General discussion

Heart failure with preserved ejection fraction (HFpEF) is widely prevalent and has a cumbersome prognosis, while its pathophysiological mechanisms are incompletely understood and specific therapeutic strategies remain uncertain. It is still debated whether HFpEF and HF with reduced EF (HFrEF) represent distinct HF phenotypes. However, prominent differences in patient demographics, risk factor profile, hemodynamic characteristics and macroscopic and myocellular remodeling coupled to disparate responses to similar HF pharmacotherapy would all seem to justify that HFpEF and HFrEF indeed represent distinct HF phenotypes. Elevated diastolic left ventricular (LV) stiffness is an important feature in HFpEF. Numerous studies, of which several are included in the present thesis, demonstrated that raised intrinsic cardiomyocyte stiffness (Fpassive) importantly contributes to high diastolic LV stiffness in HFpEF. Cardiomyocyte Fpassive is mainly determined by the giant elastic sarcomeric protein titin, which regulates myocardial passive tension, stiffness and biomechanical stress/stretch signaling. Titin spans a half sarcomere, running from the Z-disk to the M-band and functions as a bidirectional spring responsible for early diastolic recoil and late diastolic resistance to stretch. Cardiomyocyte titin-based elasticity can be adjusted through transcriptional and posttranslational modifications. At the transcriptional level, titin modulates stiffness through shifts in expression of its compliant N2BA (3.2-3.7 MDa) and stiff N2B (3.0MDa) isoforms, which are co-expressed in the sarcomere at a N2BA:N2B ratio of approximately 35:65 in the normal heart. In eccentrically remodeled explanted hearts from dilated or ischemic cardiomyopathy patients, as well as in LV endomyocardial biopsies from HFrEF patients, the N2BA:N2B expression ratio was increased, resulting in reduced myocardial stiffness. In contrast, the N2BA:N2B expression ratio was decreased in LV endomyocardial biopsies from HFpEF patients compared to HFrEF, whereas mixed results were reported in LV biopsies from patients with severe AS, ranging from either reduced or similar N2BA:N2B expression ratios compared to donor hearts. While titin-based stiffness may be altered by titin isoform switching within days or weeks, titin-based stiffness can also be modulated acutely through posttranslational modifications, such as titin isoform phosphorylation.

Both protein kinases A and G (PKA and PKG) phosphorylate the stiff titin N2B isoform thereby acutely lowering titin-based cardiomyocyte stiffness, which has been demonstrated in skinned human LV muscle strips, in isolated human LV myofibrils and in isolated cardiomyocytes from HFpEF and HFrEF patients. Two additional posttranslational
modifications of titin, both increasing titin-based stiffness, include protein kinase C α (PKC-α) mediated phosphorylation of titin’s PEVK segment (rich in Proline, Glutamic acid, Valine and Lysine) and oxidative stress-induced formation of disulfide bridges in the stiff N2B segment of titin. Contribution by other myofilamentary proteins, such as myosin heavy chain, desmin, actin, troponin T, troponin I (TnI), myosin light chain 1 and -2 was recently ruled out, suggesting that phosphorylation-mediated posttranslational modification of titin indeed represents the most likely mechanism for acute modulation of cardiomyocyte  

Intrinsic cardiomyocyte stiffness is significantly higher in patients with HFpEF than in controls, in patients with HFrEF and in patients operated for severe aortic stenosis (AS). Because the fall in cardiomyocyte resting tension following in-vitro administration of PKA and PKG is significantly larger in HFpEF than in HFrEF and AS, a larger phosphorylation deficit of presumably titin could be present in HFpEF as recently suggested. Indeed, stimulation of PKG activity through inhibited breakdown of cyclic guanosine monophosphate (cGMP) by phosphodiesterase type 5A (PDE5A) by the PDE5A inhibitor sildenafil, was shown to ameliorate diastolic dysfunction in various experimental and clinical studies. Sildenafil restored LV relaxation kinetics in mice exposed to transverse aortic constriction (TAC) and reduced diastolic LV stiffness in an old hypertensive dog model, in patients with HFrEF and in HFpEF patients with pulmonary hypertension. Administration of sildenafil to old hypertensive dogs lowered diastolic LV stiffness through restored phosphorylation of the titin N2B segment. Moreover, PDE5A inhibitors have also been shown to attenuate adrenergic stimulation, reduce ventricular-vascular stiffening, improve endothelial function, reduce pulmonary vascular resistance and enhance exercise tolerance in HFrEF. Recently, we measured PKG activity, upstream control of PKG activity and downstream effects of PKG activity in LV myocardial biopsies of HFpEF patients. To discern altered control by PKG of the myocardial remodeling process in HFpEF, measurements were compared to measurements obtained from LV myocardium remodeled concentrically by severe AS or eccentrically by nonischemic HFrEF. Our study showed that relative to both AS and HFrEF, HFpEF patients had reduced myocardial PKG activity and lower cGMP concentration, which related to higher cardiomyocyte  

Reduced PKG activity and lower myocardial cGMP concentration in HFpEF did not result from altered myocardial soluble guanylate cyclase (sGC) or PDE5A expression, which
were similar in all groups, or from unequal brain type natriuretic peptide (BNP) expression, which was comparable in HFP EF and AS. Because of comparable proBNP-108 expression in HFP EF and AS, BNP is unlikely to account for the widely different PKG activities and cGMP concentrations observed in both conditions. Lower proBNP-108 expression in HFP EF than in HFrEF also explains the low positive predictive value of BNP for the diagnosis of HFP EF. Downregulation of cGMP-PKG signaling in HFP EF was therefore most likely related to low myocardial NO bioavailability because of high nitrosative/oxidative stress, which was almost fourfold higher in HFP EF than in both HFrEF and AS. Oxidative stress is known to lower NO bioavailability and downregulate cGMP-PKG signaling.

**Importance of NO for diastolic function**

**LV distensibility**

Endothelial dysfunction plays a prominent role in the pathophysiology and prognosis of HF. Impaired endothelium-dependent vasodilation in HF patients has been attributed to reduced activity of the L-arginine-NO synthetic pathway, increased degradation of NO by reactive oxygen species (ROS) and hyporesponsiveness in vascular smooth muscle. NO is crucially important for diastolic function, since it potently enhances LV relaxation and distensibility through cGMP-PKG dependent and independent mechanisms, including reduction of myofilament calcium (Ca^{2+}) sensitivity by TnI phosphorylation and enhancing phospholamban-mediated sarcoplasmic reticular (SR) Ca^{2+} reuptake, respectively. NO is an ubiquitous intra- and intercellular signaling molecule generated in a temporally and spatially restricted manner by a family of NO synthases (NOSs), including endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS), which vary in their subcellular localization. The myocardial autocrine and paracrine effects of NO depend on where and by which NOS isoform NO is produced. Myocardial eNOS-derived NO was demonstrated to enhance LV relaxation and distensibility and to modulate cardiac β-adrenergic responsiveness. Stimulation of eNOS activity by intracoronary administration of NO donors instantaneously hastened LV relaxation, accelerated LV pressure decline and increased diastolic LV distensibility in the normal human heart as well as in patients with dilated cardiomyopathy and severe AS. The importance of myocardial NO bioavailability for diastolic LV function was recently reappraised because of close correlations between asymmetric dimethylarginine and diastolic LV dysfunction in patients with ischemic and non-ischemic dilated cardiomyopathy.
In addition, myocardial endothelial eNOS-derived NO regulates cardiac metabolism through modulation of myocardial substrate utilization and by reduction of myocardial oxygen consumption through inhibition of complexes I and II of the mitochondrial electron transport chain. Interestingly, mitochondrial biogenesis is stimulated in response to increased myocardial NO bioavailability and cGMP levels. Because cross-bridge detachment is an energy consuming process, slow LV relaxation in HFpEF could therefore also result from a myocardial energy deficit because of reduced myocardial NO bioavailability due to increased nitrosative/oxidative stress burden. Recent 31P-magnetic resonance spectroscopy studies indeed demonstrated reduced myocardial energy reserve in patients with HFpEF, HFrEF and in both diabetic and obese patients, in whom impaired myocardial bioenergetics correlated with diastolic LV dysfunction.

**NO-mediated activation of cGMP-PKG signaling**

NO activates sGC, which is present predominantly in the cytosol and in smaller amounts at the plasma membrane, via a complex interplay between binding of NO to both heme and non-heme sites of sGC. Stimulated sGC subsequently enhances cGMP generation, which activates its primary effector kinase PKG. In turn, PKG phosphorylates a vast number of target proteins, exerting a wide range of downstream effects like enhanced SR Ca\(^{2+}\) reuptake, inhibition of Ca\(^{2+}\) influx and suppression of pro-hypertrophic and fibrotic signaling as well as stimulation of relaxation and LV distensibility by phosphorylation of TnI and the stiff titin N2B segment. Because NO diffusion distances in cardiomyocytes are limited, NO-sGC-cGMP signaling is compartmentalized into functional signalosomes, coupling NO synthesis to downstream sGC-cGMP signals. For instance, in cardiomyocytes and endothelial cells, eNOS, sGC and PKG colocalize with caveolin at plasma membrane invaginations, called caveolae, which may serve as microdomains for optimized NO-sGC-cGMP signaling. Furthermore, besides being subject to intricate intracellular compartmentalization, signaling through the eNOS-NO-cGMP-PKG pathway is also characterized by considerable crosstalk with \(\beta\)-adrenergic mediated cAMP signaling, providing the heart with a complex and highly differentially regulated machinery to modulate and integrate divergent cardiovascular (patho)physiological conditions.

**Importance of cGMP-PKG signaling in myocardial remodelling**
Cardiac remodeling in HFpEF is characterized by concentric LV and cardiomyocyte hypertrophy and interstitial fibrosis\textsuperscript{5,9,68-70}. The Framingham Heart Study determined that LV hypertrophy follows aging as independent risk factor for cardiovascular morbidity and mortality\textsuperscript{71}. Stimuli for LV hypertrophy activate an array of membrane-bound receptors coupled to multiple intracellular signaling cascades, which ultimately stimulate pro-hypertrophic gene expression\textsuperscript{72,73}. Important upstream triggers for cardiac hypertrophy include mechanical stretch, pressure overload, cytokines, activation of RAAS, endothelin and catecholamines, whereas cGMP-PKG signaling exerts prominent anti-hypertrophic and anti-fibrotic effects\textsuperscript{27,72,73}. Indeed, modulation of the sGC-PKG-PDE5A axis affected myocardial remodeling with less cardiomyocyte hypertrophy and interstitial fibrosis in TAC mice exposed to sildenafil\textsuperscript{28}. Despite similar levels of LV and cardiomyocyte hypertrophy in patients with HFpEF and AS, HFpEF patients demonstrated higher cardiomyocyte F\textsubscript{passive} and lower myocardial PKG activity than AS patients\textsuperscript{14}. The finding in AS, of higher PKG activity corresponding with low F\textsubscript{passive} but not with cardiomyocyte diameter, suggests cardiomyocyte diastolic stiffness to evolve independently from cardiomyocyte hypertrophy. This concept also emerged from the VALIDD trial, in which only 3\% of hypertensives had significant LV hypertrophy despite all having diastolic LV dysfunction\textsuperscript{74}. External myocardial force signals imposed during hemodynamic load are transmitted to ECM components and the cardiomyocytes\textsuperscript{15}. Mechanical stimuli sensed at the outer cardiomyocyte cell membrane can activate cytoskeletal and sarcomeric signaling cascades, which subsequently trigger pro-hypertrophic factors\textsuperscript{15}. In addition to its prominent role in regulating cardiomyocyte stiffness, titin also acts as a dominant signaling protein, which integrates both internally generated sarcomeric forces and externally sensed biomechanical stretch/stress signals to hypertrophy regulation\textsuperscript{75}. Thus, it could be speculated that increased cardiomyocyte stiffness sensed by titin precedes titin-mediated stimulation of pro-hypertrophic signaling.

**Nitrosative/oxidative stress lowers NO bioavailability and NO signalling in HFpEF**

Reduced NO bioavailability, dysregulation of NO-mediated signaling and increased nitrosative/oxidative stress are firmly implicated in the pathogenesis of HF\textsuperscript{40,41,76-78}. Although generation of ROS (i.e. superoxide, hydroxyl radical and non-radical species such as hydrogen peroxide) is a normal component of cellular redox-based signaling, excessive ROS production causes nitrosative/oxidative stress\textsuperscript{40,41,76-79}. 
Nitrosative/oxidative stress impairs NO bioavailability and NO-mediated signaling through a number of mechanisms. First, superoxide rapidly reacts with NO to form peroxynitrite, which triggers eNOS uncoupling with subsequent generation of superoxide instead of NO, thereby augmenting oxidative stress. Second, by scavenging NO, superoxide reduces NO bioavailability and prevents NO from stimulating sGC-mediated activation of cGMP-PKG signaling\textsuperscript{37,40,41}. Finally, sGC activity is adversely affected by nitrosative/oxidative stress through several mechanisms, including superoxide and peroxynitrite mediated decrease in sGC activity and sGC heme group oxidation converting sGC to its NO-insensitive state\textsuperscript{40,63}. The lower myocardial PKG activity and reduced cGMP concentration in HFpEF patients, which was associated with increased myocardial nitrotyrosine levels\textsuperscript{14}, could therefore have resulted from upstream nitrosative/oxidative stress-induced impairments of NO bioavailability and reduced NO-mediated activation of sGC-induced cGMP/PKG signaling. Accordingly, a number of recent studies also suggested oxidative stress to be involved in the pathogenesis of diastolic LV dysfunction in HFpEF. In HFpEF rodent models, oxidative stress uncoupled cardiac NOS and induced diastolic LV dysfunction, whereas stimulation of eNOS activity reversed oxidative stress and improved diastolic LV function\textsuperscript{80,81}. Furthermore, high plasma levels of methylated L-arginine metabolites, indicative of increased oxidative stress, were strongly related to diastolic LV dysfunction in patients with HFrEF\textsuperscript{52}. Moreover, in patients with HFpEF, myocardial fibrosis and inflammation were associated with diastolic LV dysfunction and with increased oxidative stress in endomyocardial biopsies compared to controls\textsuperscript{82}. Abundant evidence demonstrates that ROS potently activate pro-hypertrophic and pro-fibrotic signaling, and by interfering with NO-sGC-cGMP-PKG signalling, ROS inhibit anti-hypertrophic and anti-fibrotic signalling cascades\textsuperscript{41,72}.

In addition, nitrosative/oxidative stress induces a state of NO/redox disequilibrium, which impairs S-nitrosylation signaling (a posttranslational protein modification process through coupling of an NO moiety to a reactive cysteine thiol). S-nitrosylation serves as a major effector of NO bioactivity and an important mode of cellular signal transduction, regulating the activity of numerous metabolic enzymes, kinases, phosphatases and respiratory proteins as well as cytoskeletal and structural components and transcription factors\textsuperscript{41,76,83}.

**Possible mechanisms for enhanced nitrosative/oxidative stress in HFpEF**

It was recently suggested that the particularly high prevalence of metabolic risk factors in HFpEF could be associated with increased inflammation and nitrosative/oxidative stress in
this condition\(^{14}\). HFpEF patients demonstrate a high prevalence of obesity and DMII\(^{84-86}\). Obesity and DMII are strongly related to insulin resistance (IR) and the metabolic syndrome (a constellation of cardiovascular risk factors, including obesity, hypertension, IR, hyperglycemia, dyslipidemia, microalbuminuria and hypercoagulability)\(^{87}\) and the frequent clustering of these metabolic risk factors causes synergistic adverse effects on myocardial structure and function\(^{88}\). The prevalence of obesity, DMII, IR and metabolic syndrome increases rapidly and is expected to reach pandemic proportions in the next few decades\(^{88-92}\), while these metabolic risk factors have all been prospectively identified as precursors of incident HF\(^{93-95}\) and are independently associated with early development of diastolic LV dysfunction\(^{96-99}\). Diastolic LV dysfunction is recognized as the earliest manifestation of DMII-induced LV dysfunction\(^{96,100-104}\), while the excessive diastolic LV stiffness of the diabetic heart has been related to myocardial fibrosis\(^{105}\) and to circulating advanced glycation endproducts (AGEs)\(^{106}\). In a recent study, DM increased diastolic LV stiffness in both HFpEF and HFrEF patients through distinct mechanisms\(^{12}\). In diabetic HFrEF, raised diastolic stiffness was associated with both increased CVF and myocardial deposition of AGES. Conversely, in diabetic HFpEF, myocardial CVF was unchanged and elevated diastolic stiffness related to increased cardiomyocyte F\(_{\text{passive}}^{12}\). Diabetes also increases LV diastolic and cardiomyocyte stiffness in patients with severe AS\(^{102}\) and elevated cardiomyocyte stiffness in diabetic AS patients related to hypophosphorylation of the titin N2B segment, which could be corrected by phosphorylation with PKA\(^{22}\).

Obesity, DMII and IR can have direct adverse effects on myocardial structure and function independently from common confounders as hypertension or CAD, which has been referred to as “obesity”\(^{107}\), “diabetic”\(^{102}\), or “insulin-resistant”\(^{108}\) cardiomyopathy. Moreover, metabolic risk factors are strongly associated with endothelial dysfunction, inflammation, oxidative stress, impaired myocardial energetics, abnormal cardiomyocyte Ca\(^{2+}\) handling, reduced NO bioavailability and maladaptive cardiac remodelling\(^{102,107,108}\).

**Metabolic risk factors interfere with NO-cGMP-PKG signaling**

Oxidative stress-mediated downregulation of NO-cGMP-PKG signaling has been demonstrated in various experimental models of obesity, diabetes, IR and metabolic syndrome\(^{109,110}\). Cultured rodent aortic smooth muscle cells subjected to hyperglycaemia demonstrated significantly reduced PKG expression and activity, which occurred through a PKC-dependent activation of NADPH oxidase-derived superoxide production\(^{109}\). In this
model, high glucose-mediated lowering of PKG levels was inhibited by a superoxide scavenger or NADPH oxidase inhibitors\textsuperscript{109}. In addition, in human diabetic cardiomyopathy, sildenafil improved measurements of LV geometry, strain and torsion determined by magnetic resonance imaging\textsuperscript{111}. Furthermore, in a rodent model of diet-induced obesity and IR, signaling through the NO-cGMP pathway was reduced, while vascular inflammation and IR were completely prevented by administration of sildenafil\textsuperscript{110}. Physiologically, insulin phosphorylates titin through activation of the phosphoinositol-3-kinase (PI3K)/Akt/eNOS pathway, while IR, FFAs and adipokines inhibit insulin-dependent PI3K/Akt signaling, resulting in reduced eNOS activity and impaired NO generation\textsuperscript{112,113}. Inflammation and oxidative stress associated with metabolic risk factors can reduce eNOS activity and NO bioavailability through a number of ways, including RAAS-mediated activation of ROS producing enzymes, increased flux through the hexosamine biosynthesis pathway, mitochondrial uncoupling, activation of the polyol pathway, lipotoxicity, glucose auto-oxidation and formation of AGEs\textsuperscript{113}. Moreover, obesity, DM and IR induce activation of multiple PKC isoforms, with PKCs being prominently involved in numerous cellular functions and signal transduction pathways\textsuperscript{114}. PKC can hamper myocardial eNOS activity and NO generation by inhibition of PI3K/Akt signaling and stimulation of NADPH oxidase activity\textsuperscript{114}, whereas PKC\textgreek{a} was recently shown to increase titin-based cardiomyocyte stiffness through phosphorylation of the PEVK segment\textsuperscript{25}. Interestingly, in a rodent diabetic HFpEF model, PKC\textgreek{b} inhibition improved diastolic distensibility, contractility and maladaptive cardiac remodelling\textsuperscript{115}. Recent evidence demonstrates that in addition to downregulation of NO-cGMP-PKG signaling, metabolic conditions such as IR, metabolic syndrome and DM also induce a state of myocardial energy deficiency with decreased mitochondrial oxidative phosphorylation and diminished mitochondrial biogenesis, which predisposes to a metabolic cardiomyopathy characterized by early development of diastolic LV dysfunction\textsuperscript{116,117}. In summary, metabolic risk factors could contribute to diastolic LV dysfunction and elevated myocardial stiffness in HFpEF through provoking myocardial inflammation and nitrosative/oxidative stress, which on its turn results in decreased NO bioavailability, downregulation of cGMP-PKG signaling, impaired myocardial bioenergetics and maladaptive remodeling. Furthermore, downregulation of cGMP-PKG signaling could subsequently result in hypophosphorylation of titin, which elevates cardiomyocyte stiffness and possibly triggers cardiomyocyte hypertrophy.
Novel treatment strategies for HFpEF
In HFpEF, the reduced myocardial PKG activity, increased nitrosative/oxidative stress and the presence of myocardial fibrosis, impaired myocardial energetics and the high prevalence of metabolic risk factors supports use of NO-donors, PDE5A inhibitors, statins, anti-fibrotic agents, such as aldosterone antagonists and optimized treatment of metabolic risk.

Stimulation of NO-cGMP-PKG signaling
NOS uncoupling has emerged as an important contributor to pathologic chamber remodeling, endothelial dysfunction and diastolic dysfunction in animal models of HF. Oxidative depletion of the essential NOS cofactor tetrahydrobiopterin (BH4), results in NOS destabilization and uncoupling subsequently causing NOS to generate superoxide instead of NO. Administration of BH4 in animal models reduces pressure-overload hypertrophy, fibrosis, NOS uncoupling and oxidative stress, while at the same time systolic and diastolic function are improved, suggesting that targeting NOS uncoupling could improve diastolic LV dysfunction in HFpEF. Exogenous administration of BH4 has also been shown to improve endothelial dysfunction in patients with hypercholesterolemia, DM and hypertension through improvement of NOS function and enhanced NO bioactivity coupled with BH4 treatment.

Acute administration of NO donors is known to improve diastolic LV function with an earlier onset of LV relaxation, lower LV peak systolic, end-systolic and end-diastolic pressures, with rightward displacement of the diastolic LV pressure-volume relation. Current ESC HF guidelines accord a class IIa indication for administration of NO donors in patients admitted for acute HF with pulmonary edema without concomitant cardiogenic shock. Unfortunately, longterm use of NO donors is frequently hampered by development of NO resistance. NO resistance largely results from a combination of scavenging of NO by superoxide and of (reversible) inactivation of sGC. Conversely, chronic use of isosorbide dinitrate combined with the antioxidant hydralazine improved outcome in V-HeFT I and A-HeFT trials. Hydralazine reduces superoxide generation by xanthine oxidase and NADPH oxidase. The clinical characteristics of the A-HeFT HFrEF patients revealed a high prevalence of obesity and DM, conditions which are also highly prevalent in HFpEF. Combined use of isosorbide dinitrate and the antioxidant hydralazine could therefore be potentially favourable in HFpEF.
An exciting promising new inroad for HFpEF treatment is represented by agents that enhance cellular cGMP signaling\textsuperscript{27}. Upstream activation of sGC by NO stimulates cGMP-PKG activation in the cytosolic and subsarcolemmal compartments, while upstream activation of particulate GC (pGC) by natriuretic peptides stimulates cGMP-PKG signaling at the plasma membrane\textsuperscript{63,65}. Several key transcription factors and sarcomeric proteins involved in hypertrophy signaling, diastolic relaxation and stiffness and vasorelaxation are favourably modified by PKG-dependent phosphorylation, suggesting that these agents may be beneficial in HFpEF\textsuperscript{13,24,27,28,48}. PDE5A inhibitors, such as sildenafil, increase cGMP levels by blocking their catabolism. PDE5A inhibitors attenuate adrenergic stimulation\textsuperscript{32}, reduce ventricular-vascular stiffening\textsuperscript{32}, antagonize maladaptive chamber remodeling\textsuperscript{28,30}, improve endothelial function\textsuperscript{34}, reduce pulmonary vascular resistance\textsuperscript{30,31,35} and lower diastolic LV stiffness in patients with HFrEF\textsuperscript{30} and in HFpEF patients with pulmonary hypertension\textsuperscript{31}.

An alternative approach to enhance cGMP-PKG signaling is through direct stimulation of sGC activity. Recently, two classes of drugs have been discovered, the sGC activators and sGC stimulators, which target two different redox states of sGC: the NO-sensitive reduced (ferrous) sGC and NO-insensitive oxidized (ferric) sGC respectively\textsuperscript{130}. sGC consists of an \(\alpha/\beta\)-heterodimeric protein with a prosthetic ferrous heme group. The presence of a reduced Fe\textsuperscript{2+} (ferrous) heme group is crucial for NO-sensing and NO-dependent sGC stimulation\textsuperscript{130}. The heme group can exist in different redox states, which may enable sGC to modulate intracellular redox homeostasis in addition to its NO-sensing capability\textsuperscript{130}. Oxidative stress favours heme-free sGC, which is unresponsive to NO. Hence, oxidative stress synergistically hampers NO-cGMP signaling through sGC oxidation and through ROS-mediated scavenging of NO\textsuperscript{38,40} thereby compromising NO-sGC-cGMP mediated signaling\textsuperscript{130-132}. The sGC stimulators have a dual mode of action; they sensitize sGC to low levels of NO and can stimulate sGC directly in the absence of any endogenous NO. Conversely, sGC activators specifically activate the NO-unresponsive, heme-free form of the enzyme irrespective of NO bioavailability\textsuperscript{130-133}.

In a nonrandomized proof of concept study, the direct NO- and heme-independent sGC activator cinaciguat was shown to acutely reduce pulmonary capillary and artery pressures, lower pulmonary and systemic vascular resistance, while improving cardiac output in patients admitted for acute decompensated HF\textsuperscript{134}. A number of phase IIb studies (COMPOSE program) evaluated the safety and efficacy of fixed low doses of intravenous cinaciguat as add-on therapy in patients hospitalized for acute HF and demonstrated cinaciguat to be associated with an excess of non-fatal hypotensive episodes without improvements in dyspnea.
or cardiac index. Recently, the sustained sGC stimulator (BAY 41-8543) was found to offset NOS inhibition and to restore acute cardiac modulation by sildenafil in adult mice, which suggests that direct sGC stimulators could be used to enhance the action of PDE5A inhibitors in settings where critical sGC-generated cGMP is inadequate.

Stimulation of cGMP-PKG signaling can also be achieved through NP-mediated activation of pGC. The synthetic NP nesiritide was shown to acutely reduce pulmonary capillary wedge pressure and systemic vascular resistance, while increasing cardiac index and stroke volume index in HFrEF patients. Acute NP administration was recently reported to lower diastolic LV stiffness and to increase myocardial titin phosphorylation in an old hypertensive HFpEF dog model but failed to improve clinical endpoints in a large, randomized clinical trial of acutely decompensated HF patients with either LVEF <40% and LVEF ≥40%.

Conversely, the ubiquitous enzyme dipeptidyl peptidase IV (DPP IV) cleaves BNP1-32 into BNP3-32, which has reduced biological activity compared to BNP1-32 and was found increased in patients with chronic HF. Interestingly, sitagliptin, a specific DPP IV inhibitor, preserved renal function, modulated stroke volume and heart rate and potentiated the positive inotropic effect of exogenous BNP in a pacing-induced pig HF model.

Modulation of myocardial remodeling and nitrosative/oxidative stress
Prominent features of HFpEF, DM, IR and obesity are cardiac hypertrophy and fibrosis. Hypertrophic stimuli, such as mechanical stretch, neurohumoral activation and inflammatory markers activate various downstream pro-hypertrophic signaling pathways, whereas NO-PKG mediated signaling exerts anti-hypertrophic and anti-fibrotic effects. Furthermore, abundant evidence demonstrates that ROS potently activate pro-hypertrophic and pro-fibrotic pathways, and by interfering with both NO-sGC-cGMP-PKG signalling and NO-mediated S-nitrosylation, ROS inhibit anti-hypertrophic and anti-fibrotic signalling cascades. Hence, in HFpEF, the combination of low myocardial PKG activity and nitrosative/oxidative stress could accelerate myocardial maladaptive remodeling, because of unopposed activation of pro-hypertrophic and pro-fibrotic signaling.

Angiotensin converting enzyme inhibitors and angiotensin receptor blockers
Angiotensin converting enzyme inhibitors (ACE-I) and angiotensin receptor blockers (ARBs) prevent new onset DMII, ameliorate IR and inhibit formation of AGEs. Moreover, ACE-I and ARBs improve maladaptive remodeling in HFrEF, reduce myocardial fibrosis and LV mass in patients with hypertensive heart disease, inhibit inflammation and NADPH oxidase-mediated ROS production and stimulate NO production.
Although ACE-I and ARBs improved diastolic LV dysfunction in hypertensive heart disease\textsuperscript{157-159}, their importance for HFpEF treatment has been seriously challenged, because of the neutral outcomes of large HFpEF trials investigating ACE-I\textsuperscript{160} and ARBs\textsuperscript{161,162}. A recent large meta-analysis demonstrated divergent stimulatory effects of ACE-I on endothelial function\textsuperscript{163}, with failure of ACE-I to improve brachial flow mediated dilation in diabetic\textsuperscript{164} and obese patients\textsuperscript{165}. Furthermore, in the ALLHAT study (Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial), lisinopril was inferior to chlorthalidone in preventing new-onset HFpEF\textsuperscript{166}, while enalapril failed to improve exercise capacity or aortic distensibility in HFpEF patients\textsuperscript{167}. The reason why ACE-I and ARBs fail to improve outcome in HFpEF patients, despite their ability to improve endothelial function remains elusive. However, other mechanisms than RAAS activation can impair NO bioavailability and PKG signaling in the setting of metabolic risk factors. Insulin resistance, FFA and adipokines inhibit insulin-dependent PI3K/Akt signaling, resulting in reduced eNOS activity and impaired NO generation\textsuperscript{113}. In addition, hyperglycemia and adipokines can hamper eNOS activity through various mechanisms, including increased flux through the hexosamine biosynthesis pathway\textsuperscript{113}. Oxidative stress in DMII, obesity and IR can be generated from other sources than RAAS-associated ROS-producing enzymes, including mitochondrial uncoupling, activation of the polyol pathway, lipotoxicity, glucose auto-oxidation and formation of AGEs\textsuperscript{113,168}. Furthermore, DM, obesity and IR induce activation of multiple PKC isoforms, with PKCs being prominently involved in numerous cellular functions and signal transduction pathways\textsuperscript{169}. PKC can hamper myocardial eNOS activity and NO generation by inhibition of PI3K/Akt signaling and stimulation of NADPH oxidase activity\textsuperscript{113,169}, whereas PKC\textsubscript{α} was recently shown to increase titin-based cardiomyocyte stiffness through phosphorylation of the PEVK segment\textsuperscript{25}. Interestingly, in a rodent diabetic HFpEF model, PKC\textsubscript{β} inhibition improved diastolic distensibility, contractility and maladaptive cardiac remodeling\textsuperscript{170}. Thus, in HFpEF, increased prevalence of (frequently co-existing) metabolic risk factors will exert complex and profound adverse effects on endothelial function and NO bioavailability, which are mediated by synergistic contributions of inflammation, oxidative stress, metabolic disturbances and deranged intracellular endothelial and cardiomyocyte signaling and which could explain why isolated treatment of ACE-I and ARBs resulted in neutral clinical outcome in the large HFpEF trials.

\textit{Aldosterone antagonists}

Aldosterone plays an important role in the pathogenesis of vascular stiffening and endothelial dysfunction, and increased plasma aldosterone levels are associated with inflammation,
oxidative stress, IR and the metabolic syndrome\textsuperscript{171-173}. In addition, elevated aldosterone levels are also related to cardiac hypertrophy, fibrosis and diastolic LV dysfunction\textsuperscript{171-173}. In HFpEF clinical trials, aldosterone antagonists improved exercise capacity and diastolic LV stiffness\textsuperscript{174}, as well as LV long axis function and cardiac geometry\textsuperscript{175}. In a recent multicenter, prospective, randomized, double-blind, placebo controlled trial, spironolactone improved diastolic LV stiffness, but failed to improve exercise capacity\textsuperscript{176}.

**AGE breaking compounds**

In small scale clinical HFpEF\textsuperscript{177} and HFrEF\textsuperscript{178} trials, the AGE breaking compound alagebrium was shown to improve diastolic function and LV remodeling, whereas it enhanced arterial compliance in aged hypertensives with vascular stiffening\textsuperscript{179}. In contrast, in a recent randomized study, alagebrium did not improve exercise tolerance, nor systolic or diastolic function in HFrEF patients\textsuperscript{180}.

**Statins**

Statins have been shown to improve endothelial function by stimulating activity and expression of eNOS and by enhancing NO production through both cholesterol-dependent and cholesterol-independent mechanisms\textsuperscript{181-183}. The “pleiotropic effects” of statins also include suppression of inflammation and oxidative stress, inhibition of neurohormonal activation, inhibition of small GTP-binding protein (Rho, Rac and Ras) signaling and attenuation of cardiac hypertrophy and fibrosis\textsuperscript{181-183}. In a prospective, observational study, statin treatment substantially improved survival in HFpEF, in contrast to ACE-I, ARBs, beta blockers or calcium channel blockers which had no effect on survival\textsuperscript{184}. More recently, two additional prospective clinical studies demonstrated statins to be associated with improved survival in HFpEF patients followed over a 5 year period\textsuperscript{185,186}.

**Life style changes and exercise training**

Standard lifestyle recommendations (exercise, smoking cessation, weight loss) improve insulin sensitivity and protect against DM development in obese, non-diabetic individuals\textsuperscript{108,187}. Obesity induces cardiac structural and functional changes characterized by concentric LV remodeling\textsuperscript{188}, altered myocardial substrate metabolism\textsuperscript{189,190} and diastolic LV dysfunction, which is independently related to the extent of visceral adiposity\textsuperscript{191}. Conversely, weight loss in obesity ameliorates cardiac hypertrophy and diastolic dysfunction and improves myocardial bioenergetics and vascular stiffness independent of changes in blood pressure and other obesity-related co-morbidities\textsuperscript{192-199}. 
Physical exercise was shown to improve both clinical outcome and insulin sensitivity in non-ischemic HFrEF patients\textsuperscript{200}. The ESC HF guidelines firmly recommend regular physical activity and structured exercise training. This recommendation is based on the fact that exercise training improves exercise capacity and quality of life, does not adversely affect LV remodeling and may reduce mortality and hospitalization in patients with mild-to-moderate chronic HFrEF\textsuperscript{123,201-203}. Exercise training could also be beneficial for improving outcome in patients with HFpEF as exercise training was shown to improve endothelial dysfunction, systemic inflammation and metabolic syndrome\textsuperscript{204-206}. Moreover, exercise training was suggested to attenuate the age-dependent decline in diastolic function\textsuperscript{207}. Recently, the randomized Exercise training in Diastolic Heart Failure Pilot Study (Ex-DHF) indeed demonstrated in HFpEF patients that exercise training improved exercise capacity and quality of life, which was associated with reverse atrial remodeling and enhanced diastolic LV function\textsuperscript{208}. Conversely, in a more recent study, HFpEF patients were allocated to a 16 weeks exercise training program and although exercise training improved peak VO\textsubscript{2} max, no significant changes were observed in systolic and diastolic function\textsuperscript{209}.

**Improved metabolic control**

Under physiological conditions, myocardial energy production is derived from a balanced metabolism of glucose utilization and FFAs. Due to a decrease in glucose transport through the sarcolemmal membrane of cardiomyocytes in diabetic patients with depletion of glucose transporters 1 and 4, energy production is shifted to the beta-oxidation of FFAs\textsuperscript{210,211}. The oxidation of FFAs is less favourable in the energy balance due to the higher oxygen demand\textsuperscript{212}, while the increase in cardiac triglyceride accumulation is associated with diastolic dysfunction\textsuperscript{213}. Partial inhibitors of myocardial fatty acid oxidation, such as trimetazidine, improved myocardial ischemia and exercise capacity in diabetic patients\textsuperscript{214}. Trimetazidine, increases myocardial efficiency by enhancing glucose metabolism and decreasing FFA metabolism through inhibiting FFA beta-oxidation\textsuperscript{108,215,216}. Trimetazidine exerts cardioprotective effects by reducing oxidative damage, inhibiting inflammation and apoptosis and improving endothelial function\textsuperscript{217-219}. Indeed, in HFrEF, trimetazidine improved myocardial function, remodeling, bioenergetics and functional class, while trimetazidine decreased HF hospitalization\textsuperscript{215,220}.

Metformin is a widely prescribed drug in DMII that improves insulin sensitivity and clinical outcome in diabetic HF patients\textsuperscript{221}, while metformin also exhibits anti-oxidant effects\textsuperscript{222}. Concerns about metformin being associated with lactic acidosis restricted its use in HF,
although clinical experience suggests that this risk is low\textsuperscript{223}. Indeed, observational data suggest that metformin may actually be beneficial for congestive HF\textsuperscript{224,225} and metformin improved insulin sensitivity and minute ventilation, while it induced weight loss in non-diabetic IR HFrEF patients\textsuperscript{226}. Since metformin is not a hypoglycemic agent, metformin could represent a suitable metabolic therapy for nondiabetic HF patients. Previously, it was shown that each 1\% increase in HbA1c was associated with an 8\% increased risk of HF in diabetic patients even after adjusting for other factors, including CAD\textsuperscript{227}. In a small observational study, insulin-based metabolic control in DMII patients, improved diastolic LV function and myocardial perfusion reserve\textsuperscript{228}. Conversely, a recent large prospective randomized trial demonstrated that long-term intensive insulin-based glycemic control aiming at a HbA1c below 6\% was associated with increased 5-year mortality in DMII patients\textsuperscript{229}. In addition, the DADD (Diabetes mellitus And Diastolic Dysfunction) trial also failed to confirm improvement of diastolic dysfunction with tight insulin-based glycemic control in DMII patients\textsuperscript{230}. A statistical type II error could have contributed to the negative outcome in DADD, because of strict DADD exclusion criteria, which resulted in patients only having subtle evidence of diastolic dysfunction with slow relaxation but normal LV distensibility\textsuperscript{231}. In addition to a type II error, this negative outcome could also suggest that in DMII, myocardial damage not only results from hyperglycemia but also from hyperinsulinemia, inflammation and oxidative stress\textsuperscript{100-102}. Indeed, a recent study observed improvement of diastolic LV function in DMII patients treated with rosiglitazone, to relate more closely to the fall in malondialdehyde, a plasma marker of oxidative stress, than to the fall in HbA1c, insulin or IL6\textsuperscript{232}. Signaling through NO-cGMP-PKG is downregulated in metabolic conditions such as obesity, metabolic syndrome, IR and DM\textsuperscript{109,110}, while in human diabetic cardiomyopathy, sildenafil improved measurements of LV geometry, strain and torsion determined by magnetic resonance imaging\textsuperscript{111}.

**Conclusion**

In HFPpEF, pathophysiological mechanisms, diagnostic and therapeutic strategies remain uncertain. This is reflected in the lack of improvement of prognosis in HFPpEF, over the last decennia despite the steady increase of HFPpEF prevalence. Advances in therapeutic strategies are only to be expected when more comprehensive insight has been gained into underlying pathophysiological mechanisms. Recent studies have contributed to improved understanding
of HFpEF pathophysiology demonstrating the importance of elevated cardiomyocyte stiffness due to reduced myocardial PKG activity and high nitrosative/oxidative stress, which could be related to the particularly high prevalence of metabolic risk factors in HFpEF. Pharmacological agents that enhance myocardial NO-cGMP-PKG signaling and reduce nitrosative/oxidative stress, in conjunction with strategies that improve overall metabolic risk could therefore have therapeutic potential in HFpEF treatment.
References

7  Borlaug BA, Redfield MM. Diastolic and systolic heart failure are distinct phenotypes within the heart failure spectrum. Circulation 2011;123:2006-2014.


Exploiting cGMP-based therapies for the prevention of left ventricular hypertrophy: NO* and beyond. Pharmacol Ther 2009;124:279-300.


Hartog JW, Willemsen S, van Veldhuisen DJ, Posma JL, van Wijk LM, Hummel YM, Hildege HL, Voors AA; BENEFICIAL investigators. Effects of alagebrium, an advanced glycation endproduct...


192 Wong CY, Byrne NM, O'Moore-Sullivan T, Hills AP, Prins JB, Marwick TH. Effect of weight loss due to lifestyle intervention on subclinical cardiovascular dysfunction in obesity (body mass index >30 kg/m2). Am J Cardiol 2006;98:1593-1598.


Summary

This thesis investigates several aspects of structural, functional and biochemical remodeling at the myocardial and cardiomyocyte level in patients with heart failure with preserved ejection fraction (HFrEF) and compared these measurements to measurements obtained in patients with HF with reduced EF (HFrEF) as well as in patients operated for severe aortic stenosis (AS). We used a clinical to basic translational “bed-to-bench” approach, relating clinical, hemodynamic and echocardiographic parameters to structural, functional and biochemical characteristics of the myocardium and cardiomyocytes through procurement of left ventricular (LV) endomyocardial biopsies. In addition, the importance of metabolic risk factors, such as diabetes mellitus type II, for diastolic LV dysfunction is investigated. According to the results of this thesis, raised diastolic LV stiffness in the different patient groups results from distinct pathophysiological mechanisms, which provides evidence that HFrEF and HFrEF actually represent distinct HF phenotypes.

Heart failure with preserved ejection fraction (HFrEF) is widely prevalent and is associated with a cumbersome prognosis, while its pathophysiological mechanisms are incompletely understood and specific therapeutic strategies remain uncertain. It is however still debated whether HFrEF and HFrEF are indeed distinct HF phenotypes despite prominent differences in myocardial remodeling and clinical characteristics. To support the clinical distinction between HFrEF and HFrEF, LV myocardial structure and function were compared in LV endomyocardial biopsy samples of patients with HFrEF and HFrEF, which is described in Chapter 2. Patients hospitalized for worsening HF were classified as having HFrEF (n=22; LVEF 62 ± 2%) or HFrEF (n=22; LVEF 34 ± 2%). None of the patients had significant coronary artery disease and transvascularly procured LV endomyocardial biopsies did not demonstrate evidence of infiltrative or inflammatory myocardial disease. Biopsy samples were analyzed with histomorphometry and electron microscopy. Single, permeabilized cardiomyocytes were isolated from the biopsies, stretched to a sarcomere length of 2.2 μm to measure intrinsic stiffness (passive force (F_{passive})), and activated with calcium (Ca^{2+})-containing solutions to measure total force. HFrEF and HFrEF patients demonstrated important differences in clinical, hemodynamic and echocardiographic characteristics. HFrEF patients were more often hypertensive and obese and tended to be older than HFrEF patients. LV peak systolic pressure, EF, LV wall thickness and myocardial stiffness were higher in HFrEF. Furthermore, HFrEF patients demonstrated concentric remodeling with increased LV mass index/LV end-diastolic volume index ratio (LVMI/LVEDVI), whereas HFrEF patients
had eccentric remodeling with lowered LVMI/LVEDVI ratio. HFpEF and HFrEF patients also demonstrated important differences in myocellular structural and functional characteristics. In HFpEF, cardiomyocyte diameter, myofibrillar density, myofilament Ca$^{2+}$ sensitivity and cardiomyocyte $F_{\text{passive}}$ were higher than in HFrEF, while myocardial collagen volume fraction (CVF) and total cardiomyocyte force were comparable. Following in-vitro administration of protein kinase A (PKA), cardiomyocyte $F_{\text{passive}}$ was reduced in both HF groups, but the drop in cardiomyocyte $F_{\text{passive}}$ was significantly larger in HFpEF, suggesting a larger myofilament phosphorylation deficit in HFpEF. The giant sarcomeric protein titin importantly determines cardiomyocyte $F_{\text{passive}}$ through shifts in expression of its stiff N2B and compliant N2BA isoforms and through shifts in isoform phosphorylation status. In HFpEF, the titin N2BA/N2B isoform expression ratio was lower than in HFrEF. We concluded that important differences exist in both macroscopic and microscopic myocardial structural and functional properties in LV myocardium of HFpEF and HFrEF patients, which suggests HFpEF and HFrEF to be associated with phenotypically distinct myocardial and cardiomyocyte abnormalities. These differences support the clinical discrimination of HF patients into HFpEF and HFrEF groups.

Chapter 3. Excessive diastolic LV stiffness is an important contributor to HF in patients with diabetes mellitus (DM). Diabetes is presumed to increase stiffness through myocardial deposition of collagen and advanced glycation end products (AGEs). Cardiomyocyte $F_{\text{passive}}$ also elevates stiffness, especially in HFpEF. We assessed the contribution to diastolic stiffness of fibrosis, AGEs and cardiomyocyte $F_{\text{passive}}$ in diabetic HF patients with preserved or reduced LVEF. In this study, LV endomyocardial biopsies were procured in both HFpEF and HFrEF patients without significant coronary disease, who were hospitalized for worsening HF. Biopsy samples were used for quantification of CVF and AGEs and for isolation of cardiomyocytes to measure $F_{\text{passive}}$. Diabetic HF patients had higher diastolic LV stiffness irrespective of LVEF. The underlying mechanisms for increased diastolic LV stiffness in diabetic HFrEF consisted predominantly of increased myocardial CVF and AGEs deposition. In contrast, increased cardiomyocyte $F_{\text{passive}}$ was the most important contributor to raised diastolic LV stiffness in diabetic HFpEF, with similar CVF and only a trend towards increased myocardial AGEs deposition in diabetic HFpEF compared to non-diabetic HFpEF patients. Furthermore, the higher $F_{\text{passive}}$ in diabetic HFpEF than in non-diabetic HFpEF cardiomyocytes was paralleled by widening of the sarcomeric Z-disc, which was significantly larger in diabetic HFpEF than in non-diabetic HFpEF cardiomyocytes. This study was the
first to report Z-disc widening in humans and because of the simultaneous elevation of $F_{\text{passive}}$, it suggests that Z-disc widening results from altered elastic properties of cytoskeletal proteins, which pull at and open up adjacent Z-discs. Raised cardiomyocyte $F_{\text{passive}}$ was acutely lowered with in-vitro administration of PKA with the decrement of $F_{\text{passive}}$ lowering being greatest in diabetic HFpEF cardiomyocytes, suggesting the largest phosphorylation deficit in this condition.

In this study, we concluded that the mechanisms responsible for the increased diastolic stiffness of the diabetic heart differ in HFpEF and HFrEF. In diabetic HFpEF patients, increased cardiomyocyte $F_{\text{passive}}$ more importantly underlies raised diastolic LV stiffness, whereas in diabetic HFrEF patients, elevated fibrosis and myocardial AGEs deposition are more important.

**Chapter 4.** In this editorial comment, the implications of transcriptional and posttranslational modifications of the giant sarcomeric protein titin for diastolic function are discussed. Titin importantly determines myocardial stiffness through modulation of cardiomyocyte $F_{\text{passive}}$. Titin spans a half sarcomere, running from the Z-disc to the M-band and functions as a bidirectional spring responsible for early diastolic recoil and late diastolic resistance to stretch. Cardiomyocyte titin-based elasticity can be adjusted through transcriptional and posttranslational modifications. At the transcriptional level, titin modulates stiffness through shifts in expression of its compliant N2BA (3.2-3.7 MDa) and stiff N2B (3.0MDa) isoforms, which are co-expressed in the sarcomere. Titin-based stiffness is posttranslationally modified by PKA and protein kinase G (PKG) mediated phosphorylation of the stiff N2B segment of titin, which acutely lowers cardiomyocyte $F_{\text{passive}}$ in skinned human LV muscle strips, in isolated human LV myofibrils and in isolated cardiomyocytes from HFpEF and HFrEF patients. This acute adjustment of titin-based stiffness by PKA and PKG mediated phosphorylation provides the cardiomyocyte with an “adaptive suspension”, regulating short term modulation of myocardial stiffness, while extracellular matrix changes provide long term modulation of myocardial stiffness.

**Chapter 5.** Transcriptional and posttranslational changes in the giant sarcomeric protein titin underlie increased cardiomyocyte $F_{\text{passive}}$, which importantly contributes to high diastolic stiffness of failing myocardium. Shifts in titin isoform expression ratio and isoform phosphorylation can alter cardiomyocyte $F_{\text{passive}}$. In LV biopsies of HF patients, AS patients and controls, we therefore related $F_{\text{passive}}$ of isolated cardiomyocytes to expression of titin
isoforms and to phosphorylation of titin and titin isoforms. Biopsies were procured by transvascular technique in HF patients and perioperatively in AS patients or from explanted hearts. All patients were free of significant coronary disease. Isolated, permeabilized cardiomyocytes were stretched to 2.2 μm sarcomere length to measure $F_{\text{passive}}$. Expression and phosphorylation of titin isoforms were analyzed using gel electrophoresis with ProQ Diamond and SYPRO Ruby stains. Cardiomyocyte $F_{\text{passive}}$ was higher in HF than in AS or controls, while in-vitro administration of PKA acutely lowered $F_{\text{passive}}$ only in HF cardiomyocytes. High $F_{\text{passive}}$ of HF cardiomyocytes also fell on administration of PKG and remained unaltered on subsequent PKA treatment. The titin N2BA/N2B isoform expression ratio was higher in HF than in controls and comparable in HF and AS patients, while a significant relation was observed between N2BA/N2B ratio and LV end-diastolic wall stress. Overall titin phosphorylation was comparable in HF and AS, but relative phosphorylation of the stiff N2B titin isoform was significantly lower in HF than in AS. Because of comparable expression of titin isoforms and similar overall phosphorylation of titin, the higher $F_{\text{passive}}$ of HF cardiomyocytes compared to AS cardiomyocytes was attributed to a shift in titin isoform phosphorylation with relative hypophosphorylation of the stiff N2B titin isoform. Contribution to $F_{\text{passive}}$ of the thin filament and crossbridge interaction were ruled out because of unaltered $F_{\text{passive}}$ after exposure to gelsolin (which removes the thin filament) and 2,3-butanedione monoxine (which prevents cross bridge interactions). Cardiomyocyte $F_{\text{passive}}$, PKA-induced decrease in $F_{\text{passive}}$ and N2B phosphorylation deficit rose from AS to HFrEF to HFpEF. A larger N2B titin isoform phosphorylation deficit in HFpEF explains both their higher $F_{\text{passive}}$ and larger PKA-induced fall of $F_{\text{passive}}$. We concluded that relative hypophosphorylation of the stiff N2B titin isoform represents a novel mechanism responsible for raised $F_{\text{passive}}$ of human HF cardiomyocytes. In HF, phosphorylation of the stiff N2B titin isoform acutely lowers cardiomyocyte $F_{\text{passive}}$, which could therefore have important therapeutic implications, as stimulation of titin phosphorylation may represent a means to relax the stiffened heart.

Chapter 6. In this review pathophysiological mechanisms for increased diastolic LV stiffness in diabetic heart failure are discussed. Increased diastolic LV stiffness is recognized as the earliest manifestation of DM-induced LV dysfunction. Mechanisms responsible for increased diastolic LV stiffness in diabetic HF include myocardial fibrosis, AGEs, metabolic disturbances, impaired Ca$^{2+}$ homeostasis, endothelial dysfunction, myocardial oxidative stress, impaired nitric oxide (NO) bioavailability, downregulated myocardial cyclic guanosine
monophosphate (cGMP) signaling and altered titin regulation. Furthermore, mechanisms responsible for the elevated diastolic LV stiffness differ in diabetic HFpEF and diabetic HFrEF patients. Myocardial fibrosis and deposition of AGEs are important determinants of increased diastolic LV stiffness in diabetic HFrEF, whereas increased \( F_{\text{passive}} \) of hypertrophied cardiomyocytes is the main determinant of increased diastolic LV stiffness in diabetic HFpEF. Downregulation of NO-mediated cGMP-PKG signaling, probably induced by vascular inflammation and oxidative stress, could account for the high \( F_{\text{passive}} \) of hypertrophied cardiomyocytes observed in diabetic HFpEF patients.

**Chapter 7.** Diabetes mellitus (DM)-induced diastolic LV dysfunction is recognized as an important determinant of morbidity and mortality in HF. In this chapter, the effects of DM on diastolic LV function and its underlying mechanisms were identified in patients with severe AS with or without DM who were referred for aortic valve replacement in the absence of significant coronary artery disease. Preoperative Doppler echocardiography and hemodynamics were implemented with perioperative LV biopsies. Myocardial CVF and AGEs deposition were quantified by histomorphometry and immunohistochemistry, whereas \( F_{\text{passive}} \) was determined in isolated, permeabilized cardiomyocytes stretched to a sarcomere length of 2.2 \( \mu \)m. Expression and phosphorylation of titin isoforms were analyzed with gel electrophoresis with ProQ Diamond and SYPRO Ruby stains. Diabetic AS patients had reduced LV end-diastolic distensibility as evident from higher LV end-diastolic pressure at comparable LV end-diastolic volume index and attributed to higher myocardial CVF, more AGEs deposition and higher cardiomyocyte \( F_{\text{passive}} \). The higher cardiomyocyte \( F_{\text{passive}} \) in diabetic AS patients was related to significant hypophosphorylation of the stiff N2B titin isoform, while in-vitro administration of PKA normalized cardiomyocyte \( F_{\text{passive}} \). We concluded that an additional impairment of LV end-diastolic distensibility is observed when severe, symptomatic AS was associated with DM. This additional impairment related to both structural and functional alterations of LV myocardium consisting of increased interstitial collagen deposition, augmented intramyocardial AGE accumulation and higher intrinsic cardiomyocyte stiffness. The contribution of increased cardiomyocyte \( F_{\text{passive}} \) to raised diastolic LV stiffness could be amenable to novel therapeutic strategies modulating posttranslational titin-based stiffness through stimulation of titin phosphorylation.

**Chapter 8.** Prominent features of myocardial remodeling in HFpEF are high cardiomyocyte \( F_{\text{passive}} \) and cardiomyocyte hypertrophy. In experimental models, both reacted favourably to
raised PKG activity. In this chapter, we assessed myocardial PKG activity, its downstream effects on cardiomyocyte \( F_{\text{passive}} \) and cardiomyocyte diameter, and its upstream control by cGMP, nitrosative/oxidative stress, and brain natriuretic peptide (BNP). To discern altered control of myocardial remodeling by PKG, HFpEF was compared with HFrEF and AS. All patients were free of significant coronary disease. More HFpEF patients were obese or had DM. LV myocardial biopsies were procured transvasularly in HFpEF and HFrEF and perioperatively in AS. \( F_{\text{passive}} \) was measured in isolated, permeabilized cardiomyocytes before and after PKG administration. Myocardial homogenates were used for assessment of PKG activity, cGMP concentration and expression of proBNP-108 and nitrotyrosine. Nitrotyrosine represents an indirect measure of nitrosative/oxidative stress. Additional quantitative immunohistochemical analysis was performed for PKG activity, nitrotyrosine expression and expression of soluble guanylate cyclase (sGC) and phosphodiesterase type 5A (PDE5A), which breaks down cGMP, thereby terminating cGMP-PKG signaling. Our study showed that relative to both AS and HFrEF, HFpEF patients had reduced myocardial PKG activity and lower cGMP concentration, which related to higher cardiomyocyte \( F_{\text{passive}} \) and increased myocardial nitrotyrosine levels, indicative of raised nitrosative/oxidative stress. In all groups, cardiomyocyte \( F_{\text{passive}} \) was acutely lowered by in vitro administration of PKG, whereas cardiomyocytes from HFpEF patients demonstrated the largest PKG-induced fall in \( F_{\text{passive}} \). Reduced PKG activity and lower myocardial cGMP concentration in HFpEF did not result from altered myocardial sGC or PDE5A expression, which were similar in all groups, or from unequal natriuretic peptide (NP) expression, which was comparable in HFpEF and AS. Downregulation of cGMP-PKG signaling in HFpEF was therefore most likely related to low myocardial NO bioavailability because of high nitrosative/oxidative stress, which was almost fourfold higher in HFpEF than in both HFrEF and AS. Oxidative stress is indeed known to lower NO bioavailability and downregulate cGMP-PKG signaling. Increased nitrosative/oxidative stress in HFpEF could have resulted from the high prevalence in HFpEF of metabolic comorbidities. We concluded that HFpEF myocardium is characterized by downregulated NO-cGMP-PKG signaling probably resulting from high nitrosative/oxidative stress. Low PKG activity raises cardiomyocyte intrinsic stiffness and could be a potential target for a specific HFpEF treatment strategy.

**Chapter 9.** In this review underlying molecular and cellular mechanisms for diastolic LV dysfunction are discussed. HFpEF is widely prevalent, associated with a dismal prognosis and incompletely understood pathophysiology, which precludes specific therapy. Prominent
features of HFpEF are impaired relaxation, increased diastolic LV stiffness and distinct cardiac remodeling processes at the macroscopic, microscopic and ultrastructural level. In HFpEF, increased diastolic LV stiffness can be explained by maladaptive changes in the extracellular matrix and by elevated cardiomyocyte stiffness, which seem to be related to myocardial inflammation, nitrosative/oxidative stress, endothelial dysfunction and downregulation of NO-cGMP-PKG signaling. NO is crucially important for diastolic function, since it potently enhances LV relaxation and distensibility through a variety of mechanisms including cGMP-PKG dependent and independent mechanisms and modulation of β-adrenergic responsiveness, S-nitrosylation signaling and myocardial bioenergetics.

Patients with HFpEF demonstrate a high prevalence of metabolic disorders, which may contribute to enhanced myocardial inflammation, nitrosative/oxidative stress, downregulation of NO bioavailability and NO-mediated cGMP-PKG signaling, thereby triggering diastolic LV dysfunction. We concluded that targeting metabolic risk and improving endothelial function, NO bioactivity and cGMP-PKG signaling can be promising strategies for specific HFpEF treatment.

**Future perspectives**

Heart failure with preserved ejection fraction (HFpEF) demonstrates an increasing prevalence, and currently approximately 50% of HF patients present with this type of HF. Although clinical outcome is slightly better for HFpEF than for HFrEF, mortality associated with HFpEF is considerable and most patients with HFpEF die from cardiovascular causes. Unfortunately, in HFpEF, pathophysiological mechanisms, diagnostic and therapeutic strategies remain uncertain and this is reflected in the lack of improvement of prognosis in HFpEF over the last decennia. Advances in therapeutic strategies are only to be expected when more comprehensive insight has been gained into underlying pathophysiological mechanisms responsible for development and progression of HFpEF. HFpEF appears a complex syndrome characterized by maladaptive changes in myocardial and cardiomyocyte structural, functional and biochemical characteristics, probably induced in a multifactorial fashion by a combination of adverse effects of myocardial inflammation, nitrosative/oxidative stress, endothelial dysfunction, downregulation of NO bioavailability and NO-mediated signaling, impaired myocardial bioenergetics, disturbed calcium handling and concentric hypertrophy. Our recent translational studies have contributed to improved understanding of HFpEF pathophysiology and exciting insights with potential direct applications for
therapeutic strategies have emerged. For instance, pharmacological agents that enhance myocardial NO-cGMP-PKG signaling and reduce nitrosative/oxidative stress, in conjunction with strategies that improve overall metabolic risk could have therapeutic potential in HFpEF treatment. Nevertheless, because of the high complexity of HFpEF pathophysiology, continuing research efforts, based on an integrative translational approach are undisputably valuable to keep on moving this exciting field forward. This importance of integrative physiology to gain insight into clinical pathophysiology in order to improve HFpEF outcome has recently been acknowledged and awarded a significant grant from the European Committee, to launch a large collaborative project, including 20 centers across Europe, coordinated by Prof Dr. W.J. Paulus. This large scale integrating project includes both experimental models of diastolic dysfunction as well as HFpEF patients and focuses on pathophysiological mechanisms, improved diagnostic modalities and potential specific HFpEF therapeutic strategies.