deserves future attention.[252,253] Beyond the macroscopic evaluation of adipose tissue, exploration of circulating metabolic intermediates in an unsupervised, unbiased manner (metabolomic approach, including lipidomics) identify activated or downregulated pathways.[254] Metabolomics could also be valuable to study intricate mechanisms of diabetes and HFpEF. Our observation that patients with better controlled diabetes were more at risk for adverse events requires

confirmation and mechanistic investigation. Similarly to sex-specific risk associated with obesity, there seem to be an excess risk of heart failure in women with diabetes compared to men. Accumulating evidence shows that women with diabetes exhibit greater endothelial, coronary microvascular, and diastolic abnormalities.[255] Further research is needed to clarify sex-specific mechanisms and to identify appropriate prevention and treatment strategies regarding both obesity and diabetes in women. A prospective study focusing on the interaction of sex with obesity and diabetes to favour the development of HFpEF with precise measurement of VAT, metabolomic analyses, indices of insulin resistance and glycated hemoglobin could be very interesting in this context.

Our preliminary results regarding nitric oxide left our curiosity intact, if not amplified. The presence of dysregulated NO signaling driving nitrosative stress is clearly part of the picture, and data from experimental models even make a case for a causal role. Still, animal models have limitations, especially in HFpEF and data from human studies are missing. Due to the high reactivity of NO in its radical form and the numerous scavengers on its path, bioavailable NO is challenging to quantify in vivo. Furthermore, NO's fate is also dictated by the location of production and by the characteristics of the surrounding milieu. Hence, a number of elements remains to be explored. Future research will probably demonstrate the excess nitrosation of other key targets and their role in the development of the disease.

### 4.2.3 Future direction in therapeutics

Probably the most important change in the management of HFpEF in the coming years will be the implementation of treatment with SGLT2 inhibitors. After years of research and numerous trials with neutral results, SGLT2 inhibitors were shown to reduce events associated with worsening heart failure. This benefit was consistent

across all prespecified subgroups, making current effort to cluster patients of lesser value. Nevertheless, other therapeutic targets relevant to the mechanisms outlined previously (inflammation, oxidative stress and nitric oxide imbalance) are still under investigation (Figure 4.1). Many other aspects of HFpEF are being studied and targeted[256] but are beyond the scope of this work.

**Myeloperoxidase inhibitors**

As explained above, myeloperoxidase (MPO) is produced during inflammation, and contributes to the vicious circle of inflammation and oxidative stress. Studies suggest that MPO-mediated oxidative stress is implicated in the progression of restrictive filling pattern, myocardial fibrosis and atrial fibrillation.[87,88,86] We and others have demonstrated that HFpEF patients display higher plasma MPO concentration.[88] MPO may thus provide a mechanistic link between inflammation, oxidative stress, vascular dysfunction, and impaired cardiac remodeling in HFpEF. In this context, the MPO inhibitor AZD4831 was developed and a clinical trial is currently ongoing (NCT03611153).

**Uric acid lowering therapy**

Hyperuricemia is predictive of the incidence of HFpEF in hypertensive patients,[257] is associated with common comorbidities and is an independent risk factor for poor prognosis in heart failure, both in HFrEF and HFpEF.[258,259] The increase in uric acid could reflect increased xanthine oxidase and myeloperoxidase activity in cardiomyocytes, resulting in abnormal energy metabolism and increased oxidative stress. However, whether uric acid is merely a marker of advanced disease or contributes to the pathophysiology remains unclear. Studies examining the effect of uric acid lowering therapy (xanthine oxidase inhibitors allopurinol or oxypurinol) in heart failure patients led to inconsistent results.[260,261] Uric acid transporter 1 (URAT1) is responsible for reabsorption of uric acid in the proximal tubule.

Inhibition of URAT1 results in increased urinary excretion of uric acid and lowering of plasmatic concentration. A study comparing the effect of the URAT1 inhibitor Verinurad with Allopurinol, on exercise capacity in patients with HFpEF is ongoing (NCT04327024).

**Anti-inflammatory strategies**

Colchicine is a potent anti-inflammatory drug. It suppresses tubulin polymerization and inflammasome inhibition, thereby reducing the production of IL-1β and IL-18. A pilot study investigating efficacy and safety of 2 dosing regimens of colchicine in patients with HFpEF was recently initiated (NCT04857931).

**β3 adrenergic receptor agonist**

β3 adrenergic receptors (β3-AR) are expressed in several human tissues, including bladder muscle, cardiac and vascular tissues. Under physiological conditions, β3-AR are expressed at low levels in myocardial tissue relative to the more abundant β1 and β2-AR. They are mainly localized in T-tubular membrane and couple to both eNOS and nNOS resulting in NO production and NO/cGMP signalling. In addition, β3-AR expressed in coronary microvascular endothelium produces NO to increase myocardial perfusion. In failing heart β3-AR are upregulated while in contrast β1-AR and β2-AR are downregulated and/or desensitized. In the short term, this β3-AR signaling may decrease inotropy but in the long term, β3-AR activation will protect from deleterious effects of β1-AR overstimulation, thereby preventing adverse remodeling, including hypertrophy.[262] In preclinical studies, activation of β3AR decreases myocardial hypertrophy and fibrosis.[263,264] For these reasons, the β3AR agonist Mirabegron is being investigated in patients with left ventricular hypertrophy and preserved EF, with or without HF symptoms (NCT02599480).

**iNOS inhibitor**

Conceptually, iNOS represents an attractive therapeutic target since its pharmacologic suppression improved the HFpEF phenotype in mice.[41] However, iNOs inhibitors have been investigated in other inflammation-associated diseases and none of them has proven effective in clinical trials. Some inhibitors even exhibited severe toxicities, which are attributed to their non-selectivity since the three isoforms have 50% to 60% structural similarities.[265] Therefore, research for selective iNOS inhibitors is still on. Meanwhile, a preclinical study demonstrated that imeglimin (a recently developed oral anti-diabetic medication) ameliorates the HFpEF phenotype and cardiac steatosis by suppressing iNOS expression and normalizing the UPR.[266] Whether or not imeglimin will have a preventive or therapeutic effect on HFpEF awaits the results of future clinical trials.

**SIRT3 activation – NAD + repletion**

Mitochondrial dysfunction is a hallmark of metabolic disorders implicated in the development of oxidative stress. A recent proteomic analysis incriminated mitochondrial dysfunction, and more specifically downregulation of protein deacetylase sirtuin-3 (SIRT3) in the development of HFpEF.[267] SIRT3 regulates several cellular processes, including mitochondrial DNA damage repair, gene expression, bioenergetics, redox balance, autophagy and apoptosis. Its important role in maintaining cardiac function has been demonstrated with SIRT3 knockout mice manifesting accelerated age-related cardiac hypertrophy and fibrosis.[268,269] Activation of SIRT3 by Resveratrol ameliorates cardiac fibrosis by inhibition of TGF-β signaling[270] and a similar effect was observed by repletion of its cofactor nicotinamide adenine dinucleotide (NAD+).[269] Oral nicotinamide riboside, correcting cardiac NAD+ deficiency, improved mitochondrial function and attenuated LVH and diastolic dysfunction in a murine HFpEF model.

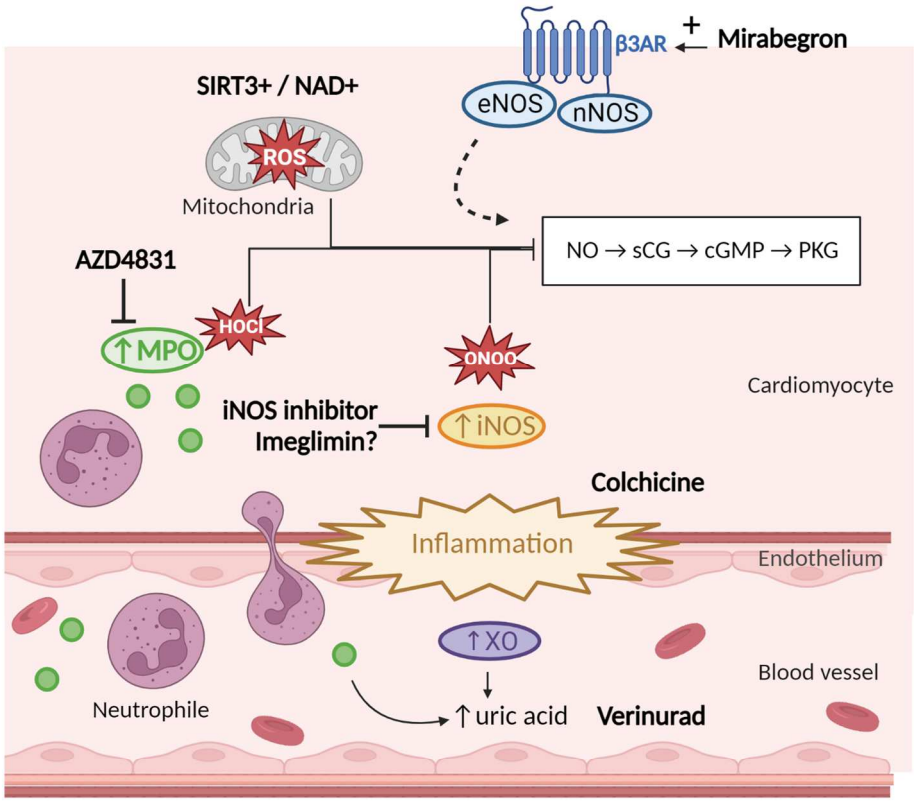



**Figure 4.1 Therapies targeting inflammation and oxidative stress under investigation in heart failure and preserved ejection fraction.**
The myeloperoxidase (MPO) inhibitor AZD4831 is expected to reduce the production of the free radical hypochlorous acid (HOCl) and decrease oxidative stress. Verinurad inhibits the reabsorption of uric acid and is tested in combination with xanthine oxidase (XO) inhibitor allopurinol to reduce hyperuricemia. Colchicine is tested for its systemic effect on inflammation. β3 adrenergic receptor agonist Mirabegron has shown a favourable effect on myocardial hypertrophy and fibrosis in preclinical studies. Research for selective iNOS inhibition is ongoing to tackle iNOS-induced nitrosative stress. Mitochondrial dysfunction can be targeted by the activation of the sirtuin 3 (SIRT3) or the repletion of its cofactor nicotinamide adenine dinucleotide (NAD+)

**Lifestyle modifications: a needed shift in mentality?**

Although benefits of exercise training and weight loss on symptoms and quality of life have been described for a while[271], this paragraph finds its place in the

perspective section since the implementation of lifestyle modifications is still largely underused in practice. Yet, exercise, and more broadly a healthier lifestyle could tackle HFpEF development at different levels by correcting comorbidities, reducing inflammation, increasing NO bioavailability, and promoting antioxidant defences.[235,272] Indeed, exercise-induced shear stress activates eNOS activity and thereby increases NO production, and improves endothelial function. Exercise also have a favourable effect on endothelial injury-repair balance through increased mobilisation of endothelial progenitor cells (EPCs) from the bone marrow.[273,274] Additionally, several sessions of exercise (training) activates sirtuins (SIRT1 and SIRT3 being the most studied), which jointly activate ATP production and the mitochondrial antioxidant function.[275] On the other hand, exercise-induced ischemic metabolites of the vascular system causes the generation and elevation of ROS. Nevertheless, the overall net effect of long-term exercise will ultimately improve the tolerance of oxidative stress and mitigate the oxidative burden.[276]

In 2010, Kitzman and colleagues[277] reported the first randomized controlled trial evaluating exercise training as a treatment for HFpEF, showing substantial improvement in exercise capacity. The SECRET trial[278] demonstrated that exercise training and weight loss significantly improved aerobic capacity, and the combination of both interventions was additive. Exercise intolerance being the primary symptom of HFpEF and an important determinant of quality of life, improvement of exercise capacity is a meaningful endpoint in this population. The diastolic or systolic functions are generally unchanged or only partially modified by the exercise, suggesting mechanisms contributing to the improvement of exercise tolerance in HFpEF patients results from complex peripheral adaptation mechanisms and the consequent increase in oxygen extraction by skeletal muscle.[279,280]

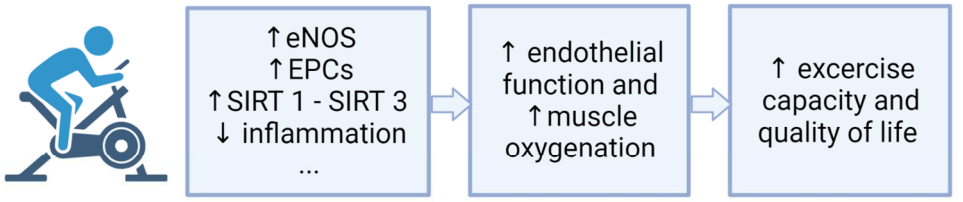



**Figure 4.2 Mechanisms underlying the favourable effect of exercise on endothelial and muscle function.** eNOS: endothelial nitric oxide synthase, EPCs: endothelial progenitor cells, SIRT: sirtuin.

Given this body of evidence, patients should be encouraged to perform at least 30 min/day of moderate-intensity physical activity (sufficient to provoke mild or moderate breathlessness) gradually increased to 60 min/day.[281]

Several challenges are associated with exercise training among patients with HFpEF. The recently published OptimEx study[282] comparing moderate continuous training and high-intensity interval training showed a significant attenuation in the beneficial effects of both interventions over long-term follow-up, coinciding with a decline in adherence. Moreover, patients with established HFpEF are generally old and have important functional limitations and comorbidities that may interfere with successful exercising. This is why lifestyle modifications are probably more powerful and valuable preventively, before the onset of heart failure. Poor dietary quality, including excess caloric intake and unhealthy food choices, low physical activity, and mental stress, are major, modifiable lifestyle factors that are likely contributing to the rapidly growing epidemiology of HFpEF.[283] Underlining this statement, there is strong evidence that lack of physical activity is associated with incident HFpEF in the general population[284], while exercise training reduced LV myocardial stiffness in patients in stage B heart failure[285]. Hence, the focus of

research and public health policies should be oriented towards preventive approaches and target patients in stage A and B, before the onset of symptoms.

To end this work on a more philosophical and personal note, I believe that global scale changes in lifestyle is the only way to achieve tangible improvements of the burden of heart failure. Endless search for molecular mechanisms and therapeutic targets is fascinating from a scientific point of view but in the end, have a relatively minor impact on patient's prognosis and quality of life. This was remarkably understood by Mr John Sharpley, a patient living with HFpEF, who wrote a letter entitled "The key to managing HFpEF? Fun, exercise, diet & sun".[286] A beautiful letter deserving to be read by every physician taking care of patients with HFpEF.

*"In their effort to help, clinicians often reach for the prescriptions pad. But in our experience less is best! […] Our HFpEF caring tips are driven by the philosophy fun, exercise, diet and sunshine […] You may come across articles and professional advice that propose "there is no therapy for HFpEF", which discourages clinicians, patients and carers. But this doesn't have to be. By working together with our GP, getting to grips with self-monitoring and building strong self-care routines, we have learnt to live our best life, in spite of HFpEF."*

Mr John Sharpley

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# 6. PUBLICATIONS

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