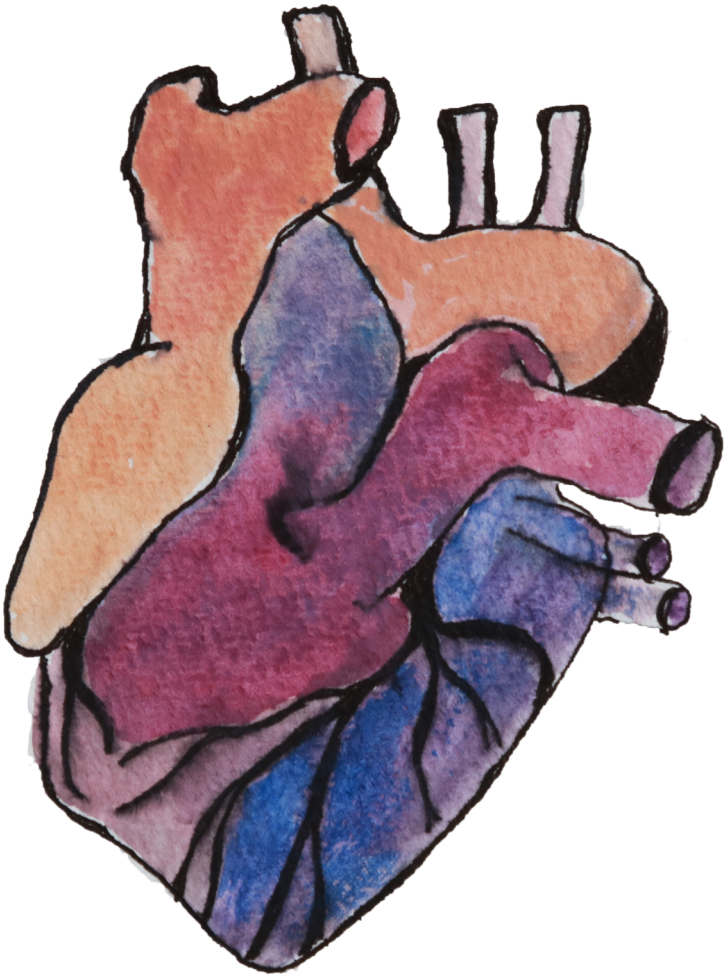
Distinctive Alterations in Microvascular Function Due to Multiple Common Morbidities



Jens van de Wouw

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Distinctive Alterations in Microvascular Function Due to Multiple Common Morbidities

Karakteristieke veranderingen in microvasculaire functie door meerdere veelvoorkomende morbiditeiten

Thesis

to obtain the degree of Doctor from the
Erasmus University Rotterdam
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and in accordance with the decision of the Doctorate Board.
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by

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born in ‘s-Hertogenbosch.

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Chapter 1

General introduction, aim and outline of this thesis

1

General Introduction, Aim and Outline of This Thesis

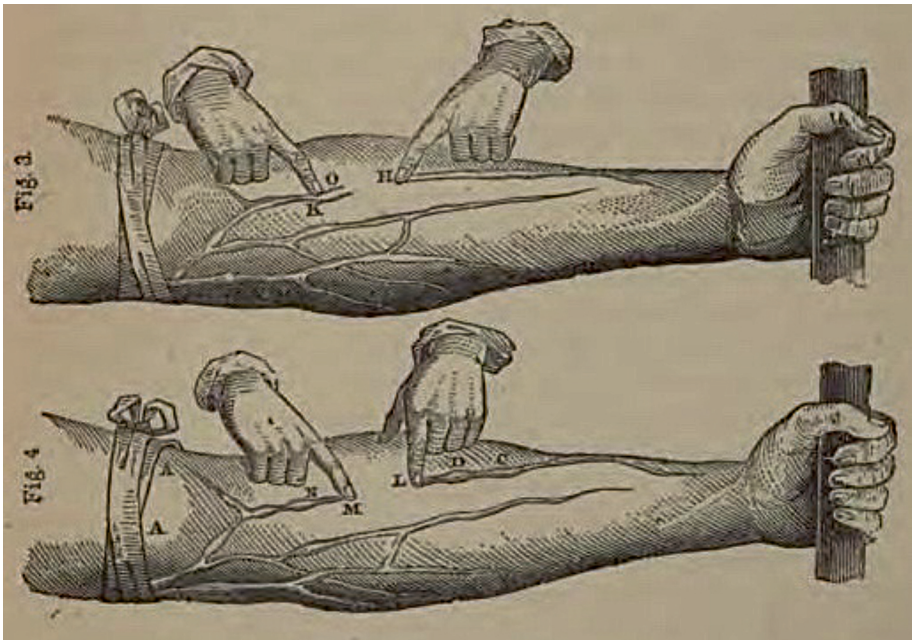
'There is no science which does not spring from pre-existing knowledge' - W. Harvey

History of The Anatomy and Physiology of the Cardiovascular System

Physiology, coming from the Ancient Greek word *physis* 'nature' or 'origin' and *logica* 'study of', focuses on the normal function of organisms, organs and cells. Therefore, it laid the foundation of the study of the development of disease (pathophysiology, coming from the Ancient Greek word, *pathos* 'suffering' and physiology) and served as a cornerstone for the modern medical sciences. In the beginning of modern cardiovascular physiology there was no differentiation between anatomy and function.[1] Thus the ancient scientist formulated hypotheses about the function of the human body based on empirical observations which fitted within the beliefs of that age and location. Hippocrates (around 400 B.C.) was one of the first to apply logical reasoning to medicine and stated that disease is caused by imbalance of the four humors (blood, water, black and yellow bile). In other words a disruption of the normal physiology results in disease.[2] Aristoteles believed that the heart was the centre of the physiological mechanisms and the origin of all blood vessels. Interestingly, only about 50 years later, Praxagoras was the first to differentiate between arteries and veins, but stated that arteries were filled with air instead of blood. Galen (born 129 A.D.) continued on these hypotheses and formulated that both the arteries and the veins are filled with blood, but the blood streams through openings in the ventricular septum. Blood in the left ventricle is oxygenated by air coming from the lungs directly and the systemic circulation was still considered an open system.[2]

During the European golden ages of anatomy in the 15th century, multiple anatomists—such as Da Vinci, Vesalius, Servetus, Columbus and Caesalpinus—described important anatomical features of the heart, lungs and circulation and how they influence cardiovascular function.[3] Their findings helped William Harvey to write 'Exercitatio Anatomica de Motu Cordis et Sanguinis in Animalibus' (Latin for "An Anatomical Exercise on the Motion of the Heart and Blood in Living Beings") which was published in 1628.[4] His description of a closed system with two separate—pulmonary and systemic—circulations was the synopsis of multiple observations done by his predecessors but was truthfully combined first by Harvey. Especially his experiments (**Figure 1**) and hypothesis about the movement of blood were of utmost importance for modern cardiovascular physiology.[2] Furthermore, the change in role of the heart, from a mythical and spiritual organ to a blunt pump, was the missing link in combining the existence of arteries, veins and a closed circulation. Unfortunately, there was one missing facet which would complete the closed circulation hypothesis by Harvey, namely the connection between the arteries and veins. This connection was not observable yet, as it was not

Figure 1 Classical experiments performed by Harvey



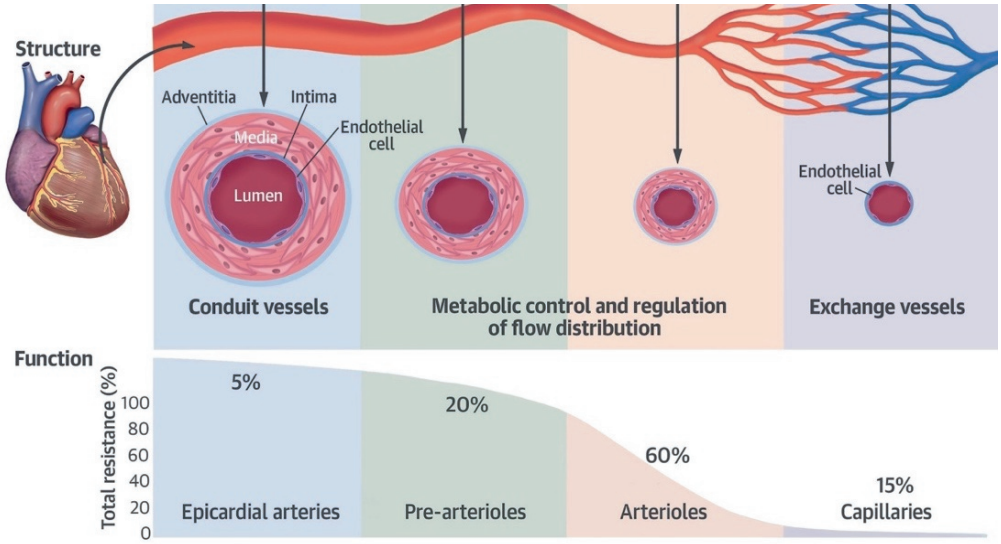
Classical experiments from 'An Anatomical Exercise on the Motion of the Heart Blood in Living Beings' performed by Harvey proving the arterial and venous system and the direction of blood flow.

possible at that time to observe vessels smaller than those visible to the eye. It was not until 1660, after Harvey's death, that Marcello Malpighi observed blood flow through the capillaries (of a frog's lung) with an early microscope and thus discovered the missing link which confirmed the closed circulation hypothesis. Since this important discovery, a large portion of evidence has been acquired about the microcirculation and its function.

The Coronary Microcirculation

The human circulation exists of vessels of different sizes, ranging from ~30mm (aorta) to <10µm (capillaries) inner luminal diameter, with an arterial and a venous system connected by capillaries. All vessels in the human body are lined inside by a layer of endothelial cells which form the barrier between the circulation and the surrounding tissue. Vascular smooth muscle cells (VSMC) and fibrotic tissue surround the endothelial cells depending on the size and location of the vessel (**Figure 2**). Whereas the large conduit arteries are responsible for blood transportation, the smaller arteries (~100-400µm), arterioles (~10-100µm) and capillaries (<10µm), forming the microcirculation, are of uttermost importance in maintaining blood pressure, regulating blood flow, tissue perfusion and maintaining tissue homeostasis.[5, 6] In the majority of humans, the arterial system of the heart, called

Figure 2 Normal structure and function of coronary macro- and microcirculation



Normal structure and function of coronary macro- and microcirculation. Most of the coronary resistance comes from the coronary microcirculation, regulating myocardial perfusion. Adapted from Taqueti and Di Carli.[9]

the coronary circulation is composed of the 3 main coronary arteries (conduit vessels) which originate from the aorta—the right coronary artery, left circumflex and left anterior descending coronary arteries. The human coronary circulation is right dominant, indicating that the right coronary artery is responsible for supplying not only the right side of the heart but also the posterior wall of the left ventricle, whereas the left anterior descending coronary artery supplies the anterolateral wall of the left ventricle, apex as well as the interventricular septum, and finally the left circumflex is responsible for supplying the posterolateral walls of the left ventricle. As the arteries branch off they form the small arteries, arterioles and eventually capillaries responsible for myocardial perfusion. This thesis will focus on the (dys)function of this last part of the circulation; the coronary microcirculation.

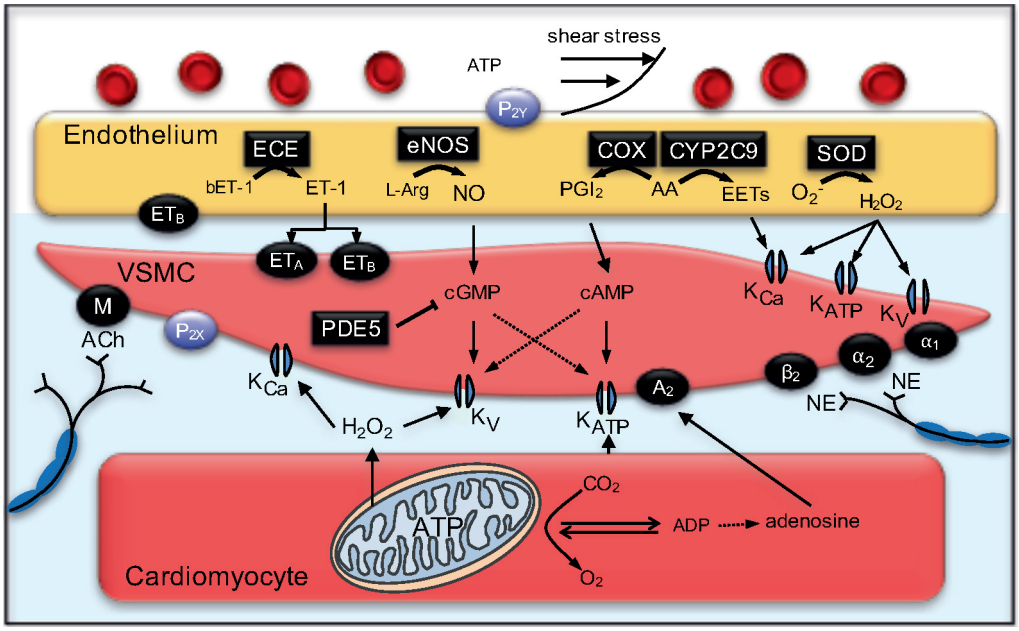
The microcirculation of the heart faces a unique challenge in that it is compressed during every heart contraction (or systole), thereby limiting blood inflow. Hence, the heart is mainly perfused during the relaxation of the cardiac muscle, the so-called diastole. [7, 8] In addition, the microcirculation of the heart is particularly crucial as the perfusion of the myocardium needs to be tightly regulated for multiple reasons. Firstly, the normal heart continuously beats about 60-70 times per minute and is therefore in constant need of supply of oxygen and nutrients as the cardiac reserves last for just about 3 heart beats.[7] Secondly, Secondly, with the heart utilizing ~70% of the oxygen supplied through

the coronary vasculature at rest, the perfusion of the heart needs to quickly adapt to increased metabolic demand as myocardial oxygen consumption can rapidly increase up to six times during exercise.[7, 8] As myocardial oxygen extraction is already high during resting conditions (60-70%) there is not much room to increase extraction during increased metabolic demand, therefore it must be met by an commensurate increase in coronary blood flow which can be increased by about 5 times in healthy humans.[7] A reduction in coronary vascular resistance is required to increase myocardial blood flow and most of the vascular resistance resides in the coronary microcirculation and thus can regulate myocardial perfusion (**Figure 2**).[9]

The regulation of coronary microvascular tone is dependent on the balance between constrictor and vasodilator influences. The coronary microvasculature is especially sensitive to vasodilator influences as it has a relative high resting tone.[10] Vessels of different sizes act in concert to adequately respond to increases in demand. Thus, the coronary smallest arterioles are mostly sensitive to metabolic and larger arterioles to myogenic factors, while upstream small arteries are flow sensitive and dilate in response to increases in flow. **Figure 3** gives an overview of the most important factors involved in the regulation of the coronary microvascular tone. Both endothelium-dependent and independent factors can induce vasodilation as well as vasoconstriction, mediated by relaxation and constriction of the VSMC.[11] Such factors comprise of neurohumoral, metabolic and endothelial factors. Important neurohumoral factors include the sympathetic and parasympathetic nervous system-derived molecules which also play an important role in regulation of cardiac function during exercise, complicating the investigation of the direct effect of the nervous system on coronary microvascular vasomotor control.[7] However, many of the factors involved in the regulation of the coronary microvascular tone are produced locally by endothelial cells and surrounding cardiomyocytes. Endothelium-derived factors include vasodilators such as nitric oxide (NO), prostaglandins, and endothelium-derived hyperpolarizing factors (EDHF) which counterbalance potent vasoconstrictors such as endothelin-1 (ET-1).[10]

NO is produced by nitric oxide synthase of which the endothelial isoform (eNOS) is most abundant in the vasculature and thus most important in the regulation of vascular tone. It is not only produced by biochemical stimulation with several agonists, but also in response to increased shear stress. Endothelium-produced NO is released luminally, where it inhibits platelet aggregation, and abluminally where it binds to its receptor soluble guanylyl cyclase in VSMC, which increases cGMP levels and thus activates protein kinase K, resulting in VSMC relaxation. Interestingly, NO as a signalling molecule in vascular biology has been discovered only about 30 years ago.[12] Since then studies have been conducted to investigate the role of NO in vascular biology and have found that the role of NO is not limited to vasodilation but NO also has anti-inflammatory, anti-oxidative and anti-proliferative

Figure 3 Overview of the main mechanisms involved in coronary microvascular vasomotor control



Schematic drawing of endothelium, vascular smooth muscle cell (VSMC) and cardiomyocyte illustrating mechanisms for control of vasomotor tone and diameter. Abbreviations: ATP adenosine triphosphate, P_{2y} purinergic receptor type 2y, SOD superoxide dismutase, O_2^- superoxide anion, H_2O_2 hydrogen peroxide, eNOS endothelial nitric oxide synthase, L-arg L-arginine, NO nitric oxide, COX cyclooxygenase, PGI_2 prostacyclin, AA arachidonic acid, CYP2C9 cytochrome P450 2C9, EETs epoxyeicosatrienoic acids, ECE endothelin-converting enzyme, bET-1 big endothelin-1, ET-1 endothelin-1, ET_A endothelin type A receptor, ET_B endothelin type B receptor, M muscarinic receptor, ACh acetylcholine, PDE5 phosphodiesterase, K_{Ca} calcium-activated K^+ channel; 5, K_V voltage-gated K^+ channel, K_{ATP} ATP-sensitive K^+ channel, A_2 adenosine receptor 2, β_2 β_2-adrenergic receptor, NE norepinephrine, α_1 α_1-adrenergic receptor, α_2 α_2-adrenergic receptor, CO_2 carbon dioxide, O_2 oxygen, ADP adenosine diphosphate. Adapted from Sorop *et al.*[10]

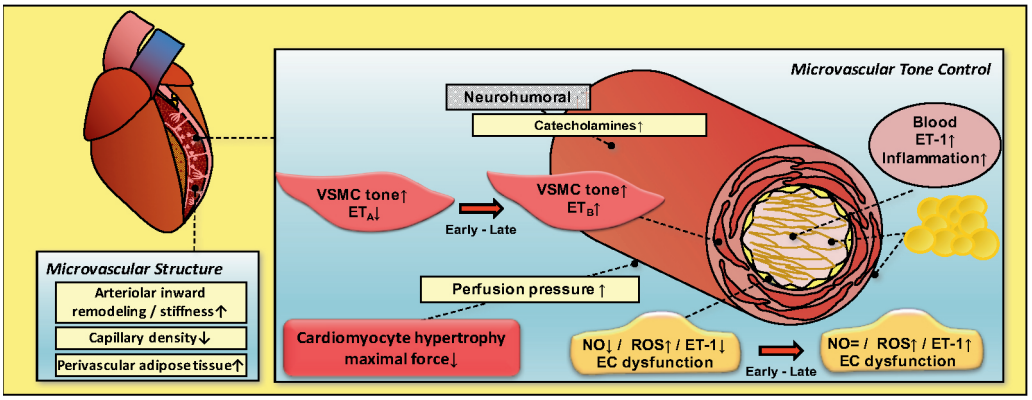
characteristics.[12] NO can act as antioxidant since it can react with reactive oxygen species (ROS), or more specifically superoxide anion (O_2^-). Scavenging of NO is one of the main mechanisms by which increased ROS can decrease vasodilation of the microcirculation.[13, 14] Moreover, ROS can uncouple eNOS and subsequently enhance ROS production by eNOS.[14] Additionally, NO has also paracrine effects on the surrounding tissue (cardiomyocytes and fibroblasts) and has an important role in maintaining tissue homeostasis. For example, NO exerts an inhibitory effect on VSMC proliferation and therefore limits vascular remodelling.[14] Therefore, it is considered as one of the most important active molecules in the (coronary) microcirculation.

EDHFs are responsible for an additional endothelium-dependent vasodilatory mechanism, which results in hyperpolarization of the VSMC and opening of calcium-sensitive potassium channels. Although the exact identity of EDHFs is still under debate, different candidates such as epoxyeicosatrienoic acid and endothelial-produced hydrogen peroxide (H_2O_2) have been proposed as potential EDHFs.[15, 16] Furthermore, the major active metabolite of the arachidonic acid—prostacyclin (PGI_2)—is a potent coronary vasodilator but has limited contribution to vascular tone control in the normal heart.[17] Adenosine—extracellular break down product of ATP released by red blood cells and cardiomyocytes— is a potent vasodilator with a limited role in resting conditions and during exercise, but regarded as a contributor to tone regulation during low oxygen pressure and myocardial ischemia, respectively, when reuptake of ATP/AMP insufficient.[7, 18]

In concert with the above mentioned vasodilators, vasoconstrictors such as ET-1 regulate tissue perfusion by increasing vascular tone in tissues with less metabolic needs and are subsequently responsible for redistribution of blood flow to the hyperaemic tissues (e.g. increased vasoconstriction in splanchnic circulation during exercise).[19] There are two main receptors for ET-1, endothelin receptor A (ET_A) and B (ET_B). Whereas ET_A is only present on VSMC, mediating vasoconstriction, ET_B is present on both VSMC—resulting in vasoconstriction—and endothelial cells, where stimulation results in vasodilation through eNOS activation, with a subsequent increase in NO bioavailability, and through clearance of ET-1.[20]

With this intricate system, which is more comprehensive and complex as discussed above, which is responsible for adequate blood supply in all tissues—and especially in high oxygen demanding tissues such as the heart—dysfunction of the microvasculature can obviously be detrimental to maintaining physiological function. Furthermore, coronary microvascular dysfunction (CMD) cannot only result from functional changes in the microcirculation but also from structural changes (e.g. medial hypertrophy, increased perivascular fibrosis and a loss of arteriolar density). Not only the microvessels are prone to be affected by various diseases, so are larger arteries. Although there are distinctive pathological changes observed within vessels of different sizes and location, e.g. large conduit arteries are most prone to atherosclerosis development, there is a central overlapping role for endothelial dysfunction in vessels of different sizes in most vascular diseases.[11] Especially metabolic derangements—such as obesity and diabetes mellitus—are well established risk factors for the development of coronary microvascular dysfunction. **Figure 4** gives an overview of the pathophysiological mechanisms involved in CMD induced by metabolic derangement.[10] CMD is comprised of alterations of multiple pathways, which can affect endothelial cells and VSMCs both on the functional and structural level. However, functional endothelial dysfunction seems to be most common as it is an important paracrine organ and it acts as a barrier between circulating factors and

Figure 4 Proposed mechanisms of coronary microvascular dysfunction induced by metabolic derangements



Microvascular dysfunction in the presence of metabolic dysregulation. Abbreviations: ET-1 endothelin-1, VSMC vascular smooth muscle cell, ET_A endothelin receptor A, ET_B endothelin receptor B, RAAS renin angiotensin aldosterone system, NO nitric oxide, ROS reactive oxygen species, EC endothelial cell. Adapted from Sorop *et al.*[10]

the surrounding tissue.[21, 22] For example, a systemic pro-inflammatory state, induced by metabolic derangements or chronic kidney disease, can induce endothelial oxidative stress, which in turn can reduce the bioavailability of vasodilators, mainly NO and EDHFs, or increase the bioavailability of vasoconstrictors (ET-1), resulting in higher vasomotor tone, reduced tissue perfusion and vascular remodelling.[21, 22] Eventually, surrounding tissues adapt to these changes in microvascular function, for example CMD can result in cardiomyocyte functional (systolic and diastolic) dysfunction and induce cardiomyocyte hypertrophy due to a loss of NO bioavailability.[22, 23]

Coronary Microvascular Dysfunction in Ischemic Heart Disease

As stated above, CMD is common in a variety of cardiovascular diseases and a classification system, composed of 4 types, has been proposed to differentiate between the different forms of CMD in cardiovascular disease (**Table 1**).[24, 25] Although the link between obstructive epicardial coronary artery disease (CAD) and myocardial ischemia has been thoroughly studied and is currently undisputed, CMD is increasingly considered an important contributor. For instance, it has been shown that obstructive CAD is accompanied by CMD in a significant portion of the patients, classified as type 3 CMD.[24, 25] Importantly, cardiovascular outcome of patients with combined myocardial infarction patients and CMD is worse than that of patients with a myocardial infarction without CMD.[26] This difference is possibly due to changes in the coronary microvascular control distal of the obstruction which further limit myocardial perfusion.[27]

Table 1. Classification of coronary microvascular dysfunction

	Clinical setting	Main pathogenic mechanisms
Type 1: in the absence of myocardial diseases and obstructive CAD	Risk factors Microvascular angina	Endothelial dysfunction SMC dysfunction Vascular remodeling
Type 2: in myocardial diseases	Hypertrophic cardiomyopathy Dilated cardiomyopathy Anderson-Fabry's disease Amyloidosis Myocarditis Aortic stenosis	Vascular remodeling SMC dysfunction Extramural compression Luminal obstruction
Type 3: in obstructive CAD	Stable angina Acute coronary syndrome	Endothelial dysfunction SMC dysfunction Luminal obstruction
Type 4: iatrogenic	PCI Coronary artery grafting	Luminal obstruction Autonomic dysfunction

CAD, coronary artery diseases; SMC, smooth muscle cells; PCI, percutaneous coronary intervention. Adapted from Crea *et al.*[24]

The main treatment of acute coronary syndromes to date is revascularization by percutaneous coronary intervention—, which is considered very effective.[28] Yet, in a significant group of patients revascularization is not met by full restoration of myocardial perfusion, this phenomenon deemed 'no-reflow'[29] which, importantly, is associated with a worse outcome.[26, 30] CMD in these patients has been classified as type 4 CMD, which encompasses iatrogenic causes of CMD.[24, 25]

In contrast to CMD in patients with obstructive CAD, a significant number of patients (40%) that undergo coronary angiography for chest pain appears to have structurally normal coronary arteries, defined as luminal narrowing <50%.[31] This phenomenon was originally named 'cardiac syndrome X' and later 'microvascular angina'.[32-34] Recently a broader syndrome was defined named 'Ischemia with No Obstructive Coronary Artery disease' (INOCA), including microvascular angina as one of its components.[32, 35, 36] INOCA fits within the description of type 1 CMD.[24, 25] These patients are more often women than men (65% versus 32% respectively) and have more comorbidities.[37] These patients are more often women than men (65% versus 32% respectively) and have more comorbidities than patients with obstructive coronary artery disease.[37] These comorbidities can lead to microvascular dysfunction with subsequent myocardial ischemia and angina.

In clinical practice, diagnosing INOCA is still challenging due to limited knowledge about the pathophysiological cascade. In 2017, a diagnostic pathway based on expert opinion has been proposed, with a central role for invasive coronary reactivity testing.[35] A reduction in coronary flow reserve (CFR), (defined as the maximal divided by the basal coronary blood flow), measured by coronary flow velocities or positron emission tomography (PET) and impaired responses to the infusion of vasodilatory agents (adenosine, acetylcholine and nitroglycerine) have been proposed as characteristic features of INOCA. Although not specific for one cause of microvascular dysfunction, this does indicate general CMD.[35] Furthermore, a reduction in CFR, in the absence of coronary artery disease, is independently associated with an increase in MACE without differences between sexes.[38] The effect size of mere CMD should not be underestimated, for example the risk of MACE in diabetic patients with reduced CFR but non-obstructive CAD is similar to non-diabetic patients with CAD.[39] When measured using a Doppler flow wire, a CFR of <2.32 best predicted outcome in INOCA patients, with a 5-year MACE rate of 27% versus 9.3% for patients above 2.32.[35] This cut-off value is lower when CFR is measured by PET, there a CFR of <2 increased the MACE rate 3 times compared to those above the cut-off value.[35] After 10 years, myocardial infarction or cardiovascular death occurred in 6.7% of the women without 'evident angiographic CAD' and in 12.8% among patients with non-obstructive CAD[40], which is comparable to the prognosis in male INOCA patients.[35]

Although the exact pathophysiological relation between the risk factor profile and INOCA is not yet known, CMD is thought to play a pivotal role.[24, 32, 35, 36, 40] A possible common pathway, is that CMD is induced by a systemic pro-inflammatory state due to multiple common cardiovascular comorbidities.[41, 42] However, as summarized in **Figure 4**, the mechanisms by which CMD is induced by comorbidities can be multifold, and therefore a tailored treatment for INOCA patients is not available to date. Further research is needed to determine the pathogenesis so that targeted therapy can be developed. Therefore, translational animal models which recapitulate these features of CMD with alterations in myocardial perfusion are needed to unravel the pathophysiological cascade and test new treatment options.

Coronary Microvascular Dysfunction in Heart Failure with Preserved Ejection Fraction

Recently, INOCA has been linked to heart failure with preserved ejection fraction (HFpEF), a multifactorial heart failure syndrome in which CMD is also considered a hallmark.[33, 43, 44] It is suggested that HFpEF and INOCA represent two extreme clinical presentations of a disease continuum but have the same 'common soil': CMD.[33, 43] However, this hypothesis is still a new concept and more research is needed to confirm the link and underlying mechanisms.[33]

HFpEF is present in about half of all HF patients and this portion is projected to rise in the coming years, as the overall world population ages.[45] In HFpEF, the heart is unable to maintain cardiac output commensurate to the metabolic demand of the body, mainly due to diastolic dysfunction (impaired relaxation) while ejection fraction is preserved (≥50%), as opposed to HFrEF—heart failure with reduced (<50%) ejection faction—wherein systolic dysfunction is the main mechanism involved.[46] HFpEF is associated with classical cardiovascular risk factors such as diabetes mellitus, hypertension and obesity. Interestingly though, HFpEF has also been associated with non-classical risk factors, such as obstructive sleep apnoea syndrome, chronic kidney disease and anemia.[23, 45, 46] Recent insights have demonstrated that chronic kidney disease especially, plays an important role in the development of HFpEF.[47-49] Especially multimorbidity is common in HFpEF as ~50% of the patients have five or more major comorbidities.[45] In part this can be explained by the fact that over 90% of the HFpEF patients are over the age of 59.[45, 46] It has consistently been shown that the risk of HFpEF is higher in women than in men, while HFrEF is more prevalent in men than in women.[50] This post-menopausal women-skewed distribution is in line with the sex-distribution seen in INOCA patients and men-skewed distribution of patients with obstructive CAD.[35]

The current HFpEF hypothesis, in which it proposed that dysfunction of endothelial cells is the driven factor, was postulated by Paulus and Tschöpe in 2013, and has received wide support from cardiovascular researchers and clinicians across the world.[23] They proposed that multiple common comorbidities induce a systemic pro-inflammatory state which subsequent coronary microvascular endothelial dysfunction with a loss of NO-bioavailability. Loss of the paracrine effect of endothelium-produced NO on cardiomyocytes results in reduced protein kinase G (PKG) activation. Normally, activated PKG has anti-hypertrophic effects and phosphorylates the large protein titin—a spring-like structure determining the passive stiffness of (cardio)myocytes—resulting in a lowering of the passive stiffness.[23] Besides impaired myocardial relaxation caused by intrinsic cardiomyocyte passive stiffness, increased interstitial fibrosis by inflammation-mediated fibroblast to myofibroblast differentiation also induces myocardial stiffness and subsequent diastolic dysfunction.[23] Both intrinsic cardiomyocyte stiffening and extra cellular matrix expansion can occur in patients with HFpEF, and they might represent distinctive phenotypes which require a tailor-made therapeutic approach.

Additionally, besides the clear cardiac phenotype of HFpEF, extra-cardiac changes in microvascular function contribute to its morbidity, for example through impairments in muscle function and pulmonary vascular disease.[51] Especially the pulmonary circulation is of importance, as pulmonary congestion and subsequent dyspnoea are the main symptoms in patients with heart failure and contribute the most to the disease burden.[52] Although the physiological function of the pulmonary circulation is quite different as compared to the coronary circulation, risk factors for HFpEF

might also directly induce pulmonary vascular dysfunction.[51] As stated before[53], due to the systemic pro-inflammatory state, microvascular dysfunction is often not limited to one organ or one circulation. Therefore, researchers need to consider to investigate multiple organ systems in the same model, as differences and similarities in pathophysiology are important to address, especially in diseases of which it is known that multiple organ systems are affected, such as HFpEF.

Although some clinical studies confirm intrinsic cardiomyocyte stiffness and extracellular matrix expansion due to microvascular oxidative stress in HFpEF pathogenesis[54, 55] suggesting possible therapeutic targets, there is still no evidence-based proven treatment for HFpEF specifically.[52] In part this is due to the heterogeneity of HFpEF patient populations[52] but also due to the lack of a good translational model which recapitulates the complexity of HFpEF patients, especially with regards to multimorbidity.[56] Therefore, an animal model which not only recapitulates diastolic dysfunction but also the underlying comorbidities is needed to unravel HFpEF pathophysiology and testing of new compounds to treat HFpEF.

Porcine Model for Coronary Microvascular Dysfunction

Notwithstanding the undisputable merits of experimental animal models, we need to carefully consider the choice of a specific animal model. It is imperative to acknowledge that no single animal model perfectly emulates the human disease (CMD, INOCA and/or HFpEF), nor has a perfect translational capacity to the clinical setting.[57, 58] A significant portion of all therapeutic candidates emerging from basic research fails to translate into a clinical available therapy, referred to as the translational gap.[59] For a part this is attributable to the lack of expertise into translation of both basic researchers as well as clinicians.[59] Another part it is due to the use of animal models which are relatively healthy and mimic only the investigated disease but do not mimic the comorbidities as present in the patients. Therefore, there is a clear need for translational models of cardiovascular disease, which show high resemblance to human disease but also take in account comorbidities which might be present in patients ultimately receiving treatment.

Swine pose a very valuable animal model in cardiovascular research specifically, given their resemblance to human cardiovascular anatomy and physiology. Furthermore, the size of swine enables the use of imaging modalities also available in humans, therefore improving the translational value to the clinical setting.[60] In addition, in large animal models such as swine, chronic implementation of catheters allows for continues or repeated measurements of disease development in awake animals. The latter has been shown to be important as anaesthesia can influence cardiac as well as vascular function, possibly masking involved pathophysiological mechanisms. Chronic implantation of catheters also allows for measurements during exercise-induced stress of the

cardiovascular system.[61] As described below, cardiovascular stress testing has been proven a valuable diagnostic and prognostic tool, especially for heart failure and INOCA research.

Exercise as Physiological Stressors of Cardiovascular Disease

To differentiate between different causes—e.g. cardiac or extracardiac—of dyspnoea or exercise intolerance, cardiopulmonary exercise testing (CPET) should be used. Furthermore, most initial symptoms in patients with cardiovascular disease occur during exercise, with symptoms occurring at rest less often and/or with more advanced disease. This underlines the importance of investigating changes in cardiovascular health not only in 'static' conditions, but also during physiological stressors of the cardiovascular system (e.g. exercise). This is confirmed by findings in multiple large human studies conducted in both HFpEF patients and patients with pulmonary hypertension (PH). At the Mayo clinic (Rochester, MN), extensive work has been done in HFpEF patients showing that CPET has the ability to unmask patients with early HFpEF before overt left ventricular backward failure occurs.[62, 63] Reduced exercise intolerance (reduced peak VO_2) is a common feature in—but is not limited to—HFpEF.[62] Such reduction in exercise capacity is partially due inadequate cardiac output generation during increasing metabolic demand.[64, 65] The latter is in line with the reduced peripheral vascular function which limits peak VO_2 as commonly seen in HFpEF.[51, 65, 66] Indeed, peak VO_2 especially could help to differentiate HFpEF from non-cardiac dyspnoea, with a proposed cut-off value of <14 ml min^{-1} kg^{-1} reflecting HFpEF.[63] In addition, pulmonary congestion is thought to play an important role in the reduced exercise capacity.[62] Interestingly, the correlation between peak VO_2 and peak filling pressures, as a measure for pulmonary congestion, is observed in HFpEF but not in HFrEF.[63] Although pulmonary congestion due to left ventricular diastolic dysfunction is a key feature of HFpEF, direct pulmonary vascular alterations and right ventricular dysfunction have also been observed[67, 68], possibly due to direct effects of the comorbidities on pulmonary (endothelial) function and structure.[69] This hypothesis is confirmed by a unique response to CPET in HFpEF patients with isolated pre-capillary PH—due to pulmonary vascular disease, (PVD)—as compared to non-PH and combined-capillary PH HFpEF patients.[70] Whereas, in the HFpEF guidelines, CPET is relatively new and is only recommended in a selective group of patients with an intermediate diagnostic algorithm score[71], in PH or pulmonary arterial hypertension, more specifically, exercise testing (both 6-minutes walking test and CPET) has been investigated extensively and is included into the guidelines as an important prognostic marker.[72] Additionally, isolated exercise-induced PH has not been included in the current PH-guidelines[72], but it is currently under debate, as it has been shown that an increased pulmonary arterial pressure and pulmonary vascular resistance during CPET can unmask PH in patients with normal resting pulmonary arterial pressure (<20mmHg), which is thought to be of prognostic value.[73]

The value of exercise testing in determining cardiovascular (dys)function is therefore beyond any debate and should be included in research, both clinical and preclinical.[74-76]

Aim and Outline of This Thesis

As outlined above, clarification of the pathophysiology and treatment of multimorbidity-induced cardiovascular diseases, in particular INOCA and HFpEF, are unmet clinical needs. The general aim of this thesis is to study how cardiovascular risk factors, specifically diabetes mellitus, dyslipidaemia and chronic kidney disease, impair cardiovascular function, with a focus on microvascular function and left ventricular diastolic function. For this purpose, we exposed swine to multiple risk factors and utilized novel sensitive methods to extensively characterized cardiac and (micro)vascular (dys)function in these models. This thesis is divided into 2 parts.

Part I of this thesis focuses on the effect of metabolic derangements on coronary microvascular function as well as myocardial function. **Chapter 2** provides an overview of the main proposed effects of metabolic derangements, such as obesity and diabetes mellitus, on microvascular function in different vascular beds. **Chapter 3** presents an overview of the various animal models of CMD, pointing towards swine as a translationally highly relevant experimental animal. In **Chapter 4** we investigate the effect of metabolic derangements, by induction of diabetes mellitus and dyslipidaemia, in Göttingen miniswine on left ventricular function and structure using echocardiography, molecular and histological techniques. In **Chapter 5** we utilize the same Göttingen miniswine model as well as farm swine with metabolic derangement to investigate the relation between microvascular endothelial dysfunction, atherosclerosis and the circulating coagulation proteins von Willebrand Factor and Factor VIII.

In **Part II** we investigate the combination of chronic kidney disease, as non-classical risk factor for cardiovascular disease, and metabolic derangements on cardiovascular function. In **Chapter 6** we reviewed the link between chronic kidney disease and HFpEF, previously conducted clinical trials and novel therapeutic options for HFpEF, with a focus on the coronary microcirculation. In **Chapter 7** we introduce a novel swine model with left ventricular diastolic dysfunction induced by multiple cardiovascular risk factors—diabetes mellitus, dyslipidaemia and chronic kidney disease. In this chapter, we extensively phenotype this swine model using MRI, *in vivo* cardiac function measurements and multiple *in vitro* techniques. **Chapter 8-10** focus on *in vivo* cardiac and vascular function measurements in awake resting swine and during exercise using the same swine model. **Chapter 8** describes the coronary microvascular, systemic and left ventricular function and myocardial oxygen balance in awake swine at rest and during exercise, additionally cardiac and vascular structure were determined by histology. In **Chapter 9** we elucidate the mechanisms underlying the in **Chapter**

8 observed perturbations in coronary microvascular vasomotor control, by investigating the nitric oxide signalling pathway, both *in vivo* at rest and during exercise, as well as *in vitro* using molecular techniques and isolated vessel experiments. In **Chapter 10** we investigated underlying mechanisms of the pulmonary vascular dysfunction observed at rest and during exercise, in this swine model with multiple morbidities, as well as right ventricular function and structure. In **Chapter 11** we discuss the findings of this thesis and provide a general conclusion.

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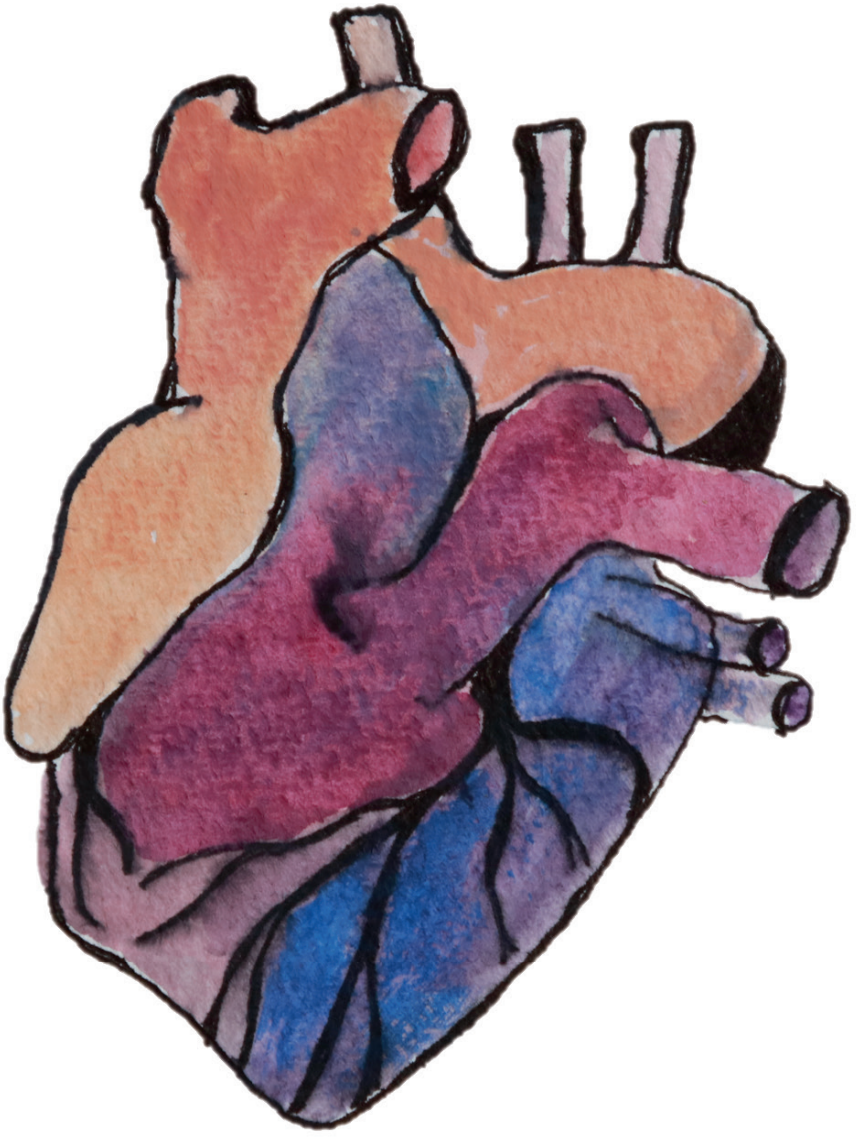
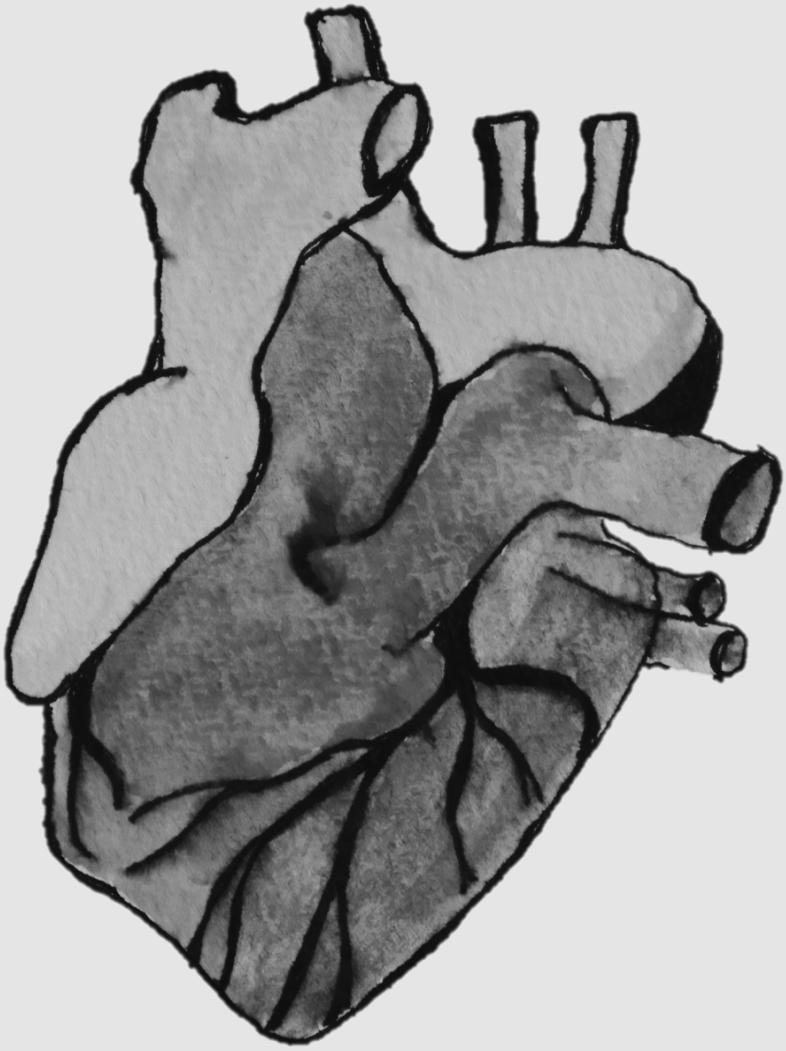


Part I

Microvascular and myocardial dysfunction in metabolic derangements



Chapter 2

The microcirculation: a key player in obesity-associated cardiovascular disease

Sorop O, Olver TD, **van de Wouw J**, Heinonen I, van Duin RW, Duncker DJ, Merkus D.

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Abstract

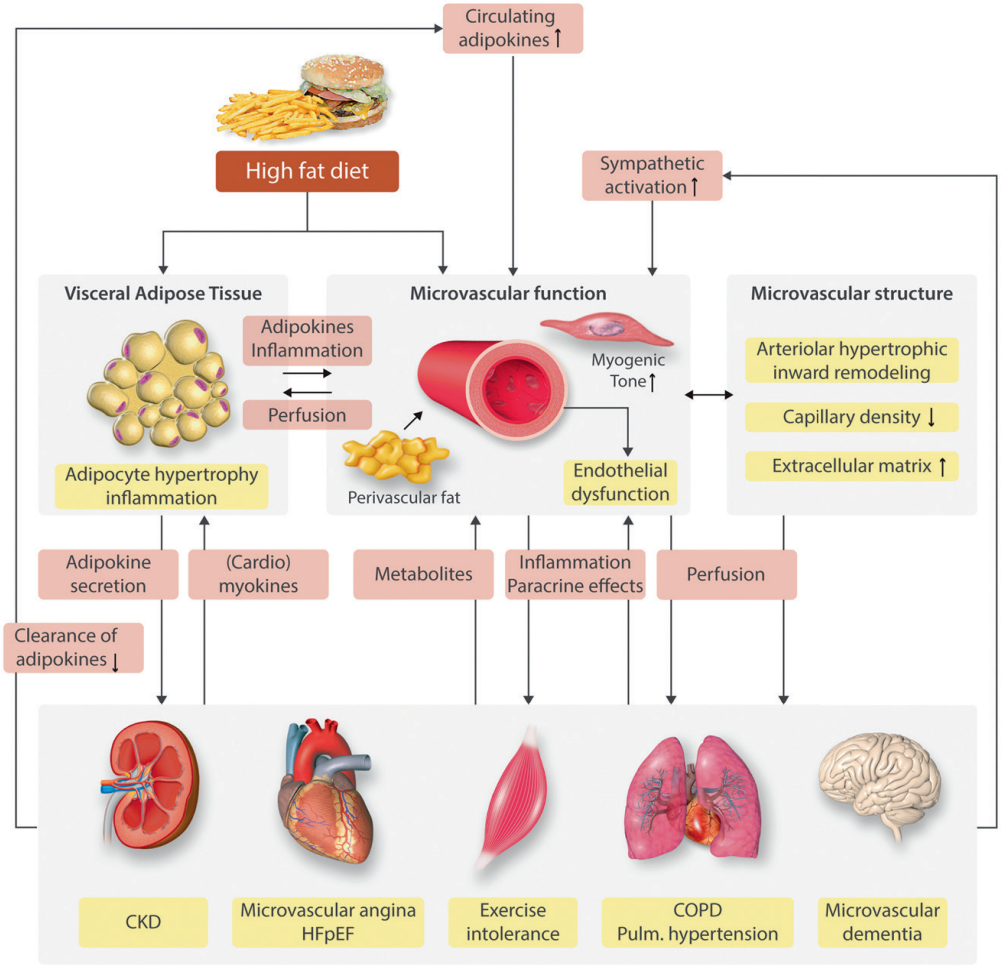
It is increasingly recognized that obesity is a risk factor for microvascular disease, involving both structural and functional changes in the microvasculature. This review aims to describe how obesity impacts the microvasculature of a variety of tissues, including visceral adipose tissue, skeletal muscle, heart, brain, kidneys, and lungs. These changes involve endothelial dysfunction, which in turn (i) impacts control of vascular tone, (ii) contributes to development of microvascular insulin resistance, (iii) alters secretion of paracrine factors like nitric oxide and endothelin, but (iv) also influences vascular structure and perivascular inflammation. In concert, these changes impair organ perfusion and organ function thereby contributing to altered release and clearance of neurohumoral factors, such as adipokines and inflammatory cytokines. Global microvascular dysfunction in obese subjects is therefore a common pathway that not only explains exercise-intolerance but also predisposes to development of chronic kidney disease, microvascular dementia, coronary microvascular angina, heart failure with preserved ejection fraction, chronic obstructive pulmonary disease, and pulmonary hypertension.

1. Introduction

A large body of evidence has accumulated over the years, from both clinical and experimental studies, indicating that obesity is associated with endothelial dysfunction and development of atherosclerosis, and that obesity has become one of the most important risk factors for cardiovascular disease including coronary artery disease, heart failure, and stroke.[1] In addition to atherosclerosis in the larger arteries, obesity is also a risk factor for microvascular disease.[2–4] Interestingly, a single high fat meal already perturbs endothelial function in the brachial artery,[5] and reduces flow reserve in the coronary vasculature,[6] illustrating how a single exposure to a high circulating lipid load has an impact, albeit transient, on the microvasculature. Regular exposure to high circulating lipid loads, even prior to the onset of overt obesity, leads to an inflammatory response that is accompanied by microvascular dysfunction,[7,8] the severity of which correlates with the amount of visceral adipose tissue present in the body.[9] Eventually, obesity and the associated inflammation not only impact function, but also structure of the microvasculature (**Figure 1** and **Table 1**).

The microcirculation regulates the supply of oxygen and nutrients by determining flow to the tissue through regulation of vascular resistance and exchange at the capillary level. Acute regulation of resistance to blood flow is accomplished by changes in microvascular tone, i.e. in contraction of vascular smooth muscle, through integration of multiple signals from the perivascular nerves, the surrounding tissue, the endothelium as well as circulating factors (**Figure 1**).[10] The central nervous system contributes to regulation of vascular tone through modulation of the balance between activation of the sympathetic and parasympathetic nervous system. In obesity, the sympathetic nervous system is activated by leptin,[11] but the impact of sympathetic nervous system activation on the regulation of tone in the different organs depends on their innervation pattern. The endothelium produces both vasodilators [nitric oxide (NO), prostacyclin, and hydrogen peroxide (H_2O_2) and other endothelium-derived hyperpolarizing factors] and vasoconstrictors [endothelin (ET), vasoconstrictor prostanoids and superoxide],[12] as well as factors, including uridine adenosine tetraphosphate (Up_4A), of which the vasoactivity depends on the vascular bed studied.[13] It is increasingly recognized that many of these endothelial factors not only influence smooth muscle tone, but also act in a paracrine fashion on the surrounding parenchymal tissue.

Figure 1 Proposed mechanisms of obesity-related microvascular dysfunction predisposing to multi-organ disease



High fat diet on a regular basis changes the composition of visceral adipose tissue, and induces a low grade local inflammatory response, which together modify the secretion of adipokines. Simultaneously, high fat diet results in endothelial dysfunction throughout the body, which not only alters vascular tone, and contributes to development of microvascular insulin resistance, but also influences vascular structure and perivascular inflammation. In concert, these microvascular changes impair organ perfusion and organ function thereby further contributing to altered release and clearance of metabolites and neurohumoral factors, like adipokines, inflammatory cytokines as well as (cardio)myokines. Global microvascular dysfunction in obese subjects therefore is a common pathway that contributes to exercise-intolerance and predisposes to development of chronic kidney disease, microvascular dementia, coronary microvascular angina, COPD and pulmonary hypertension. CKD, chronic kidney disease; HFpEF, heart failure with preserved ejection fractioan; COPD, chronic obstructive pulmonary disease.

The balance between vasodilators and vasoconstrictors shifts in response to mechanical stimuli such as an increase in shear stress, but also in response to endocrine factors such as insulin. In the endothelial cells, insulin can activate PI3-kinase, resulting in eNOS activation, NO production, and vasodilation. Conversely, insulin can also activate the ERK1/2 pathway, leading to increased production of ET and resulting in vasoconstriction.[8,14] In the healthy vasculature, insulin-induced activation of eNOS and epoxyeicosatrienoic acids (EETs) predominate, which serves to facilitate glucose uptake in tissue, whereas in obesity insulin-mediated activation of the PI3K pathway in endothelial cells is selectively impaired while the insulin-mediated activation of ET and vasoconstrictor eicosanoids remains intact.[8,15] This shift in the resistance vessel response to insulin impairs downstream capillary recruitment, which plays a critical role in the development of insulin resistance.[8,9,16] The fat surrounding the blood vessels, i.e. perivascular fat, likely plays an important role in determining insulin resistance. Thus, insulin-mediated capillary recruitment is increased by adiponectin, whereas it is decreased by free fatty acids as well as by inflammatory cytokines, including TNFα, that can be released from the perivascular fat.[8,9,16] In obesity, there is a shift in factors secreted by perivascular fat, that either directly, or via an increase in oxidative stress, tilts the vasomotor balance towards vasoconstriction and insulin resistance.[12,17] At the same time, these inflammatory cytokines in conjunction with oxidative stress upregulate adhesion molecules on the endothelium, and causing the microcirculation to serve as an entry point for inflammatory cells into the tissue,[9,11] that can then further contribute to microvascular and ultimately organ dysfunction.

As stated, the primary function of the microcirculation in tissues such as skeletal and cardiac muscle and the brain is to supply oxygen and nutrients, and remove carbon dioxide and waste products. Hence, microvascular dysfunction, resulting in impaired oxygenation and low grade inflammation in these tissues, likely contribute to exercise intolerance as well as to the pathogenesis of coronary microvascular angina[18] and cerebromicrovascular disease (i.e. microvascular dementia; **Figure 1**).[19,20] The microcirculation serves a different purpose in various other highly specialized tissues, including adipose tissue, kidneys, and lungs. Within adipose tissue, the microcirculation serves as an exchange site allowing storage of fat in times of nutrient excess, and re-uptake of fat when the body experiences nutrient shortage. The pulmonary microvasculature serves to clear carbon dioxide and oxygenate the blood in the alveolar capillaries. Similarly, the microcirculation in the kidneys is designed for clearance not only of waste products like urea and creatinine, but also of adipokines.[21] Microvascular dysfunction in these organs therefore impacts the removal of waste products from the body, thereby prolonging circulation time of carbon dioxide (lungs), or adipokines and uremic toxins (kidneys) that may further damage the microvasculature of other organs (**Figure 1**). Given the variety of specialized tasks in different organs, it is not surprising

that microvascular control mechanisms differ markedly between vascular beds, and that the impact of obesity on the microcirculation varies between organs (**Table 1**).

In this review article, we will discuss the functional and structural changes in the microvasculature of different organs, that are associated with obesity—and how these changes contribute to organ dysfunction—with a particular focus on evidence obtained in humans and large experimental animals

2. Adipose tissue microcirculation in obesity

Two main types of adipose tissue can be distinguished in the body, which have distinctly different functions both in healthy and obese subjects being white and brown adipose tissue (WAT and BAT respectively). WAT is the primary site of fat accumulation, and not only allows efficient fat storage, but also quick mobilization of fat stores to meet energy demands of the body.[22] WAT comprises both subcutaneous and visceral adipose tissue (VAT). The main role of BAT is thermogenesis.[22,23] Thermogenesis in BAT is activated by the sympathetic nervous system. High expression of uncoupling protein-1 on the inner membrane of BAT mitochondria results in uncoupling of mitochondrial respiration so that heat is generated instead of ATP.[23] In adult humans, BAT is mainly located in cervical, supraclavicular, mediastinal, paravertebral, suprarenal, and peri-renal areas.[22] In addition, epicardial and perivascular adipose tissue have a phenotype that more closely resembles BAT than WAT. Both WAT and BAT contain dense microvascular networks, but microvascular density is higher in BAT as compared to WAT, with 3 vs. 1 capillary per adipocyte, respectively.[22] The microvasculature in WAT serves as the exchange site for fat deposition and mobilization, while in BAT it is required for both delivery of fuel for and dissipation of heat produced during thermogenesis.[7,22,23] Metabolism, perfusion and function of both WAT and BAT are affected by obesity.

Table 1 Effect of Obesity on Microvascular Function and Structure in different tissues.

	Adipose Tissue			Skeletal muscle	Coronary	Cerebral	Renal	Pulmonary
	Perivascular	WAT	BAT					
Functional changes								
Metabolic coupling				=Rest, ↓Exercise	=Rest, ↓Exercise	↓	Hyperfiltration	NA
Endothelial Factors								
NO		↓		↓	↓ Early,=Late	↓	↑Early, ↓Late	↑Early, =/↓Late
Prostacyclin				↓		↓		
ET				↓	↑		↑	↓
EDHF				↑				
Insulin signalling								
Insulin sensitivity		↓	↓	↓		↓		
PI3K-NO		↓	↓	↓		↓		
Erk1/2-ET				↑		↑		
Inflammatory cytokines	↑	↑	↑	↑	↑		↑	↑
ROS	↑	↑	↑	=Rest, ↑Exercise	↑	↑	↑	
PDE5				↓				=
Adipokines								
Adiponectin	↓	↓	↓					
Leptin/ resistin	↑	↑	↑	↑	↑			
Sympathetic control			↓	↑Rest, =Exercise				
β-adrenergic vasodilation				↓				
α-adrenergic vasconstriction				↑Rest, ↓Exercise				
Structural changes								
Medial Thickening/ Inward remodeling					↑	↑	↑	↑
ECM deposition	↑	↑	↑		↑	↑	↑	
Capillary density		↓	↓	↓	↓	↓	Early: Leaky	
							Late ↓	
Consequences/disease	Whitening	Hypertrophy	Whitening	Exercise	Diastolic	Microvascular	Renal	Pulmonary
				intolerance	dysfunction	dementia	dysfunction	inflammation
							Uremic toxins	

WAT: White adipose tissue; BAT: Brown adipose tissue, NO: Nitric Oxide; ET: Endothelin-1; EDHF: endothelium-derived hyperpolarizing factor;
ROS: Reactive oxygen Species; PDE5: Phosphodiesterase type 5

Obesity, by definition an excessive accumulation of fat mass, results in expansion of particularly WAT. Ingestion of a single high fat meal induces upregulation of P-selectin on the venular side of the visceral adipose microvasculature, thereby forming an anchoring point for leucocytes. The leucocytes infiltrate the VAT and initiate an inflammatory response. Hence, low grade inflammation particularly in visceral adipose tissue may precede excessive fat accumulation, increase oxidative stress, and cause chronic microvascular dysfunction.[7] Interestingly, blood flow to VAT increases following meal ingestion in lean but not obese subjects.[24] Moreover, diet-induced obesity is accompanied by decreased eNOS-expression and activity, while eNOS overexpression protects against diet-induced obesity (**Table 1**).[25] These observations suggest a key role for microvascular function in adipose tissue homeostasis. Besides this paracrine interaction between adipocytes and the microvasculature, it should be noted that fat accumulation in VAT results in an increase in adipocyte-size from 50 µm up to 150–200 µm, which is beyond the diffusion distance for oxygen, while the accompanying reduction in capillary density will further decrease adipose tissue oxygenation. Indeed, chronic hypoxia has been shown to be present in expanded VAT.[26] Similar to WAT, brown adipocytes hypertrophy in obesity. Intriguingly, it has recently been shown that capillary rarefaction, leading to focal hypoxia in BAT, is sufficient to induce 'whitening' of BAT, which is associated with reduced beta-adrenergic signaling, mitochondrial dysfunction, loss of thermogenic capacity and further accumulation of lipid droplets.[27,28]

Hypoxia *per se* induces a reduction in adiponectin and an increase in leptin release from isolated adipocytes.[26] Moreover, chronic hypoxia results in sustained inflammation thereby further modulating the secretion of adipokines from both WAT[26,29] and BAT[23]and contributing to metabolic derangement in obesity.[26,30,31] In healthy subjects, the secretion of the anti-inflammatory adipokine adiponectin predominates, whereas in obese subjects, there is a shift toward pro-inflammatory adipokines such as leptin, resistin, TNFα, IL-6, and IL-18 (**Table 1**).[32] Thus, adipose tissue hypoxia and inflammation are centrally involved in the pathophysiology of obesity, and can, through release of vasoactive and/or inflammatory adipokines, modulate microvascular function throughout the body (**Figure 1**).

3. Skeletal muscle microvasculature in obesity

Although some studies in young adult humans suggest that skeletal muscle blood flow is relatively well-maintained in obesity[33] even during exercise,[34] others show a reduction in flow normalized for muscle mass both at rest and during exercise.[34–38] These findings seem to be independent of age and

vascular bed, as the reduction in flow is present in children,[36,38] and adults,[35,37] both in the forearm[35–37] and upper leg.[34,37,38] Similarly, the exercise-induced increase in systemic vascular conductance is blunted in obese swine as compared to lean swine, consistent with a decrease in flow to exercising muscle.[39] This decrease in flow is compensated by an increase in oxygen extraction to fulfill the oxygen-requirement of skeletal muscle.[39,40]

In skeletal muscle, close coupling of blood flow to metabolic activity is required and besides substances released from nerve terminals, the endothelium and the contracting muscle, also involves mechanical interaction between the contracting muscle and the vasculature.[10] The nervous system contributes to exercise hyperemia in skeletal muscle via activation of sympathetic vasodilator fibers, vasodilation elicited by acetylcholine spillover from active motor nerves as well as functional sympatholysis in active muscle.[10] Resting muscle sympathetic nerve activity (MSNA) is significantly higher in obese patients with metabolic syndrome, but it does not further increase during exercise.[41] Interestingly, in the presence of β-blockade, exercise resulted in larger increase in forearm blood flow and conductance in obese as compared to lean men. Together with the observation that the exercise-induced increase in skeletal muscle blood flow is reduced or at best preserved in obesity, these data suggest that β-adrenergic vasodilation is reduced in obesity. Furthermore, in the presence of β-blockade, α_2- but not α_1-stimulation resulted in a larger decrease in forearm vascular conductance in obese vs. lean subjects at rest and during exercise. Similarly, there was a tendency towards a larger increase in conductance with α-blockade with phentolamine at rest in obese vs. lean subjects, but a reduced increase in conductance upon α-blockade during exercise.[41] These data suggest that obesity results in a shift in the balance of neurogenic control of skeletal muscle blood flow, with increased α-adrenergic constriction at rest, that is withdrawn during exercise, thereby compensating for a loss of β-adrenergic vasodilation (**Table 1**).

In humans, endothelium-dependent skeletal muscle microvascular vasodilation in response to acetylcholine is either preserved [34,42] or reduced[43] in obesity, while eNOS expression is unaltered.[42] However, the contribution of both NOS-[42] and cyclooxygenase (COX)-[44] dependent vasodilator mechanisms to acetylcholine-induced vasodilation is reduced, which is compensated by NOS- and COX-independent vasodilator mechanisms, potentially an increase in endothelium-derived hyperpolarizing factors such as EETs and/or H_2O_2.[12] An increase in H_2O_2 may result from superoxide dismutase (SOD)-mediated conversion of superoxide, particularly in more active obese individuals.[43] In addition, a reduction in NO-bioavailability has been shown to be counterbalanced by reduced phosphodiesterase 5 (PDE5) activity,[40] as well as by a reduced vasoconstrictor influence of ET (**Table 1**). The latter is mediated through a reduction in ET-sensitivity of the skeletal muscle

arterioles together with a decrease in circulating ET, reflecting a decrease in local ET production.[39] Interestingly, a study in rodents suggests that basal differences exist in endothelial cell phenotype between arteries perfusing slow-twitch and those perfusing fast-twitch muscle fibers, with the former being less susceptible to endothelial dysfunction.[45]

Alterations in endothelial function may also play a role in insulin-dependent modulation of microvascular tone. In healthy individuals, insulin-induced vasodilation serves to facilitate glucose delivery and uptake in skeletal muscle. As outlined in the introduction, insulin resistance is associated with a shift from insulin-induced vasodilation to vasoconstriction. Indeed, the change in femoral vascular conductance upon glucose ingestion is smaller in obese as compared to lean women, which is associated with impaired body glucose uptake.[46] Moreover, flow to the quadriceps muscle of obese men is lower in the presence of insulin both at rest and during exercise,[47] and insulin-induced vasodilation is converted into vasoconstriction in skeletal muscle arterioles isolated from obese women.[16] The latter is mediated, at least in part, by alterations in perivascular adipose tissue (PVAT), that displays a pro- inflammatory phenotype in obesity.[16]

Although low grade inflammation may lead to an increase in oxidative stress, TBARS (as index of systemic oxidative stress) do not appear to be different between normal and obese young adults.[48,49] Conversely, ROS production by NADPH-oxidase and Xanthine oxidase in skeletal muscle is increased in overtly obese, but not mildly obese individuals.[43] In accordance with this observation, infusion of the NADPH-oxidase inhibitor and antioxidant apocynin augmented the acetylcholine-induced increase in flow to skeletal muscle in obese but not lean subjects.[43] However, despite a significant inverse correlation between TBARS and vasodilator responses, the antioxidant ascorbic acid augmented acetylcholine-induced vasodilation to a similar extent in normal and obese subjects under resting conditions.[48] Nevertheless, the significant correlation between waist-to-height ratio and TBARS post-exercise,[48] together with a negative correlation between catalase and BMI,[49] suggests that anti-oxidant capacity may fall short during stress in obese subjects.

The obesity-induced decrease in exercise capacity (VO_2max) closely correlates with capillary density in skeletal muscle, indicating that besides functional, also structural changes in the skeletal muscle microvasculature are present (**Table 1**).[50] A study in rodents suggests that capillary rarefaction in obesity occurs in two phases, of which the first one is mediated by an increase in oxidative stress, and the second one by a decrease in NO-bioavailability.[51]

Taken together, obesity moderately reduces skeletal muscle blood flow at rest. The mechanisms of this reduction are incompletely understood but may involve factors released from

perivascular fat that modulate insulin sensitivity and endothelial function as well as an increased vasoconstrictor influence caused by an increase in MSNA. In addition, structural changes in the skeletal muscle microvasculature may contribute to the decreased resting blood flow. Because exercise hyperemia involves many redundant regulatory mechanisms, it is relatively well-maintained. However, the impairments in blood flow and its distribution are likely to become more severe when the metabolic disturbance persists for a longer time or when other co-morbidities such as hypertension, hyperlipidemia, and hyperinsulinemia co-exist in the same patient,[33,52,53] which may contribute to exercise intolerance in obese people.

4. Coronary microcirculation in obesity

Similar to skeletal muscle, coronary microvascular function plays an important role in coupling of myocardial perfusion to cardiac metabolism.[54] Clinical studies have shown that in conditions such as obesity and hypercholesterolemia the coupling of coronary blood flow to the myocardial metabolic demand is altered.[2] Thus, coronary flow velocity of obese patients during dobutamine stress-echo is impaired, with the impairment becoming even more evident when obesity is associated with other risk factors.[55] Also, myocardial blood flow measurements with PET indicate that increases in myocardial blood flow in response to cold-pressor testing are diminished in obese patients.[56] Interestingly, female sex and the volume of visceral fat are associated with a reduction in myocardial perfusion at peak dose of dobutamine, as measured by MRI.[57] These clinical observations are supported by studies in obese Ossabaw swine with metabolic syndrome and swine with severe familial hypercholesterolemia (FH), demonstrating that coronary blood flow regulation and myocardial oxygen balance are altered in particular during treadmill exercise.[58,59]

Several studies have reported reduced coronary flow reserve and increased minimal vascular resistance in patients with obesity and hypercholesterolemia as assessed by either PET, Doppler echocardiography or MRI.[60–64] Such abnormalities can be the result of either changes in microvascular function, i.e. the regulation of vascular tone, or structural changes within the coronary microcirculation, such as vascular rarefaction or inward remodeling.[54,65] Alterations in control of coronary microvascular tone are generally characterized by a loss of endothelial vasodilator influence (such as NO) as well as by increased neurohumoral (angiotensin II) and endothelium-derived vasoconstrictor (ROS, ET, prostanoids) influences resulting in a shift in the vasomotor balance towards increased vasoconstriction (**Table 1**).[2,3,66] Indeed, several studies in humans and animal models have demonstrated that obesity is associated with alterations in the vasodilator-vasoconstrictor balance

controlling coronary microvascular tone.[2–4,67] Thus, hyperoxia-induced vasoconstriction in the coronary microvasculature is enhanced in obese adolescents,[68] while bradykinin-induced vasodilation is impaired in isolated coronary arterioles from obese patients due to increased tissue angiotensin-converting enzyme activity.[69] Similarly, in swine with hypercholesterolemia, endothelial dysfunction of isolated small coronary arteries due to impaired NO biovailability is noted early in the disease process (2.5 months after start of the high fat diet).[70] In contrast, at 15 months of diet, NO signaling is restored but the constriction to ET-1 is exacerbated, this response being mediated by ET_B-mediated vasoconstriction, indicating that disturbances in the balance between vasodilators and vasoconstrictors are modulated during progression of the disease (**Table 1**).[71] The observations that eNOS activity is impaired by adipokines secreted by perivascular fat[72] and that coronary microvascular dysfunction correlates with increased inflammation[62] suggest that inflammation and fat-derived cytokines in obesity are also important determinants of coronary microvascular dysfunction. This is consistent with the correlation of fat deposits with the decline in coronary microvascular function.[57,63]

In addition to the functional changes in the control of vascular tone, obesity can also result in structural remodeling of the coronary microcirculation. Histological analysis of left ventricular tissue biopsies obtained during coronary bypass surgery show significantly lower capillary densities in obese patients (**Table 1**).[73] Importantly, both arteriolar remodeling and capillary rarefaction likely contribute to the reduced coronary flow reserve in obese patients as reported in several,[60–62] though not all studies.[66] Similarly, hypertrophic inward remodeling of coronary arterioles, increased stiffness as well as capillary rarefaction are reported in animal models of obesity and hypercholesterolemia.[58,71,74]

It is increasingly recognized that the factors released by the coronary endothelium also impact the function of the surrounding cardiomyocytes in a paracrine fashion. Loss of NO, increased oxidative stress and the ensuing tissue inflammatory response are thought to play a key role in development of left ventricular diastolic dysfunction through altering relaxation of the cardiac myocytes and increasing collagen fraction in the extracellular matrix.[75,100]

In conclusion, both clinical and experimental evidence indicate that obesity is an independent risk factor for coronary microvascular dysfunction, with both functional and structural alterations in the coronary microcirculation contributing to the impairments in coronary flow regulation and having a negative impact on the coronary flow reserve in these patients. Some, but not all, of these changes can be alleviated by weight loss and physical exercise.[68,76] However, in order to be able to specifically address the different aspects the obesity-associated coronary microvascular dysfunction, future studies should focus on revealing the underlying mechanisms that drive the obesity-associated coronary microvascular abnormalities.

5. Cerebral microvasculature in obesity

Similar to the heart, the brain relies on a continuous supply of blood flow, with regional alterations in brain activity requiring corresponding changes in brain flow distribution via metabolic vasodilation, which is referred to in the brain as neurovascular coupling. Obesity-induced changes in brain microvascular structure and function have been proposed to result in disruptions in neurovascular coupling, thereby leading to vascular cognitive impairment.[20,77] Importantly, the brain microvasculature is only surrounded by neurons and glia as there is no perivascular adipose tissue. Therefore, the effects of obesity likely manifest as the result of changes in neural/glia-vascular (metabolic) interactions, and hemodynamic (i.e. blood pressure) or circulating factors (hyperglycemia and dyslipidemia) that have a direct vasoactive effect or act indirectly via influencing neurons and glia.

Both pre-clinical[78,79] and clinical[80,81] obese human populations exhibit reduced brain blood flow and impaired vasodilation during hypercapnia. The mechanisms responsible likely relate to microvascular rarefaction,[19,20,82] decreased contribution of NO to basal cerebral microvascular tone control, altered release of vasodilator prostanoids, and/or a direct effect of H^+ on vascular smooth muscle ion channels.[83] Impaired cerebral vasoreactivity in obesity occurs independently of clinical insulin resistance,[78,84] but may also worsen with accompanying hypertension and poor glycemic control.[80,81] Obese Zucker rats display impaired endothelium-dependent NO-mediated middle cerebral artery (MCA) vasodilation as well as depressed insulin-stimulated vasodilation, potentially due to increased PKC- and MAPK-activation, combined with eNOS uncoupling resulting in augmented superoxide production.[82,85] Indeed, depressed vasodilator responses to hypoxia and NOS-dependent dilators, as well as enhanced constrictor responses to 5-hydroxytryptophan[1] reflect potential pathological adaptations that impair neurovascular coupling. Decreased insulin-mediated vasodilation potentially contributes to impaired microvascular insulin delivery in the brain (**Table 1**).[86] The physiological significance of this remains incompletely understood, but similar to skeletal muscle, altered insulin signaling in the brain (i.e. brain insulin resistance) can link microvascular and metabolic dysfunction, thereby leading to cognitive impairment. Collectively, the data from humans[81] and rodents[82,85] suggest that obesity induces endothelial dysfunction with impaired NO production/bioavailability, resulting in altered cerebral vasoreactivity.

Obesity also affects the structure of small arteries, arterioles and capillaries in the brain, with many of these changes reflecting the development of cerebral microvascular disease.[20,77] Indices of cerebral microvascular disease, including cerebral microbleeds, lacunas and microlacunas, increase the vulnerability to neurodegeneration,[20,77] and occur more commonly in obese individuals with

insulin resistance,[87] dyslipidemia,[87] and central adiposity.[88] In addition, genetic predispositions may also contribute to this relationship.[77] Beyond these preliminary observations, the underlying mechanisms responsible for obesity-induced cerebrovascular remodeling in humans remain elusive. However, cortical microvascular density decreases,[89] and the MCA undergoes eutrophic inward remodeling and progressive arterial stiffening during the progression of metabolic syndrome in obese Zucker rats.[82] In diet-induced obesity in Sprague–Dawley rats, similar MCA adaptations are observed in conjunction with increased MMP-2 activity, collagen I expression, and reduced MMP-13 expression, suggesting that increases in collagen deposition contribute to vascular stiffening (**Table 1**).[90] In the aforementioned studies,[82,89,90] inward MCA remodeling coincided with the development of hypertension, while pharmacological treatment of hypertension ameliorated the remodeling.[82] This suggests that obesity-induced hypertension, and not metabolic dysfunction,[91] serves as the primary stimulus responsible for inducing inward remodeling of the MCA.

Although pharmacological treatment of hypertension ameliorates MCA remodeling, it does not improve cortical microvascular rarefaction in obese Zucker rats.[89] Also in Rhesus monkeys, diet-induced obesity causes cortical capillary rarefaction, but without concurrent changes in blood pressure.[92] In the latter study, cortical capillary rarefaction occurred alongside decreased VEGF, increased von Hippel-Lindau protein (which degrades HIF-1α) and (paradoxically) increased expression of FOXO3, eNOS, and eNOS uncoupling. In contrast to inward remodeling in the MCA, it appears that obesity-induced metabolic dysfunction and oxidative stress, and not hypertension, is responsible for the observed microvascular/capillary rarefaction (**Table 1**). Therefore, while inward remodeling may prevent hypertension-induced cerebral hyperperfusion, inward remodeling and microvascular rarefaction may limit cerebrovascular reserve and impair brain blood flow control.

Functional and structural microvascular deficits resulting in impaired neurovascular coupling likely reflect an acquired, and not programmed feature of obesity, suggesting that these abnormalities can be environmentally induced, prevented or even reversed.[93–95] Indeed, short-term diet-induced obesity reduces prefrontal cortex blood flow in mini-pigs,[94] and impairs metabolic vasodilation and precedes neuronal loss in rodents.[95] Furthermore, individuals with an elevated BMI exhibit reduced basal flow[79] and post-prandial vasodilation[93] in the prefrontal cortex, but formerly obese individuals do not display this defect.[93] Reversal of such adaptations is of growing importance as the early signs of cerebral microvascular disease can manifest very early in life, as seen in an obese 2-year-old child.[96]

In conclusion, the cumulative effect of cerebral inward remodeling, microvascular rarefaction, and impaired vasodilator capacity, likely contribute to obesity-related impairments in brain flow control and neurovascular coupling. Such obesity-induced changes can occur rapidly and in

young individuals, but it appears these pathological adaptations are modifiable. Gathering more knowledge about mechanisms of obesity-related changes in brain microvascular structure and function along the (micro)vascular tree is essential in understanding the pathology of disease progression and developing effective prevention and treatment strategies.

6. Renal microvasculature in obesity

Obesity is associated with an increased risk for chronic renal failure. In a Swedish case-control study, particularly diabetic nephropathy, nephrosclerosis, and glomerulonephritis were associated with obesity,[97] suggesting that obesity negatively impacts the renal microvascular bed. Indeed, clinical studies show that in obesity, afferent arteriolar vasodilation results in an increased renal blood flow (RBF), that causes a state of hyperfiltration.[98] Results in experimental animals are equivocal with some laboratories showing that RBF and glomerular filtration rate (GFR) are increased 3–4 months[99,100] of high fat diet, whereas others show no change in RBF and GFR.[101–105] The reported increase in RBF is mostly due to an increase in renal cortical volume, vascular volume fraction and cortical perfusion, whereas filtration fraction, medullary size, and medullary perfusion showed no difference.[99,100,106]

Obesity is linked to increased peri-renal fat deposition,[100,107] which in turn leads to low grade inflammation and renovascular endothelial dysfunction. Indeed, endothelium-dependent vasodilation to acetylcholine was impaired both *in vivo*[98,102,103,108] and *in vitro* in renal arteries of obese swine,[100] while endothelium-independent vasodilation to the NO donor sodium nitroprusside (SNP) was unaltered *in vitro* (**Table 1**).[100] Although renal eNOS-expression may initially increase,[99] prolonged exposure to high fat diet reduces expression of eNOS and promotes eNOS-uncoupling and activation of xanthine oxidase resulting in impaired bioavailability of NO in obese swine.[102] Antioxidant capacity in obesity is further reduced by a decrease in SOD activity.[103,108] Moreover, increased NAD(P)H-oxidase and LOX-1 expression further contribute to increased oxidative stress, which together with upregulation of iNOS, may have led to increased nitrotyrosine levels.[99,102,104,105,108,109] ROS in turn, induce upregulation of renal pre-pro ET-1 and ET_A receptors thereby promoting vasoconstriction (**Table 1**).[102–104]

Interestingly, incubating renal arteries of lean swine with peri-renal fat of obese animals transferred the impaired endothelial function to those arteries.[100] This response appears to be the result of fat derived inflammatory molecules, and not of oxidative stress, as the endothelial dysfunction could only be reversed by neutralizing TNF-α, but not by the free radical scavenger

Tempol, *in vitro*.[100] Furthermore, anti-inflammatory treatment with thalidomide *in vivo*, abolished the renal increase in TNF-α, and improved endothelial function without altering oxidative stress, suggesting that increased levels of TNF-α play a vital role in impaired function of the endothelium.[102]

In addition to renovascular dysfunction, structural alterations in the kidney produced by obesity have also been reported (**Table 1**). Interestingly, obesity is associated with a change in the balance of angiogenic and anti-angiogenic factors in the kidney that favors angiogenesis. Thus, VEGF and its receptor Flk-1[108] as well as Angpt2[110] are increased. Moreover, protein expression of the angiogenesis inhibitor TSP-1 is decreased as a result of increased oxidative stress,[99] although this is not a unanimous finding.[104] Indeed, both arteriolar and capillary density in the outer cortex of the kidney are increased in animals with obesity.[99,108] However, these newly formed vessels are more tortuous and erythrocyte exudation has been shown, suggesting that the newly formed vessels are leaky and immature.[99,102,103,108,109] Furthermore, glomerular density is decreased in animals with metabolic derangement,[109] while glomerular hypertrophy due to matrix hyperplasia and glomerular swelling are also observed in obese swine.[99,109–111] Taken together, these data suggest that the newly-formed microvasculature does enhance glomerular filtration, but is rather damaged and dysfunctional. Vascular remodeling in obesity is facilitated by dynamic processes in the extracellular matrix, as an increased MMP expression, which promotes extracellular matrix (ECM) degradation was noted at 10 weeks,[99] but not at 16 weeks[104] of high fat diet. Furthermore, in obese swine, renal expression of tissue transglutaminase is increased, causing extracellular matrix crosslinking and vascular remodeling especially in conditions of sustained vasoconstriction.[103] Moreover, microvascular media-to-lumen ratio is increased,[104,108] and perivascular as well as tubulointerstitial fibrosis is observed in obese swine.[104,108] The increased presence of renal M1-macrophages, and increased NF-κB expression, plasma/renal levels of TNF-α as well as activation of the TGFβ-system in the kidneys of obese animals, suggests that inflammatory cells play a central role in these processes.[98–100,103,104,106]

In conclusion, the increased fat or lipid deposition in and around the kidney acts as a promotor of a pro-inflammatory state with oxidative stress, endothelial dysfunction and microvascular remodeling as a consequence. Although these changes are initially reversible by switching to a healthy life style,[104] as well as by interventions that lower lipids and/or oxidative stress,[105,108] modulate the immune-system[102] or prevent vasoconstriction,[98,103] prolonged exposure results in irreversible alterations in the renal microvasculature. Indeed, although diabetes mellitus and hypertension are still the main causes of chronic kidney disease (CKD),[112] it has been shown that obesity independently increases the risk of CKD and end-stage renal disease even in the absence of

these known cardiovascular risk factors or nephropathy.[113] As the kidney is important in clearing waste products and adipokines from the body, renal dysfunction in obesity and the ensuing increase in so-called uremic toxins, can further contribute to microvascular dysfunction in other organs and thereby contribute to development and aggravation of cardiovascular disease.[114]

7. Pulmonary microvasculature in obesity

Functional and structural changes in the pulmonary microvasculature as a result of obesity are less well studied as compared to their systemic counterparts. The comparison of the effect of obesity in the pulmonary and systemic microvasculature is of interest as the pulmonary vasculature receives the same cardiac output as the systemic vasculature and is exposed to the same circulating factors, such as glucose, cholesterol, adipokines, and inflammatory factors. Indeed, obesity is also associated with a variety of lung diseases including obstructive sleep apnea, hypoventilation syndrome, chronic bronchitis, asthma, and pulmonary embolism.[115] Many of these diseases have an inflammatory component and it is likely that the change in circulating adipokines with obesity facilitates this pulmonary inflammation. Adiponectin exerts anti-inflammatory and protective effects against inflammatory lung diseases.[115] In contrast, leptin is pro-inflammatory and leptin receptors are present on all inflammatory cell-types in the lung. An increase in leptin primes leucocytes for increased secretion of inflammatory cytokines and reactive oxygen species.[115] However, exactly how a change in adipokine profile impacts pulmonary microvascular structure and function remains to be established.

An autopsy study in 1982 revealed a strong correlation between the size of atherosclerotic plaques in the aorta and the pulmonary artery.[116] In rabbits on a high fat diet, the rate of cholesterol accumulation in the pulmonary artery exceeded that in the aortic arch initially, but at later stages of atherogenesis, the rate of cholesterol accumulation slowed in the pulmonary artery ultimately falling below accumulation rates in the aortic arch.[117] Obesity is also associated with structural changes in the pulmonary microvasculature (**Table 1**). Increased medial thickness of both pulmonary small arteries and veins, and increased muscularization of pulmonary arterioles were observed in obese humans compared to controls at autopsy.[118] Similarly, an increased wall to lumen ratio was found in pulmonary arterioles of obese rats as compared to lean control rats.[119] Interestingly, adiponectin deficiency has been shown to result in pulmonary microvascular remodeling, with an increased muscularizaton of pulmonary microvessels.[120]

These structural pulmonary microvascular changes in obesity resemble those found in post-capillary pulmonary hypertension. Indeed, the prevalence of pulmonary hypertension (PH) is increased in obese subjects, and is associated not only with sleep apnea and hypoxemia, but also with left ventricular diastolic dysfunction, resulting in increased left atrial pressure.[121] The association between obesity and elevated pulmonary artery pressures has also been found in both obesity prone and overtly obese Zucker rats on a high fat diet[119] and in obese FH-swine.[39,40] Similar to humans, the elevated pulmonary artery pressure in the obese swine was mainly due to an increase in left atrial pressure as pulmonary vascular resistance was only mildly elevated.[39,40] However, despite the mildly elevated pulmonary artery pressure and pulmonary vascular resistance at rest, the pulmonary vasodilator response to exercise was preserved in swine with hypercholesterolemia, at a time when systemic vasodilation was reduced.[39,40]

The effect of obesity and high fat diet on pulmonary vascular function has only been assessed in experimental animals and may depend on the duration of exposure and the stimulus used to assess vascular function. Thus, pulmonary artery vasodilation in response to methacholine is enhanced in rabbits given a 2% cholesterol diet over a period of 2 weeks.[122] Exposure of Zucker rats to high fat diet for 18 weeks has no effect on eNOS expression or acetylcholine-induced NO production in either conductance or resistance pulmonary arteries.[123] Similarly, the increase in pulmonary vascular resistance in response to eNOS inhibition is comparable in healthy swine and in FH-swine on a high fat diet for 6 months, both at rest and during exercise, although ATP-induced NO mediated vasodilation was reduced.[40] Also, the pulmonary vasodilator response to SNP is maintained in both obese Zucker rats[123] and FH-swine[40] exposed to high fat diet. In the latter group, vasodilation to PDE5-inhibition is also preserved. Altogether, these data suggest that pulmonary vasodilation through the NO pathway is well-preserved in obesity (**Table 1**).

In general, vasoconstrictor responses in the pulmonary microvasculature are reduced in obesity. Thus, vasoconstriction to KCl, phenylephrine, serotonin, and hypoxia are reduced in pulmonary resistance, but not conductance arteries of obese Zucker rats.[123] In FH swine, endothelin receptor blockade did not reduce pulmonary vascular resistance, while it produced pronounced vasodilation in normal swine (**Table 1**).[39] This loss of ET-mediated vasoconstriction was accompanied by slightly lower plasma ET-levels. These data suggest that reducing the effect of vasoconstrictors may serve as an early compensatory mechanism to maintain pulmonary vascular resistance as low as possible, to limit the workload of the right ventricle.

8. Obesity paradox

The term obesity paradox refers to the observation that although obesity is a well-known risk factor for the development of cardiovascular disease, mortality rate in many cardiovascular disorders, once established, is lower in obese patients.[1] Thus, there is evidence suggesting that when CKD is present, mortality is higher in underweight patients and lower in patients with obesity class I, but not with class II or III.[124] Similarly, in patients with established coronary artery disease,[125] heart failure,[126] or stroke,[127,128] there are large cohort studies suggesting that mortality is reduced in patients with obesity class I, particularly in the short term. Also in patients with established PH, a recent study shows a strong inverse correlation between obesity and mortality, in that both in pre-capillary and out-of proportion post-capillary PH, obese patients had a significantly lower mortality (46% vs. 10% mortality in pre-capillary and 40% vs. 11% in post-capillary PH for lean and obese subjects). Moreover, BMI was the strongest predictor of mortality in a COX hazard analysis, followed by NYHA functional class.[129]

However, there is also concern about selection bias, insufficient control for cardiorespiratory fitness, inadequate determination of body fat localization (i.e. visceral vs. subcutaneous), and underweight vs. normal weight subjects, in these studies. Indeed, the overall consensus is that losing weight by for example exercise trainings confers protection against mortality in cardiovascular disease.[1] A large part from these benefits of exercise training can be ascribed to improved microvascular function, and reduced inflammation. Another intriguing possibility is that exercise training results in secretion of 'myokines' from skeletal muscle and 'cardiomyokines' from cardiac muscle such as FGF21 and irisin, that act in an endo- and/or paracrine fashion on perivascular and epicardial adipose tissue, and induce a 'browning' phenotype.[22]

9. Summary and conclusion

Obesity is a well-established risk factor for microvascular dysfunction throughout the body (*Figure 1*). This microvascular dysfunction is likely initiated by transient elevations of circulating free fatty acids, and perpetuated by changes in adipokines and inflammatory cytokines released from visceral as well as perivascular adipose tissue. These factors contribute to endothelial dysfunction as well as insulin resistance in the microvasculature, thereby affecting function of different organs not only by impairing tissue perfusion, but also through altering the release of paracrine factors from the endothelial cells. Thus, although the mechanisms can differ between regional vascular beds (**Table 1**), microvascular dysfunction is a central common pathway that may explain exercise-intolerance as well as the higher

prevalence of chronic kidney disease, microvascular dementia, coronary microvascular angina, heart failure with preserved ejection fraction, and pulmonary hypertension in obese subjects.

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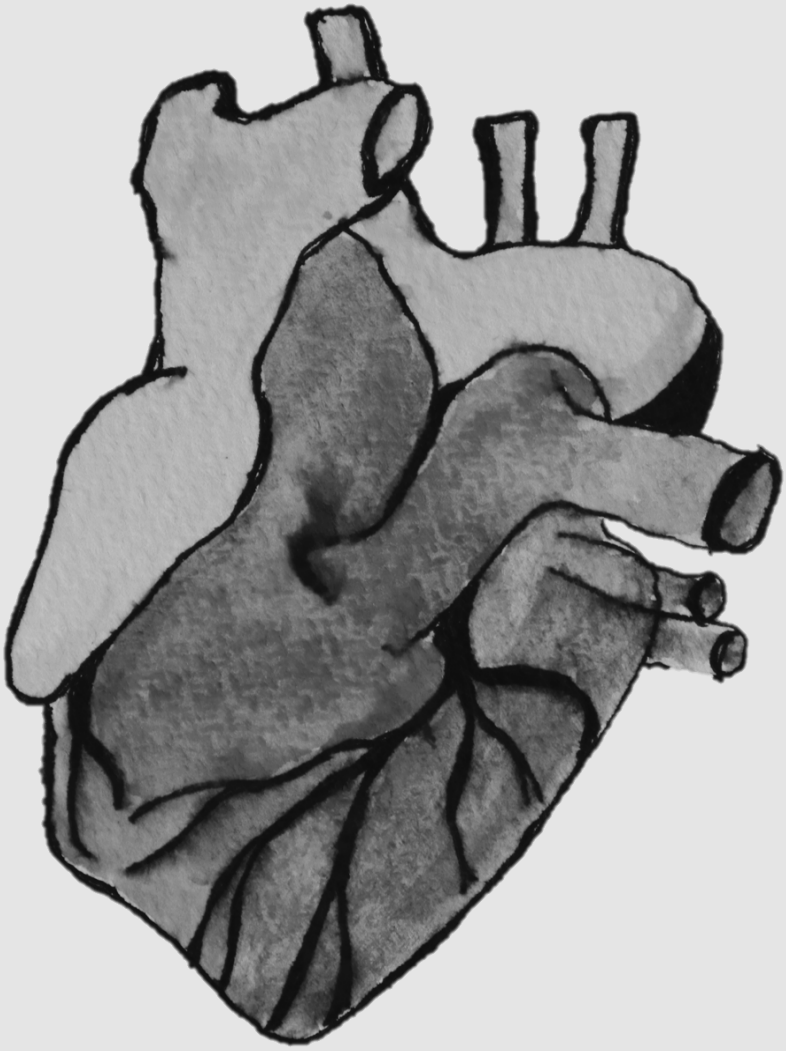

Chapter 3

Experimental animal models of coronary microvascular dysfunction

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Abstract

Coronary microvascular dysfunction (CMD) is commonly present in patients with metabolic derangements and is increasingly recognized as an important contributor to myocardial ischemia, both in the presence and absence of epicardial coronary atherosclerosis. The latter condition is termed 'ischemia with non-obstructive coronary arteries' (INOCA). Notwithstanding the high prevalence of INOCA, effective treatment remains elusive. Although to date there is no animal model for INOCA, animal models of CMD, one of the hallmarks of INOCA, offer excellent test models for enhancing our understanding of pathophysiology of CMD and for investigating novel therapies. This article presents an overview of currently available experimental models of CMD – with an emphasis on metabolic derangements as risk factors – in dogs, swine, rabbits, rats and mice. In all the available animal models, metabolic derangements are most often induced by a high fat diet and/or diabetes mellitus via injection of alloxan or streptozotocin, but there is also a wide variety of spontaneous as well as transgenic animal models which develop metabolic derangements. Depending on number, severity and duration of exposure to risk factors — all these animal models show perturbations in coronary microvascular (endothelial) function and structure, similar to what has been observed in patients with INOCA and co-morbid conditions. The use of these animal models will be instrumental in identifying novel therapeutic targets and for the subsequent development and testing of novel therapeutic interventions to combat ischemic heart disease, the number one cause of death worldwide.

1. Introduction

Common risk factors for cardiovascular disease, including diabetes mellitus (DM), dyslipidaemia, hypercholesterolaemia, and chronic kidney disease (CKD), are independently, but especially in combination, well-known risk factors for the development of coronary artery disease (CAD) of both large epicardial arteries and smaller coronary arteries.[1–4] While it is well-established that obstructive CAD is a major cause of myocardial ischaemia,[5] there is increasing evidence that coronary microvascular dysfunction (CMD) also contributes to myocardial ischaemia, not only in the presence of obstructive CAD[6–8] but also in patients without obstructive CAD, a situation referred to as 'ischaemia and no obstructive coronary artery disease' (INOCA).[2,9,10] Clinical studies have shown that INOCA is present in approximately one-third of men and two-thirds of women undergoing angiography for suspected ischaemic heart disease.[11,12] Importantly, cardiovascular death or myocardial infarction occurred in 6.7% of the patients without any signs of CAD and in 12.8% of patients with non-obstructive CAD.[11,12]

Since INOCA has only recently been recognized as a separate clinical entity, its exact definition and the underlying pathophysiology are not well-established yet.[9] The potential multitude of factors underlying ischaemia in these patients underscores the complexity of the disease and simultaneously presents a diagnostic and therapeutic challenge.[13] The current diagnostic workup for chest pain is not optimized for determining the different INOCA aetiologies, and INOCA is currently mainly used as a diagnosis *per exclusionem* in patients with non-obstructive CAD. Recently, the 'CORonary MICrovascular Angina (CorMicA)' study and the working group of INOCA of the American College of Cardiology have proposed a similar diagnostic flowchart.[9,13] According to these experts, a diagnostic flowchart for INOCA encompasses a three-step approach, including invasive coronary angiography for the evaluation of coronary obstructions with invasive diagnostic fractional flow reserve (FFR) if needed, coronary flow reserve (CFR) measurements for the evaluation of microvascular dysfunction, and a vasoreactivity test to acetylcholine and a nitrate for the assessment of endothelial dysfunction with/without vasospasm. Such an approach could discriminate patients with epicardial vasospastic angina vs. microvascular angina and enables evaluation of a tailored treatment between these groups. Although studies with non-invasive techniques, including positron emission tomography (PET), transthoracic echo-Doppler, and cardiac magnetic resonance imaging, have shown some promising results, invasive testing is currently still considered the gold-standard.[9]

An important limitation of patient studies is that the disease is only diagnosed when patients present with overt complaints, and hence the differentiation into the various angina subtypes, based on coronary function, typically occurs at a later stage, at a time when the (potentially synergistic)

contributions of individual risk factors, including diabetes, hypercholesterolaemia, CKD, hypertension, and even sedentary lifestyle are difficult to assess. Longitudinal, mechanistic invasive studies considering individual comorbidities, age and sex, should be performed to identify the different patient subgroups. However, such studies in patients are very difficult given the complexity of the disease and co-occurrence of risk factors. In addition, structural microvascular alterations, including arteriolar remodelling and capillary rarefaction that can contribute to impaired CFR and myocardial oxygen delivery, are also difficult to assess in clinical studies. For this purpose, animal models are instrumental, as influences of metabolic factors, genetic predisposition, sex and age on the development of perturbations in coronary microvascular function and structure, as well as the progression of CMD, can be thoroughly studied.

In this review, we focus on the different animal models for CMD, which is a critical hallmark of INOCA. Since each animal model has its advantages and disadvantages, the specific research question should be the prime determinant of the animal model of choice. It is therefore important to take the (pitfalls for) translation to the clinical setting into account when selecting an animal model. Here, we present an overview of different models for studying CMD in the setting of metabolic derangements in commonly used animal species. We discuss the different ways to induce metabolic derangement, the resulting microvascular dysfunction, and the underlying mechanisms for each individual model. Subsequently, the models are evaluated and compared with respect to their translational capacity for the study of INOCA.

2. Animal models: anatomical and metabolic considerations

A variety of animal species and models has been employed to study the effects of different risk factors on the development and progression of CMD. The cardiovascular system of each species has evolved differently in order to meet the demands of that species and has specific similarities and differences with the human cardiovascular system. In this section, we will provide an overview of the most important similarities and differences in terms of coronary anatomy and body and myocardial metabolism.

2.1 Anatomical considerations

Large animal species (canine and porcine) have been widely used to study ischaemic heart disease. Dogs, pigs, and humans have been shown to vary with respect to the anatomic distribution of their coronary arteries. In all these species, the coronary arteries and their main branches run on the epicardial surface. The coronary vasculature in humans is mostly right dominant, implying that in most

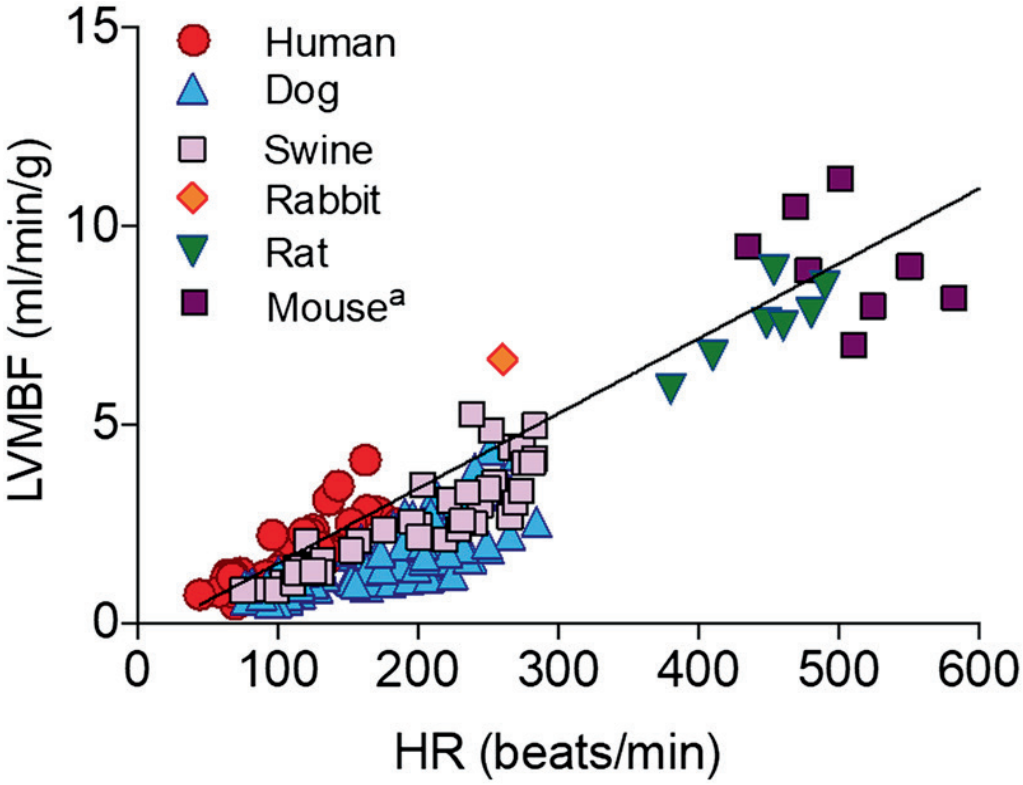
humans the right coronary artery supplies the right ventricle as well as the posterior wall of the left ventricle with blood, whereas the anterior and lateral wall of the left ventricle are perfused via the left anterior descending coronary artery and the left circumflex coronary artery. Humans have no separate septal artery, and the ventricular septum is supplied through perforating vessels that originate from the interventricular branches.[14] Similarly, the porcine coronary circulation is right dominant, while dogs are left dominant.[15] Humans and swine are also similar with respect to the vascularization of the interventricular septum, which is supplied by anterior and posterior septal branches arising from the left and right coronary arteries, respectively. In addition, the atrioventricular node and the bundle of His in both humans and swine are irrigated predominantly by the posterior septal branch.[16] This anatomical resemblance implies that ischaemia-associated injury to the conduction system of the swine heart is analogous to that in humans, contrary to the canine model, in which the blood supply originates from the anterior septal artery.[17] Importantly, while an innate coronary collateral circulation is negligible in humans and swine,[18,19] there is an extensive pre-existing collateral circulation in the dog heart, which—depending on the dog breed—can supply as much as 40% of the blood flow distal to an occluded coronary artery.[20] In rabbits, the left coronary artery is always the dominant artery, from which the septal artery originates. In rodents, the coronary anatomy differs markedly from that in large animals. Thus, coronary arteries run deeper in the myocardium and contain fewer layers of smooth muscle cells. In the mouse, the heart is supplied by two coronary arteries, left and right, each perfusing the corresponding ventricle. Studies have demonstrated a single major septal coronary artery arising either from a separate ostium from the right sinus of Valsalva or as a proximal branch of the right coronary artery.[21] The mouse septal coronary artery courses along the right side of the interventricular septum and provides perfusion to this region of the myocardium.[21] In the rat, the coronary anatomy is similar to that in mice, although coronary angiography in Lewis rats indicates that the septal artery branches off either from the proximal part of the left coronary artery (60% of the animals) or the right coronary artery (40%).[22] Furthermore, coronary collateral blood flow capacity is low, but not negligible, in rabbits,[23] rats,[19] and mice.[24]

2.2 Metabolic considerations

Blood pressure is similar among mammalian species, independent of body weight.[25] The heart is the most energy-requiring organ of the body, with heart rate being the most important determinant of oxygen consumption. There are significant intrinsic differences between rodents and the large mammals in terms of heart rate, but also oxygen consumption, and metabolic activity, partly mediated by species-specific activity of thyroid hormones.[26] Humans, dogs, and pigs have similar heart rates,

60–80 b.p.m. in rest up to 200 b.p.m. (humans) or 300 (dog, pig) during maximal exercise,[27] while resting heart rates are 200–250 b.p.m. in rabbits,[28,29] 350–400 b.p.m. in rats,[25] and 500–600 b.p.m. in mice.[30,31] Dynamic exercise increases left ventricular coronary blood flow (CBF) in proportion to the increase in heart rate (**Figure 1**).[30–36] CBF measurement during exercise is very difficult to perform in mice, and to our knowledge, no exercise blood flow data are available. However, the few studies performed in anaesthetized mice[30,31] as well as in awake rats[35] indicate high flow values already under resting conditions, i.e. five to six times higher than in humans or large mammals, which appears to be entirely due to the higher resting heart rates (**Figure 1**).[35]

Figure 1 Relation between HR and LVMBF at rest and during exercise in dogs, swine, rats, mice, and humans



For awake rabbits only a single resting measurement is available.[34],[a]There are no measurements available in awake mice. Note that the regression line is based on the human data points. Adapted with permission from Duncker *et al*.[35] HR, heart rate; LVMBF, left ventricular myocardial blood flow.

In adult fasting mammals, 60–80% of the cardiac energy metabolism relies on the oxidation of free fatty acids, the rest being accounted for by glucose, lactate, and ketone bodies.[37] However, there are species differences, as mice rely more on glucose, lactate, and ketone bodies and much less (30–40%) on fatty acids.[38] In addition, also in terms of lipid transporters in the blood—with low-density lipoprotein (LDL) and high-density lipoprotein (HDL) being the predominant carriers of cholesterol—there are major species differences in the proportion of LDL and HDL particles in plasma. Thus, pigs and rabbits transport most of the cholesterol in LDL particles—as do humans (~60% LDL, ~40% HDL)—while rodent species carry the majority of cholesterol in HDL, making rodents virtually resistant to atherosclerosis and less appropriate as models for dyslipidaemia and atherosclerosis.[39]

In line with the high heart rate in mice, basal metabolic rate per gram body weight is also seven times greater in mice than in humans. Such differences in metabolic rate have a major effect on reactive oxygen species (ROS) production in these species.[40,41] One of the consequences of high metabolism in mice is that, in response to tissue hypoxia, mice are capable of reducing their oxygen utilization by down-regulating the activity of mitochondrial uncoupling proteins (responsible for a significant part of overall O_2 consumption), without affecting ATP production. Hence, interventions targeting or affecting cellular metabolism in mouse models may not predict efficacy in humans.[42] Rats have a larger blood volume when compared with mice, however, they are particularly resistant to oxidative stress due to their high activity of tissue antioxidant enzymes. Furthermore, nitric oxide (NO) metabolites in blood are 10–20 times higher than those in humans, potentially limiting translational impact of studies involving NO and ROS in this species.[43] Given the importance of NO and oxidative stress in the regulation of coronary microvascular function, such interspecies differences are highly relevant in the choice of animal model for the study of CMD.

3. Large animal models

3.1 Canine models of CMD

The dog represents a large animal model that has traditionally been employed to study CBF regulation in health and disease[32,44,45] including in studies pertaining to the influence of cardiovascular risk factors on CBF and regulation of coronary microvascular tone, (see Supplementary material online, Table S1). To induce metabolic derangement in dogs, alloxan-induced DM and/or HFD-induced obesity that is associated with dyslipidaemia, hypertension, and insulin resistance, have been used. These canine models are highly relevant for the study of metabolic derangements and their influence on microvascular function.

3.1.1 Canine models with alloxan-induced DM

Insights in some of the alterations produced by longer exposure to metabolic derangement is offered by studies in mongrel dogs with alloxan-induced DM. A strength of this model is that measurements can be performed before and after DM induction, allowing for assessment of DM effects within the same animal. Alloxan is typically administered in a dose of 40–60 mg/kg i.v., which results in robust hyperglycaemia and hypoinsulinaemia without the need of insulin treatment.[46] Following the induction of DM, which stabilizes within 1 week of alloxan infusion, resting CBF gradually decreased to ~60% of its pre-DM value, at 5 weeks of follow-up.[46] Vasodilation in response to adenosine and acetylcholine was impaired in the absence of narrowing of the large coronary arteries, suggesting the presence of coronary microvascular endothelial dysfunction. Tune *et al*.[47] studied the haemodynamic alterations after only 1 week of alloxan-induced DM at rest and during graded treadmill exercise. Although resting CBF was unaltered 1 week post-alloxan, the exercise-induced increase in CBF was progressively impaired. The limitation of myocardial oxygen delivery during exercise elicited an increase in myocardial oxygen extraction, thereby resulting in lower coronary venous oxygen tensions at each level of exercise.[47] The authors demonstrated in a subsequent study that α-adrenoceptor blockade augmented the exercise-induced increase in CBF and attenuated the decrease in coronary venous oxygen tension to a greater extent in DM than in non-DM dogs, indicating that α-adrenoceptor-mediated coronary vasoconstriction was increased in diabetic dogs, particularly during increased metabolic demand.[48] Adenosine triphosphate-sensitive potassium (K_{ATP}) channel blockade with glibenclamide[49] blunted the coronary hyperaemia during exercise in DM, but not in healthy dogs, suggesting that K_{ATP} channels exert an increased coronary vasodilator influence in DM dogs, acting to maintain CBF during increased myocardial metabolism and increased sympathetic activation during exercise.

3.1.2 Canine models with diet-induced metabolic derangement

Several studies in dogs of either sex have investigated the effects of chronic metabolic derangement produced by 5–6 weeks of HFD on the coronary microvasculature. Such exposure to HFD resulted in features of the metabolic syndrome (MetS) with obesity, moderate hypertension, dyslipidaemia, and insulin resistance. These factors impaired myocardial perfusion, especially during exercise, by decreasing coronary vascular conductance, although myocardial ischaemia was absent.[50] The tonic constriction of the coronary vasculature in this setting was mediated by metabolic derangement-associated neurohumoral alterations, including increased vasoconstriction mediated by angiotensin II (ANGII),[51] activation of the sympathetic nervous system,[52] and the endothelium-derived

vasoconstrictor, endothelin 1 (ET-1),[53] as tested in a series of consecutive studies in male and female dogs.

Zhang *et al*.,[51] investigated the involvement of prediabetic metabolic derangement-associated activation of the renin–angiotensin–aldosterone system (RAAS) on CBF regulation. Metabolic derangement was associated with increased plasma renin activity and elevated ANGII levels, resulting in a significantly increased ANGII-induced vasoconstriction, mediated by angiotensin II receptor type 1 (AT_1) receptors in isolated arterioles. Further interrogation of this mechanism *in vivo* supported the *in vitro* data, showing an impaired exercise-induced increase in CBF that was alleviated by AT_1 receptor blockade. Taken together, these data suggested that in the canine model of prediabetic metabolic derangement, chronic activation of the RAAS contributes to coronary vascular dysfunction, via increase in circulating ANGII and/or increases in coronary arteriolar AT_1 receptor density. The increase in sympathetic activity associated with the metabolic derangement and its effects on coronary circulation were investigated in the same model by Dincer *et al.*[52] Although baseline CBF was not altered *in vivo*, increased plasma epinephrine concentrations were associated with augmented α_1-adrenoceptor-mediated coronary vasoconstrictor responses in anaesthetized dogs. Sensitization of α-adrenoceptor signalling represents a potentially important contributor to impaired control of CBF. A third important vasoconstrictor involved in the control of CBF and shown to be increased in metabolic derangement is ET-1. Knudson *et al.*[53] tested the hypothesis that prediabetic metabolic derangement augments endothelin receptor A (ET_A)-mediated vasoconstriction, thereby limiting coronary perfusion. Interestingly, despite a reduction in ET_A expression in the coronary microvasculature, coronary vasoconstrictor responses to ET-1 were not different between HFD-fed dogs and normal dogs, while circulating ET-1 levels were unaltered, suggesting either a sensitization of the ET_A receptors or an increased contribution of endothelin receptor B (ET_B)-mediated vasoconstriction. The reduction in ET_A receptor expression may represent an early compensatory mechanism acting to maintain CBF in the face of increased ANGII- and α_1-adrenoceptor-mediated coronary vasoconstrictor influences.

Adipokine production is altered in the MetS, and to investigate the potential contribution of adipokines to CMD, Tune and colleagues performed a series of experiments in which they investigated the coronary microvascular responses to a variety of adipokines. Payne *et al*.[54] investigated the effect of various endogenous adipose-derived factors on coronary endothelial function, by infusing adipose-tissue-conditioned buffer in healthy, lean dogs. Although baseline CBF remained unaltered, coronary endothelial dysfunction was observed both *in vivo* and *in vitro*. Endothelial dysfunction was the result of reduced NO bioavailability, possibly via selective inhibition of endothelial nitric oxide synthase

(eNOS), independent of oxidative stress. Although the specific adipokine(s) responsible for the alterations in the latter study were not identified, these data show that adipokine administration does result in acute endothelial dysfunction, by altering different endothelial vasodilator mechanisms, suggesting that chronic exposure to circulating adipose-tissue-derived factors can potentially result in vascular dysfunction. To further delineate the precise adipose-tissue-derived factor(s), Dick *et al*.[55] set out to test whether acute infusion of resistin, an adipokine implicated in endothelial dysfunction in obesity affected CBF *in vivo* in healthy dogs, by increasing oxidative stress. At concentrations observed in obese, type 2 DM (T2DM) patients, resistin did not affect CBF. However, it did produce endothelial dysfunction, both *in vivo* and in isolated coronary arterioles, which was likely endothelium-derived hyperpolarizing factor (EDHF)-mediated, as the bradykinin-induced vasodilation was impaired, while acetylcholine (ACh)-induced response was maintained. Furthermore, this response was independent of oxidative stress. Knudson *et al*.[56] investigated the direct effects of leptin, an adipokine involved in several biological processes including glucose metabolism and inflammation, on the coronary circulation and specifically on coronary endothelial function in healthy dogs as well as dogs with metabolic derangement. In normal healthy dogs, leptin produced coronary microvascular endothelial dysfunction characterized by a blunted vasodilator response to acetylcholine. In contrast, and despite increased leptin plasma concentration, prediabetic high-fat-fed dogs had normal coronary microvascular vasodilator responses to acetylcholine. Interestingly, the coronary vasodilator response to ACh was not affected by leptin. These findings suggest that resistance to leptin-induced endothelial dysfunction may represent a protective mechanism at this early stage of the disease.

3.1.3 Summary: canine models of CMD

Taken together, alloxan-induced DM or exposure to HFD in dogs of either sex, for up to 5–6 weeks results in altered coronary microvascular tone control, involving increased coronary vasoconstrictor mechanisms such as α-adrenoceptor-mediated and ANGII-mediated coronary vasoconstriction. These increased vasoconstrictor influences result in perturbations in CBF regulation and myocardial oxygen delivery but are partly mitigated by compensatory reductions in ET_A receptor density and by increased leptin resistance and K_{ATP} channel activation (**Table 1**). Future studies are required to determine whether long-term exposure to these cardiovascular risk factors produces progressive perturbations in coronary microvascular function during disease progression.

Table 1 Main mechanisms involved in CMD per animal model

	Functional							Structural	
	Endothelial-dependent				Neurohumoral		VSMC	Arteriolar	Capillary
	↓NO	↑ROS	↑ET-1	↓PGI_2	↑RAAS	↑SNS	↓function	↑ Media thickness	↓Density
Canine models									
Alloxan	+	NA	NA	NA	NA	+	+	NA	NA
High fat diet	NA	NA	-	NA	+	+	-	NA	NA
Acute	~	-	NA	-	NA	NA	-	NA	NA
Porcine models									
Induced domestic	+	+	~	NA	NA	NA	~	NA	+
Induced Yucatan	~	NA	NA	-	NA	NA	+	NA	NA
Rapacz FH	+	NA	NA	NA	NA	NA	-	NA	NA
Ossabaw	~	NA	NA	NA	NA	NA	~	NA	+
Rabbit models									
Alloxan	+	NA	NA	+	NA	NA	-	NA	NA
High fat diet	~	+	+	NA	NA	+	~	+	NA
WHHL	NA	NA	NA	NA	NA	NA	-	NA	NA
Rat models									
Streptozotocine	~	-	NA	-	+	NA	~	~	~
High fat diet	+	NA	NA	NA	+	NA	~	NA	-
Zucker	~	+	-	~	NA	~	-	-	NA
OLETF	~	+	+	NA	+	NA	-	+	NA
GK	~	NA	NA	-	NA	+	+	+	-
Murine models									
db/db	+	+	NA	NA	+	NA	~	~	~
ob/ob	+	NA	NA	NA	NA	NA	-	NA	-
Induced T1+2DM	+	+	+	NA	NA	+	+	+	NA
apoE	+	+	NA	-	NA	NA	-	NA	NA

Overview of models of coronary microvascular dysfunction per species and what features are present (+), absent (-), ambiguous results (~), or not investigated (NA; not assessed). VSMC vascular smooth muscle cell, NO nitric oxide, ROS reactive oxygen species, ET-1 endothelin 1, PGI_2 prostacyclin, RAAS renin angiotensin aldosterone system, SNS sympathetic nervous system, FH familial hypercholesterolemia, WHHL Watanabe heritable hyperlipidemic rabbit, Zucker Zucker obese and diabetic fatty rats, OLETF Otsuka Long-Evans Tokushima Fatty rat, GK Goto-Kakizaki non obese diabetic rat, db/db leptin receptor deficient mouse, ob/ob leptin deficient mouse, T1+2DM Type 1 and 2 diabetes mellitus, apoE apolipoprotein E knockout mouse

3.2 Porcine models of CMD

Swine are widely used in translational cardiovascular research, as they share many similarities with humans with respect to coronary and cardiac anatomy and physiology. Furthermore, swine are omnivores and have a human-like myocardial metabolism.[57] Compared with dogs, domestic swine have the advantage of lower cost and societal pressure. Unfortunately, the fast growth rate of domestic swine limits study duration and favours utilization of juvenile swine, for a disease that occurs in an ageing human population. These pitfalls can be circumvented by the availability of different strains of mini-swine. Different porcine models of CMD have been generated over the past 20 years, typically by exposure to risk factors that are common in patients with ischaemic heart disease including dyslipidaemia, DM, CKD, and hypertension. In addition, several swine strains with increased genetic susceptibility to developing metabolic derangement—particularly when exposed to these risk factors—have been identified, characterized, and inbred and are now being used in a variety of experimental studies into coronary microvascular function in health and disease. Finally, a transgenic porcine model of diabetes has recently been established.[58]

3.2.1 Domestic swine

For over 40 years, domestic swine have been employed in studies focusing on the regulation of CBF in health and ischaemic heart disease.[32,45] Studies pertaining to the effects of different risk factors on coronary microvascular function have for the most part been published in the past 10 years (Supplementary material online, Table S2). In these studies, different methods to induce risk factors have been used, including high-fat/high-sugar diets either as single intervention or in combination with streptozotocin (STZ)-induced hyperglycaemia and/or CKD-associated hypertension.

Gerrity *et al.*[59] and Ditzhuijzen *et al.*[60] demonstrated that in young (12 weeks of age) male domestic swine, <20 weeks of HFD (1–1.5% cholesterol and 15-25% lard), or a combination of HFD and STZ-induced DM did not result in flow-limiting coronary lesions. We also observed that although 10 weeks of HFD and DM by low-dose STZ in domestic swine did not induce coronary arterial lesions, CMD was already present at this early timepoint.[61] Thus, epicardial conduit artery function was unaltered, but isolated coronary small arteries (~300 μm in diameter) showed impaired endothelial function in DM swine that were fed a HFD. CMD was characterized by impaired NO bioavailability, while EDHF-dependent mechanisms were not affected, and the overall vascular smooth muscle cell (VSMC) sensitivity to NO was preserved.[61] The endothelial dysfunction-associated reduction in vasorelaxation was accompanied by a markedly reduced ET_A-receptor-mediated vasoconstrictor response to ET-1, possibly serving as a compensatory mechanism for the increase in circulating ET-1 levels and the loss

of NO bioavailability, at this early stage of the disease. Furthermore, 10 weeks of DM+HFD resulted in increased microvascular passive stiffness, potentially further contributing to perturbations in coronary microvascular function *in vivo*.[61] Indeed, Mannheim *et al.*[62] observed impaired CBF responses to intravenous adenosine in swine after approximately 3 months of HFD, suggesting that the early alterations in coronary microvascular control mechanisms as observed *in vitro* can indeed translate into impaired CBF responses *in vivo*.

Interestingly, in our initial study, no changes in coronary microvascular responses to bradykinin or ET-1 were observed in non-DM animals fed the same HFD.[61] The latter observation appears to be in contrast to an early study by Hasdai *et al.*,[63] who reported increased vasoconstriction of small arteries (~500 µm diameter) to ET-1 *in vitro*, in swine after 10–13 weeks of HFD. These different results are not readily explained but may well stem from differences in vessel size (300 µm[61] vs. 500 µm[63]), sex (male[61] vs. female[63]), or the diet composition (1% cholesterol, 25% saturated fats, 20% fructose/20% sucrose[61] vs. 2% cholesterol, 20% lard, and 1% hog bile extract[63]).

In a subsequent study, we subjected male swine with or without STZ-induced DM to HFD for 15 months and observed significant functional and structural alterations in the coronary vascular bed in both large and small arteries.[64] Thus, at this stage of the disease, plaques were found in epicardial conduit arteries (albeit not flow-limiting; i.e. <30% plaque burden) and in coronary small arteries, while the latter also showed increased passive stiffness. Moreover, microvascular tone control studies showed a normal endothelium-dependent bradykinin-induced vasorelaxation that was accompanied by an enhanced ET_B-receptor-mediated vasoconstrictor response to ET-1.[64] Interestingly, these alterations were principally the result of the HFD and independent of the presence of DM. Taken together these two studies reveal a surprising shift from an early blunting of endothelium-dependent vasorelaxation at 10 weeks[61] towards a late 'normalization' of endothelium-dependent vasorelaxation at 15 months,[64] that was accompanied by a shift from an early blunting[61] to a late augmentation[64] of the vasoconstrictor responses to ET-1. These results underscore the importance of performing longitudinal studies, as the mechanisms of microvascular dysfunction were highly dependent on the duration of exposure to the cardiovascular risk factors.

When CKD was combined with hypercholesterolaemia and metabolic derangement for 4–5 months, sustained inflammation and oxidative stress were associated with impaired coronary vasodilation to adenosine and bradykinin, suggestive of endothelial dysfunction.[65,66] These early microvascular functional alterations were accompanied by a reduced myocardial capillary density, and together may lead to impaired CBF and oxygen delivery, thereby contributing to INOCA.

Further studies directed at a complete characterization of CMD at different stages of the disease are needed, to potentially provide new therapeutic strategies aiming at alleviating microvascular disease and improving myocardial perfusion. However, such studies are difficult to conduct in domestic pigs, as their body growth limits follow-up time. Mini-swine may offer an alternative due to their limited growth rate and size at maturity.

3.2.2 Yucatan mini-swine

Yucatan mini-swine are commonly used for the study of CAD, due to their ability, similar to domestic pigs, to reproduce the neointimal formation and thrombosis as observed in humans.[67] As with domestic swine, metabolic derangement can be induced using chemical destruction of the pancreatic beta cells and/or a HFD.[68] Twenty weeks of HFD resulted in dyslipidaemia in male Yucatan mini-swine without changes in plasma glucose.[69] At this time point, isolated coronary arterioles (~100 µm in diameter) showed only very limited endothelial dysfunction as assessed by the responses to bradykinin, adenosine diphosphate (ADP) and flow-mediated dilation, despite increased microvascular spontaneous tone in HFD animals and lower eNOS protein content. These modest microvascular perturbations were alleviated by exercise training.[69]

The addition of alloxan-induced DM as an additional cardiovascular risk factor resulted in significant decreases in both basal and hyperaemic CBF in response to adenosine and resulted in endothelial dysfunction as assessed with bradykinin *in vivo*.[70] While exercise training alleviated the impairment in basal perfusion and CFR, endothelial dysfunction was not affected by exercise training.[70] These studies show that Yucatan mini-swine subjected to DM in combination with a HFD are an excellent model for long-term studies of the pathophysiology of and therapeutic interventions for CMD.

3.2.3 Ossabaw swine

Ossabaw swine represent a unique model for the study of MetS and CAD. These swine have a 'thrifty genotype' (propensity to obesity) that enabled them to survive long periods of scarce food conditions on Ossabaw Island off the coast of Savannah, Georgia. Consumption of excess kcal (i.e. HFD) causes these animals to manifest components of the MetS, including central (intra-abdominal) obesity, insulin resistance, impaired glucose tolerance, dyslipidaemia, and hypertension, and progress towards T2DM and eventually coronary atherosclerosis.[71] When studied in parallel, male Ossabaw swine had higher glucose-intolerance and insulin resistance after 43 weeks of HFD vs. male Yucatan swine.[68] Furthermore, CFR in response to intracoronary adenosine was impaired and endothelial dysfunction, as evidenced by the response to intracoronary bradykinin infusion, was greater in Ossabaw compared with Yucatan swine either on normal chow or on HFD. In addition, Ca^{2+} efflux was

impaired in coronary smooth muscle cells from HFD fed Ossabaw vs. Yucatan swine. These coronary vascular alterations were accompanied by diffuse CAD in Ossabaw but not in Yucatan swine on HFD.[68]

Also in 7–10 weeks old male Ossabaw swine, a short (9 weeks) exposure to a high-fat, high-fructose diet resulted in early-stage MetS, with obesity, hyperglycaemia and dyslipidemia.[72] However, at this early stage, CBF both at baseline and during intracoronary adenosine infusion remained unchanged, as was the response of isolated coronary arterioles (~100 µm in diameter) to adenosine or the NO donor sodium nitroprusside, suggesting preserved VSMC function despite early alterations in adenosine A_2 receptors and K_{ATP} channels expression.[72] With prolongation of diet duration, myocardial perfusion became significantly impaired.[73] The contribution of the voltage-gated potassium (K_V) channels to metabolic control of CBF at rest and during exercise was studied in lean Ossabaw swine vs. obese Ossabaw swine with MetS produced by 4 months of HFD.[73] MetS swine showed a 30–35% reduction in CBF both at rest and during treadmill exercise and a reduction in coronary vascular conductance. This CMD was the result of a blunted contribution of K_V channels to CBF control during increased metabolic demand in MetS.[73] Subsequent studies revealed that coronary large conductance Ca^{2+}-activated potassium (BK_{Ca}) channel dysfunction (both *in vivo* and *in vitro* in isolated arterioles ~100µm in diameter) was associated with increased L-type Ca^{2+} channel-mediated constriction, which also contributed to CMD after 3–6 months of MetS.[74] Furthermore, after 6 months of HFD in Ossabaw swine, isolated coronary microvessels showed increased myogenic tone, which was associated with inward hypertrophic remodelling, indicating that longer-term MetS can also result in structural changes in the coronary microvasculature.[75] In addition, capillary rarefaction was present which may have further contributed to the impaired CFR.[75] The addition of renovascular hypertension (by unilateral renal artery stenosis) to this model, resulted in further impairment of maximal myocardial perfusion, as the MetS and hypertension synergistically suppressed the adenosine-induced hyperaemic response almost completely.[76] This response was associated with impaired eNOS expression and hypertrophic remodelling in the coronary microvasculature and resulted in left ventricular diastolic dysfunction, making this animal model with multiple cardiovascular risk factors also suitable for the study of microvascular involvement in heart failure with preserved ejection fraction.[76]

3.2.4 Rapacz familial hypercholesterolaemic swine

Downsized Rapacz familial hypercholesterolaemic (FH) swine have been inbred at the University of Wisconsin Swine Research and Teaching Center by Drs Rapacz and Hasler-Rapacz to yield a swine model of high plasma cholesterol levels and accelerated atherosclerosis. This was achieved by a spontaneous mutation in the LDL-receptor gene on chromosome 2; the protein product of this gene

normally removes LDL from the circulation.[77] This model is especially suitable for the study of coronary vascular dysfunction associated with high levels of circulating LDL, as seen in FH patients prior to or in the presence of obstructive coronary artery lesions. In 20-month-old Rapacz FH swine, 5 months of HFD resulted in marked hypercholesterolaemia and diffuse coronary atherosclerosis, associated with CMD.[78] This study demonstrated once again the presence of microvascular endothelial dysfunction both *in vivo* and in isolated coronary arterioles (~100 μm in diameter) prior to obstructive plaque development. The endothelial dysfunction appeared to be mediated by impaired EDHF-dependent vasodilation as well as by impaired NO bioavailability that was compensated for by an increased sensitivity to NO. These perturbations in the regulation of microvascular tone resulted in impaired CBF and myocardial oxygen delivery—especially during exercise—that was associated with a shift towards anaerobic myocardial metabolism particularly during increased myocardial metabolic demand.[78]

3.2.5 Summary: porcine models of CMD

In conclusion, several swine models of CMD in the presence of comorbidities are currently available, showing clear evidence of CMD depending on the duration of exposure to cardiovascular risk factors. The mechanisms involved in the development and progression of CMD are summarized in **Table 1**. While domestic pigs are readily available and cheap, young animals are typically used in cardiovascular studies which makes chronic treatments, including exercise training, difficult to assess due to rapid body growth of the animals, thereby limiting follow-up time. The use of inbred mini-pigs, including Yucatan, Ossabaw, and Rapacz enables the study of adult animals, prolongation of diet duration and mechanistic studies at different time points, opening opportunities for the development of therapeutic targets aimed at alleviating CMD.

3.3 Rabbit models of CMD

For nearly a century, rabbits have been utilized to investigate the pathophysiology and therapy of atherosclerosis including endovascular stents.[79] Both in terms of cardiac physiology and body size, rabbits represent an intermediate between large animals (pigs and dogs) and small rodents (rats and mice), that are large enough to place human endovascular stents while still relatively easy to house and handle. Although the rabbit has a higher metabolic rate, lipid profiles and chemical composition of plasma in rabbits show greater resemblance to human lipid metabolism, making them especially suitable for atherosclerosis research.[79] Most commonly used are the domestic rabbit breeds, which

all originate from the European rabbit (*Oryctolagus cuniculus*) including Japanese White rabbits and New Zealand White rabbits.[80] In several of these models, investigators have studied the effects of cardiovascular risk factors on coronary microvascular function and structure (Supplementary material online, Table S3).

3.3.1 Spontaneous hyperlipidaemic rabbit models

The Japanese veterinarian, Yoshio Watanabe, discovered that a male Japanese White rabbit developed spontaneous hyperlipidaemia on normal chow, due to an inherited recessive trait. This mutation results in a defective LDL-receptor, resembling human-like familial hypercholesterolaemia. Selective inbreeding was used to generate the Watanabe heritable hyperlipidaemic (WHHL) rabbit strain, which has 8- to 14-fold higher serum cholesterol levels than normal Japanese White rabbits and develops hypertriglyceridaemia (300–600 mg/dL).[81] A different inbred strain was developed at the St. Thomas Hospital, which also shows hypercholesterolaemia but with normal LDL-receptor function and mildly elevated levels of triglycerides. This model resembles the human familial hyperlipidaemia more closely and appears therefore more suitable for evaluating the relation between hypertriglyceridaemia and insulin resistance.[82] Both models are used in combination with normal chow as well as a high (~1%) cholesterol diet. Whereas, to date, these models have principally been used to study atherosclerosis, they also represent interesting models for the assessment of CMD in response to hyperlipidaemia, although an early study in the WHHL rabbits did not reveal any changes in either basal CBF or CFR.[83]

3.3.2 Transgenic rabbit models

To study alterations in lipid compositions other than the specific phenotype of spontaneous hyperlipidaemic rabbit models, transgenic rabbit models with modifications in the genes involved in lipid metabolism have been developed. The transgenes include human apolipoproteins (hapo), specifically the A-I/C-III/A-IV gene clusters (hapoA-I/C-III/A-IV gene cluster), hepatic lipase (hHL), lecithin: cholesterol acyltransferase (hLCAT), lipoprotein lipase (hLPL), and scavenger receptor class B type I (hSRB-I).[79,84] Similar to the inbred strains, levels of cholesterol and/or triglycerides are increased.[84] However, to our knowledge, these transgenic rabbit models have not yet been used in studies of CMD.

3.3.3 Rabbit models with induced metabolic derangement

Given the limited availability of spontaneous or transgenic rabbit models for dyslipidaemia, induction of risk factors has been used to model metabolic derangement in rabbits. Similar to other animals,

induction of risk factors such as DM or dyslipidaemia can be achieved in multiple ways. Supplementary material online, Table S3 presents an overview of the studies that induced hyperlipidaemia to study alterations in coronary microvascular function. In addition, DM has been created through injection of alloxan. Alloxan was used because older studies showed that rabbits, similar to guinea pigs, are resistant to the diabetogenic effects of STZ, and thus is not feasible to use STZ in rabbits as opposed to other animal models.[85,86] In rabbits with alloxan-induced DM, the isolated perfused heart setup was used to study coronary microvascular function *ex vivo*.[87] Nine to 12 weeks of exposure to type 1 DM (T1DM) had no effect on baseline CBF or CFR and showed similar vasodilator responses to papaverine compared to euglycaemic conditions. However, the responses to serotonin and adenosine were attenuated in hyperglycaemic and hyperosmotic conditions. In addition, the coronary vasodilator response to hypoxia was reduced, which was not due to alterations in adenosine-mediated vasodilation but was mediated through an altered contribution of cyclooxygenase products.[87]

Multiple studies have used a HFD to induce metabolic derangement to study coronary (micro)vascular function in rabbits. These studies vary in the cholesterol content of the diet (ranging from 0.8% to 2%) as well as duration of exposure (ranging from 4 to 16 weeks). Endothelial dysfunction was evidenced by impaired relaxation to acetylcholine, substance P, and ADP in the hypercholesterolaemic group in most[88–90] but not all[91,92] studies. Conversely, smooth muscle responsiveness to NO was preserved after 8–12 weeks of hyperlipidaemia.[89–92] In addition, vasoconstriction to norepinephrine as well as serotonin was enhanced in coronary arteries from hypercholesterolaemic animals.[88] Furthermore, the vasodilator response to acidosis was impaired which was due to an impairment upstream of the K_{ATP} channels, as the response to the K_{ATP} channel opener levcromakalim was unaltered.[92] In line with these findings, Pongo *et al.*[93] showed that the effect of protein kinase C—acting through K_{ATP} channels—on vasomotor control in hypercholesterolaemic coronary arterioles was lost but was restored after farnesol supplementation. Finally, the response to ischaemia-induced paracrine vasodilator factors was attenuated in hypercholesterolaemic animals, as a result of increased oxidative stress.[91] Histological examination of small coronary arteries and arterioles of rabbits with hypertension (induced by removal of the left kidney and partial ligation of right renal artery) and hypercholesterolaemia (by 0.8% cholesterol diet for 16 weeks), showed structural changes such as hyalinization and/or intimal hyperplasia.[94]

3.3.4 Summary: rabbit models of CMD

Taken together, the usage of rabbits in studying CMD in metabolic derangement is viable and has distinct advantages when compared with rodent models. Genetically modified models are available, although they have not been used yet to specifically investigate CMD. Induction of metabolic

derangement is possible and although previous research utilized a large variety of diet compositions and durations, all studies report the presence of CMD (**Table 1**).

4. Rodent models of CMD

4.1 Rat models for CMD

A variety of rat models, exhibiting a range of comorbid conditions, have been utilized to interrogate mechanisms of CMD (Supplementary material online, Table S4). The most prevalent models include the STZ-induced model of T1DM, the HFD or western diet (WD)-fed and obese Zucker rat (OZR) models of obesity and insulin resistance, the Zucker diabetic fatty (ZDF) rat, the Otsuka Long-Evans Tokushima Fatty (OLETF) rat model of obesity, progressive insulin resistance, and T2DM and the Goto-Kakizaki (GK) non-obese model of T2DM. Each of these models typically exhibit hyperglycaemia and some recapitulate additional aspects of human metabolic disease. Specifically, the HFD/WD-fed rat and OZR models exhibit obesity, hyperinsulinaemia, and hypercholesterolaemia. The ZDF and OLETF rat models are also obese with hypercholesterolaemia, however, hyperglycaemia in these models is associated with insulin resistance in younger rats followed by progressive impairment of insulin secretion. Lastly, the GK rat exhibits hypercholesterolaemia and, such as the ZDF and OLETF rats, hyperglycaemia owing to insulin resistance and eventual impairment of insulin secretion independent of obesity. With several exceptions,[95–100] available studies evaluating coronary microvascular function in these models report similar blood pressures between rats with metabolic derangement and matched controls.[101–114]

Despite the various metabolic phenotypes of these models, available evidence suggests similarities in the nature of the associated CMD. A common finding across these models is altered coronary endothelial function, however, important distinctions in the manifestation of this dysfunction are worth noting. First, significant impairment of endothelium-dependent vasodilation (typically to acetylcholine) occurs early in some models or later in the disease process of others due to compensatory changes in the underlying mechanism of vasodilation. For instance, most studies in STZ rats, HFD/WD-fed rats, and young OZR report maintained coronary vasodilation to acetylcholine.[95,101,102,105,106,115,116] Further examination of mechanisms of coronary endothelium-dependent vasodilation in these models, however, has revealed compensatory up-regulation of BK_{Ca} channel expression/activity,[105,115] up-regulation of endothelial small (SK_{Ca}) and intermediate (IK_{Ca}) conductance K_{Ca} channel expression/activity,[106] increased basal phosphorylation of eNOS and Akt,[108] and increased VSMC soluble guanylate cyclase activity[116] and reduced phosphodiesterase 5 activity[109] in models of metabolic derangement. Thus, early endothelial dysfunction in these models

is masked by compensatory mechanisms not seen in the OLETF or GK rat models of metabolic derangement. Indeed, impaired endothelium-induced vasodilation has been reported at the earliest time points examined in OLETF[111] and GK[117] rats. An interesting point in this regard relates to the OZR model in which insulin-induced vasodilation, also endothelium-dependent, is impaired earlier than dilation to acetylcholine.[98,107,118] Therefore, insulin-induced vasodilation may serve as a functional biomarker of early CMD in metabolic disease and vascular insulin resistance may be an early mechanism of dysfunction in these disease states.[119] Furthermore, increased basal and stimulated coronary or cardiac oxidative stress is reported in these models[97,107,112,118,120,121] and may serve as an initial trigger of dysfunction/compensation. Scavenging of reactive O_2 species restores impaired endothelium-dependent vasodilation in OZR and ZDF rats.[98,118] Taken together, when using rat models to evaluate endothelial function, it should be kept in mind that different rat models represent different disease stages. Therefore, rat models need to be carefully selected based on whether the hypothesis being tested is focused on 'early' or 'late' coronary endothelial dysfunction with the genetic models (i.e. OLETF, GK, and older OZR) appearing to represent later stages of disease with more pronounced endothelial dysfunction.

Studies evaluating the impact of metabolic derangement on coronary microvascular VSMC function in rats are quite disparate and perhaps context- or agonist-dependent. For instance, coronary vasoconstrictor responses in OZR are similar in most,[105,107,108,120,122] but not all,[109,123,124] studies compared with lean Zucker rat as controls. Several studies report reduced vasoconstrictor responses to several agonists (e.g. ET-1, KCl) in this model.[123,124] In addition, in endothelium-denuded coronary arterioles from OZR, vasoconstrictor responses to insulin,[118] and hydrogen peroxide (H_2O_2)[120,121] are similar, however, H_2O_2-stimulated production of cyclooxygenase-2-derived prostanoids and H_2O_2-induced VSMC Ca^{2+} entry and mobilization are increased.[120,121] Conversely, in the OLETF rat model, evaluation of ET-1-induced coronary vasoconstriction reveals progressively increased vasoconstriction with age.[111] In addition, older GK rats exhibit impaired coronary myogenic vasoconstriction due to defective Rho-kinase activity.[114] Together, these data demonstrate a lack of correlation between the endothelial and VSMC phenotypes underlying CMD in rat models of metabolic derangement. Interestingly, in the STZ rat model of T1DM, inhibition of NOS/COX *in vivo* has been reported to reveal focal stenosis and segmental vasoconstriction by *in vivo* synchrotron imaging that was alleviated by Rho-kinase inhibition.[95,101] Thus, these data may suggest altered coronary VSMC function possibly preceding oxidative stress and impaired endothelial function in this particular model.

Beyond changes in vascular cell function in metabolic diseases, rat models have been utilized to examine changes in cardiac perfusion and flow control associated with CMD. In general, when viewed across studies, available evidence suggests that changes in cardiac perfusion or coronary flow control occur after the development of oxidative stress and endothelial dysfunction in metabolic diseases. In the OLETF rat model, for instance, a time course study demonstrated reduced CFR *in vivo* at 15 weeks of age,[100] while a separate study reported endothelial dysfunction as early as 5 weeks of age in this model.[111] Impaired metabolic hyperaemia was reported in OZR at 8–12 months of age[97] but the relationship to endothelial dysfunction is unclear as no earlier time points were assessed. The impact of HFD and WD feeding on cardiac perfusion is ambiguous as both a trend for increased baseline perfusion[104] and a reduction in estimated perfusion[103] have been reported, respectively. Results in these models likely differ depending on the composition of the experimental diet and the length of feeding utilized. Lastly, both reduced baseline coronary flow[125] and impaired CFR[126] have been reported in GK rats and this dysfunction may be greater in females.[127] To our knowledge, very few studies have examined sex differences in the coronary microvascular phenotype of rat models of metabolic derangement. This is a critical gap in knowledge in light of evidence that intermediate-to-high-risk women, but not men, with reduced CFR experience greater cardiovascular events.[128]

The pathogenic mechanisms initiating CMD, which may serve as appropriate therapeutic targets, in these rat models remain incompletely understood. However, one approach that has been examined in several models with promising results is inhibition of the RAAS. Indeed, angiotensin-converting enzyme (ACE) inhibition prevented reduced capillary length density in STZ rats[96] and acutely restored bradykinin-induced vasodilation in coronary arterioles from HFD-fed rats.[129] Furthermore, inhibition of aldosterone-binding mineralocorticoid receptors (MRs) reversed coronary vasodilator dysfunction in OZR[107] and OLETF[111] rats, however, enhanced vasoconstriction in OLETF rats was not altered by MR blockade. Increased acetylcholine-induced vasoconstriction and coronary perivascular fibrosis (i.e. transforming growth factor β1, plasminogen activator inhibitor 1, collagen I and III, and fibrin expression) in the OLETF rat were improved by angiotensin receptor inhibition.[99,130] These data are supported by clinical evidence that ACE inhibition and MR blockade improve CFR in patients with DM.[131–133] An additional intervention that successfully prevented coronary vasomotor dysfunction in the STZ rat is chronic *in vivo* inhibition of the sodium-hydrogen exchanger.[134] Much work remains, however, to better understand the precipitating mechanisms of CMD in these rat models as well as their translatability to mechanisms implicated in human disease.

4.1.1 Summary: rat models of CMD

There is wide variety of rat models that show CMD including non-genetic (DM and HFD) and genetic (Zucker, OLETF, and GK) models. All these models display CMD, the underlying mechanisms being summarized in **Table 1**.

4.2 Murine models of CMD

The use of murine models for the study of the coronary microcirculation in health and disease has gained interest over the past 20 years. Since some of the initial reports of endothelial dysfunction in leptin receptor deficient (db/db) and leptin deficient (ob/ob) mice,[135,136] there have been several publications showing the impact of risk factors on endothelial and vascular function in mice. Supplementary material online, Table S5 summarizes several of these murine models that have been used in the study of the coronary microcirculation.[30,135–148]

Db/db mice, such as the OZR model, have a deficient leptin receptor and as a consequence display polyphagia and obesity, resulting in T2DM. Several studies in this mouse model of obesity have demonstrated reduced acetylcholine[135,137,138,149] and flow-mediated[135,138,149] vasodilator responses, and either maintained[135,149] or reduced[138] sensitivity to NO. In addition, there is evidence of inward hypertrophic remodelling of arterioles,[150] with variable effects on microvascular densities.[150,151] Ob/ob mice are deficient in leptin production and as a result display the same phenotype as the db/db mice. Studies in this mouse model have shown maintained basal coronary flow velocities but reduced hyperaemic coronary flow velocities and coronary flow velocity reserve.[152,153] Bender *et al.*[140] used a WD to produce obesity and T2DM in wild-type mice, and observed reduced baseline coronary vascular resistance in isolated perfused mouse hearts that was accompanied by reduced NO bioavailability and blunted VSMC sensitivity to NO. Trask *et al.*[154] compared T1DM—induced with STZ—and T2DM in db/db mice, and observed inward coronary arteriolar remodelling in T2DM but not in T1DM. Finally, ApoE knockout mice are mostly used for studies of atherosclerosis but also display coronary endothelial dysfunction as demonstrated in isolated arterioles[155] or isolated buffer perfused hearts,[156] with maintained VSMC sensitivity to NO (Supplementary material online, Table S5).

Mouse models have both advantages and disadvantages—like any preclinical model—but one major advantage is the ability to use genetically modified animals, which creates a unique platform enabling investigators to address very precise questions. For example, Saitoh *et*

al.[157] reported that the drug 4-aminopyridine (4-AP) attenuated coronary metabolic dilation in a large animal model. 4-AP is an antagonist of voltage-gated potassium channels, leading the authors to conclude that these channels were involved in metabolic coronary hyperaemia. However, the K_v-channel family is large with 40 genes encoding for 12 K_v channel families. Although 4-AP may have some preferential antagonism for certain K_v channels, it is impossible to unequivocally establish the particular channel responsible for metabolic vasodilation using a standard pharmacological approach. This limitation underscores the rationale for using murine models, to engender a more precise conclusion and, in this situation, to enable determination of the specific K_v channel (or channels) that are linked to metabolic coronary vasodilation. Within this context, Ohanyan *et al.*[30] demonstrated in a genetically modified murine model that $K_{v1.5}$ channels are critical to coronary metabolic dilation. Thus, deletion of these channels compromised the connection between cardiac work and myocardial blood flow. Because the knockout was global, the investigators also created a reconstituted rescue model that had the $K_{v1.5}$ channel (on the null background) expressed only in vascular smooth muscle upon induction with a tetracycline. This model, with the reconstituted channel, restored normal metabolic dilation, i.e. re-established the connection between myocardial blood flow and cardiac work.[30]

4.2.1 Summary: murine models of CMD

Genetic and non-genetic mouse models of metabolic derangement support the concept of CMD as an early abnormality in the disease process, with clear coronary microvascular endothelial dysfunction (**Table 1**). There is no question that the murine model has its limitations—including a high heart rate, high rate of metabolism, and sympathetic dominance—but it provides a route enabling the interrogation of specific questions in coronary microvascular physiology and pathophysiology. Genetically modified mice have enhanced our understanding of the control of the coronary microcirculation and will enable further 'proof of concept experiments' whereby genes that are linked to a human pathology can be expressed in a mouse to determine if the particular gene is causal in the process.

5. Translational value of the different animal models of CMD and concluding remarks

5.1 Translational value to clinical setting

Since INOCA is more often diagnosed in post-menopausal women than in men, studies of sex differences in pathophysiology are important and animal studies of CMD and INOCA should preferably take sex differences into account. Although rodents are more often studied in a comparative manner (young vs. old and male vs. female) than large animals, most studies in the field of metabolic dysregulation and its effects on coronary perfusion have been performed in young animals (e.g. 8–12 weeks old mice, 3–6 months old pigs, Supplementary material online, Tables S1–S5) and have principally investigated a single sex. Yet, obesity and T2DM are strongly associated with maturation and ageing, and several differences have been reported between young and old animals, as well as between males and females. Thus, beta-cell replicative capacity strongly decreases with age in mice, and young rats do not develop insulin resistance in response to nutrient infusion, whereas older animals do.[158] Also in male Gottingen minipigs studied from 6 to 24 months of age, increases in plasma glucose, fructosamine, and triglycerides were observed with age, while plasma cholesterol levels decreased with age.[159] Importantly, oestrogen is known to have positive effects on metabolism and to be protective against the development of obesity, insulin resistance and hyperglycaemia,[158] as well as against endothelial dysfunction,[160] so that the use of animals of only one sex and one menopausal state in case of female animals is likely to introduce a bias. These limitations should be kept in mind when choosing an animal model, and limitations such as young age and single sex should, when possible, be circumvented in the study of INOCA, especially when testing novel therapies.

Another important aspect pertaining to the translational value of animal models is their ability to utilize clinically relevant methodological approaches to diagnose and treat the disease. PET studies and invasive microvascular function measurements have recently been proposed to be the best approach in stratifying patients with INOCA for a tailored therapy.[9] Such measurements, especially when involving invasive techniques, are not easily performed in smaller animal models (Supplementary material online, Tables S3–S5). However, large animal models may also pose some challenges. For example, while FFR measurements and acetylcholine and adenosine infusions can easily be performed in dogs and swine, these tests need to be adapted from the clinical protocol. For instance, in the porcine coronary circulation acetylcholine induces muscarinic vasoconstriction requiring use of another endothelium-dependent vasodilator. Finally, while the use of isolated small arteries/arterioles is very instrumental for the study of perturbations in specific mechanisms

regulating microvascular tone, it should be noted that—irrespective of the chosen animal model—isolated small arteries/arterioles represent only one segment of the coronary microvasculature and interactions with the surrounding myocardium are lacking. Hence, while specific mechanisms involved in CMD can easily be studied in isolated coronary small arteries or arterioles, the evaluation of the coronary microcirculation as a whole, including microvessels of all sizes, is impossible using an *in vitro* technique. Hence, *in vivo* studies are ultimately required to assess coronary function in an integrated manner.

5.2 Concluding remarks

Animal models of coronary microvascular disease yield important insights into the genetic and environmental basis of human coronary pathophysiology in ischaemic heart disease and provide translational models for preventive and therapeutic interventions (see **Figure 2**). In the past 50 years, animal models have been instrumental in advancing our knowledge pertaining to CBF regulation in health and ischaemic heart disease. Notwithstanding the undisputable merits of experimental animal models, researchers need to carefully consider the choice of a specific animal model.[161,162] It is imperative to acknowledge that no single animal model perfectly emulates the human disease, nor has a perfect translational capacity to the clinical setting (**Table 1** and **Figure 2**). In addition, there are a number of financial and logistical considerations that need to be considered, including costs, infrastructure, and the requirement for specialized personnel. Small animal models have the advantage of being relatively cheap, easy to breed and handle, have short reproductive cycles and large litter sizes, a well-defined genome and a relative ease of genetic modification to explore pathophysiological mechanisms with great molecular precision. Disadvantages include different metabolic and lipoprotein profiles compared to humans and resistance to atherosclerosis development, requiring genetic modification, and technical challenges to study the coronary microcirculation particularly *in vivo*. Conversely, large animal models have the advantage of human-sized hearts and coronary blood vessels, allowing the application of clinical diagnostic and therapeutic tools. Moreover, from a coronary anatomical and physiological perspective, large animals, especially swine models, approximate the human heart and its coronary circulation more closely, have similar lipoprotein profiles and develop similar metabolic derangement. In addition, the possibility of awake measurements in large animals is of great importance, as neurohumoral and cardiac dysfunction especially in early disease—possibly not detectable at rest or under deep anaesthesia—might be unmasked by exercise. As summarized here, several animal models have been developed for the study of CMD and characterized in detail. These models—depending on number and severity of, and duration of exposure to risk factors—show perturbations in coronary microvascular (endothelial)

function and structure, similar to what has been observed in patients with non-obstructive CAD and comorbid conditions. The use of these animal models, with careful selection based on the specific research question, will be instrumental in identifying novel therapeutic targets and for the subsequent development and testing of novel therapeutic interventions to treat CMD.

Supplementary material: Supplementary material is available at *Cardiovascular Research* online. (https://academic.oup.com/cardiovascres/article/116/4/756/5700718)

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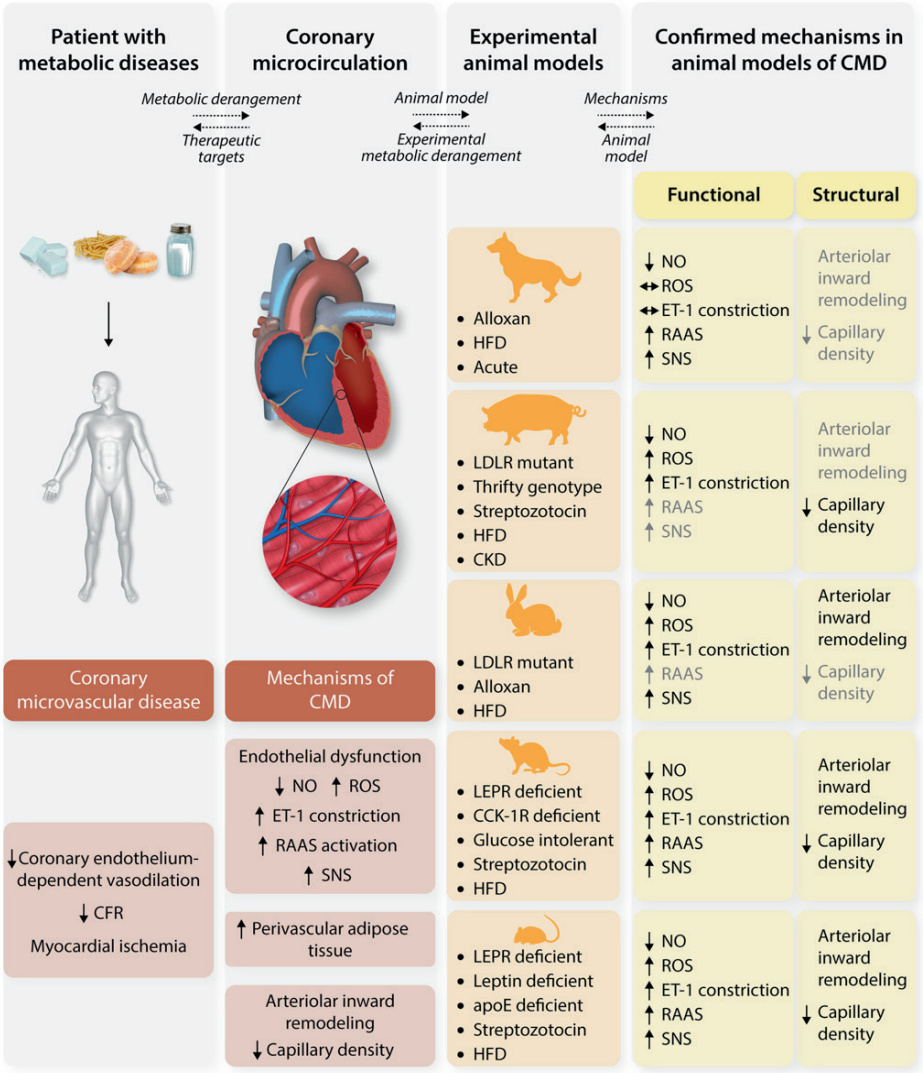
Figure 2 Animal models for CMD in the presence of metabolic derangement



Large and small animal models for CMD, either spontaneous, inducible, or inbred/engineered for the development of metabolic risk factors have been employed to study the mechanisms leading to CMD. Alterations in microcirculatory endothelial function leading to a disturbed vasodilator/vasoconstrictor balance as well as structural modifications both at the arteriolar and capillary level have been described, mimicking the human pathology including reduced basal and maximal coronary perfusion and myocardial ischaemia. apoE, apolipoprotein E; CBF, coronary blood flow; CCK-1R, cholecystokinin-1 receptor; CFR, coronary flow reserve; CKD, chronic kidney disease; ET-1, endothelin-1; HFD, high-fat diet; LDLR, low-density lipoprotein receptor; LEPR, leptin receptor; NO, nitric oxide; RAAS, renin–angiotensin–aldosterone system; ROS, reactive oxygen species. Right-sided panel presents the mechanisms of CMD which have been recapitulated in at least one animal model/species (black), which have been investigated but were not found to be present (black ↔) or which have not been investigated (grey) in the various species.

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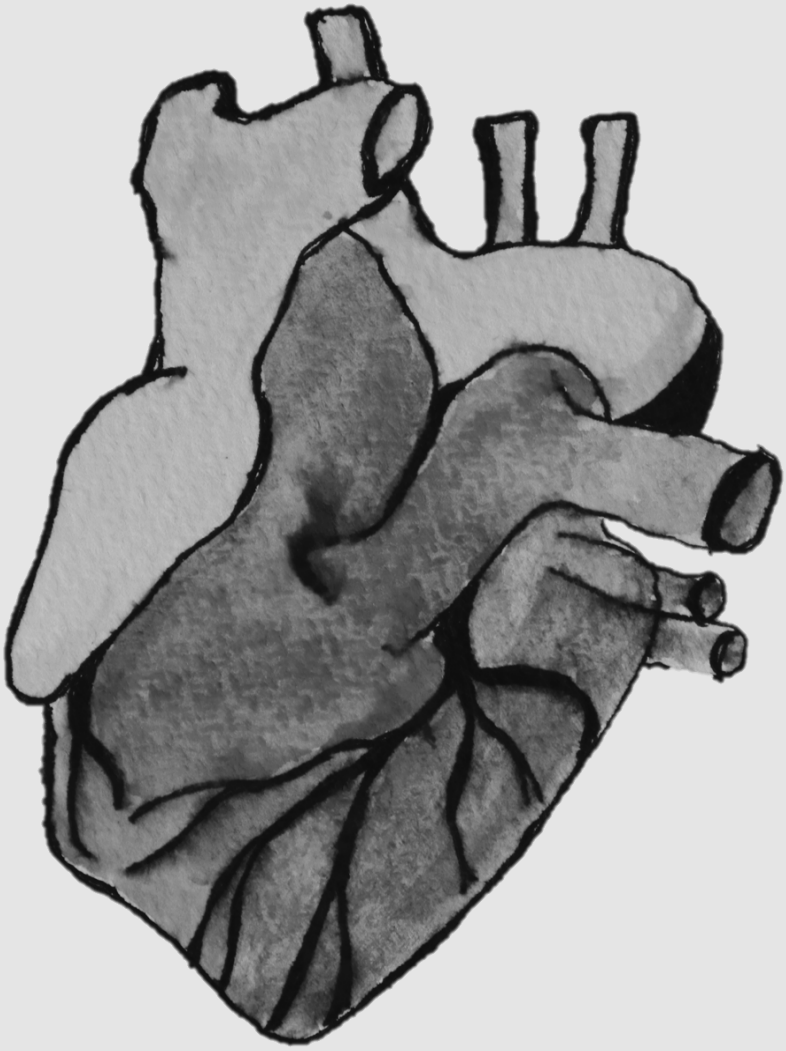

Chapter 4

Cellular, mitochondrial and molecular alterations associate with early left ventricular diastolic dysfunction in a porcine model of diabetic metabolic derangement

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Abstract

The prevalence of diabetic metabolic derangement (DMetD) has increased dramatically over the last decades. Although there is increasing evidence that DMetD is associated with cardiac dysfunction, the early DMetD-induced myocardial alterations remain incompletely understood. Here, we studied early DMetD-related cardiac changes in a clinically relevant large animal model. DMetD was established in adult male Göttingen miniswine by streptozotocin injections and a high-fat, high-sugar diet, while control animals remained on normal pig chow. Five months later left ventricular (LV) function was assessed by echocardiography and hemodynamic measurements, followed by comprehensive biochemical, molecular and histological analyses. Robust DMetD developed, evidenced by hyperglycemia, hypercholesterolemia and hypertriglyceridemia. DMetD resulted in altered LV nitroso-redox balance, increased superoxide production—principally due to endothelial nitric oxide synthase (eNOS) uncoupling—reduced nitric oxide (NO) production, alterations in myocardial gene-expression—particularly genes related to glucose and fatty acid metabolism—and mitochondrial dysfunction. These abnormalities were accompanied by increased passive force of isolated cardiomyocytes, and impaired LV diastolic function, evidenced by reduced LV peak untwist velocity and increased E/e'. However, LV weight, volume, collagen content, and cardiomyocyte cross-sectional area were unchanged at this stage of DMetD. In conclusion, DMetD, in a clinically relevant large-animal model results in myocardial oxidative stress, eNOS uncoupling and reduced NO production, together with an altered metabolic gene expression profile and mitochondrial dysfunction. These molecular alterations are associated with stiffening of the cardiomyocytes and early diastolic dysfunction before any structural cardiac remodeling occurs. Therapies should be directed to ameliorate these early DMetD-induced myocardial changes to prevent the development of overt cardiac failure.

Introduction

According to a recent report by World Health Organization, age-standardized global prevalence of diabetes has nearly doubled since 1980, rising from 4.7% to 8.5% in the adult population.[1] An estimated 422 million adults suffered from diabetes mellitus in 2014, compared to 108 million in 1980, which is the result of concomitantly increased prevalence of obesity and overweight globally.[1] As diabetes is associated with increased risk of cardiovascular diseases in particular, a deeper understanding of its cardiac pathophysiology is essential for the development of novel targeted treatments. Previous pre-clinical studies have addressed the consequences of diabetes in myocardial tissue, showing that the pathophysiology of diabetes-induced cardiac damage is a complex and multifactorial process, in which oxidative stress has been postulated as a key player.[2,3,4,5,6] Indeed, increased reactive oxygen species production and reduced antioxidant defenses have been associated with diabetic cardiomyopathy.[2,3,4,5] However, the majority of these studies have been performed in rodents[2,3,4] and it remains uncertain whether these findings can be translated to a larger, human-like heart.

Consequently, in the present study we employed a translationally relevant large animal model with Diabetic Metabolic Derangement (DMetD), having similar anatomic, physiologic and metabolic characteristics of the cardiovascular system with the human situation. We characterized, in adult swine, the early DMetD-induced left ventricular (LV) changes at the mRNA, protein, cellular, tissue and organ function levels. For this purpose, DMetD was produced in adult male Göttingen miniswine by streptozotocin injections, to result in partial destruction of the pancreatic β-cells leading to impaired insulin production, combined with a high-fat, high sucrose and high fructose diet. LV function was assessed at baseline and 5 months after induction of DMetD, and myocardial tissue was comprehensively analyzed for oxidative stress and nitric oxide (NO) production, mitochondrial function, cardiomyocyte function, collagen content, and global LV remodeling and function, as well as for genome-wide gene expression profile. Our study shows that DMetD results in myocardial oxidative stress, endothelial nitric oxide synthase (eNOS) uncoupling and reduced NO production together with mitochondrial dysfunction and alterations in metabolic gene expression profile. These abnormalities are associated with impaired LV relaxation, at a time when cardiac structural remodeling at either the global LV or myocardial tissue level is still absent.

Methods

Animals and DMetD induction

Studies were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals (8th edition, National Research Council. Washington, DC: The National Academies Press, 2011) and were approved by the Animal Care Committee at Erasmus University Medical Center, Rotterdam, The Netherlands. A total of 17 adult male Göttingen minipigs were enrolled in the study and followed for 5 months. Diabetes was induced in 9 swine with intravenous injections of streptozotocin (25 mg/kg/day), over three days7. One week later, a high fat and high sugar diet (25% saturated fats, 1% cholesterol, 10% sucrose and 15% fructose) was gradually introduced to the diabetic swine (DMetD group, N = 9), whereas the healthy control swine (Control, N = 8) continued on normal pig chow. Swine were group-housed with a separate individual access to food for 1 h/meal, twice daily, and ad libitum access to water. Fasting mixed central venous blood samples were obtained at baseline and sacrifice (5-month time point), and analyzed for glucose, triglyceride and cholesterol.

Fitness test

To objectively measure physical fitness, animals were subjected to an incremental endurance treadmill test until exhaustion. The test started at a pace of 1.5 km/h and speed was increased by 0.5 km/h every five minutes and running time was recorded as an indicator of maximal aerobic endurance capacity.

Echocardiography

Echocardiography was performed at baseline, and after 5 months. Animals were sedated with an intramuscular injection of Zoletil (tiletamine/zolazepam; 5 mg/kg) and xylazine (2.25 mg/kg). Two-dimensional echocardiographic images (iE33, Philips, Best, the Netherlands) were acquired in harmonic mode from a right lateral decubitus position using a broadband (1–5 MHz) X5-1 transducer. All acquisitions and measurements were performed according to the current guidelines8. We performed pulsed-wave Doppler examination from the (apical) 4-chamber view, to obtain peak mitral inflow velocities at early (E) and late (A) diastole and E deceleration time. Tissue Doppler imaging was performed to measure myocardial tissue velocity at the septal and lateral mitral annulus at early diastole (e'). E/A ratio and the E/e' ratio were calculated. Left atrial volume was measured using the modified biplane area-length method. Left atrial volume index was calculated by dividing left atrial volume by body weight.

Hemodynamic assessments and cardiac tissue sampling and analyses

At 5 months follow up, hemodynamic measurements were perfomed under anesthesia and animals were terminated. Sedation was induced with Zoletil (tiletamine/zolazepam; 5 mg/kg), xylazine (2.25 mg/kg) and atropine (2 ml i.m.), and animals were anesthetized with an intravenous continuous infusion of pentobarbital (20 mg/kg), intubated and artificially ventilated. Arterial and venous access was obtained by placing 9F sheaths in the left carotid artery and the jugular vein for the measurement of mean aortic pressure, LV pressure (using a Millar catheter), as well as pulmonary artery and pulmonary capillary wedge pressure, cardiac output (by thermodilution) and for blood sampling. After placing a pressure-volume catheter in the LV (PV loop catheter, CD Leycom, The Netherlands), the pressure–volume loops were recorded and end-diastolic pressure–volume relationships were constructed, during baseline hemodynamic conditions, preload reduction (by complete obstruction of the inferior vena cava by a Fogarty balloon, 8/10F, Edwards Life sciences, Amsterdam, The Netherlands) and preload increase (by saline infusion, 20 ml/kg i.v. within 7 min). Thereafter, a sternotomy was performed, hearts were arrested, and quickly excised, washed in cold saline solution and then rapidly cut and frozen in liquid nitrogen and prepared and stored for later analyses.

Myocardial reactive oxygen species (ROS) and nitric oxide (NO) production measurements

Cardiac oxidative stress was evaluated by lucigenin-enhanced chemiluminescence (Sigma Aldrich; 5 µmol/l) as previously described7. To this end, both basal and NADPH-stimulated (300 µM NADPH) superoxide generation were measured in homogenized, frozen sub-endocardial tissue samples of the anterior LV wall. NOS-dependent superoxide production was determined by incubating the samples for 20 min with the NOS inhibitor L-NAME (Sigma Aldrich; 1 mmol/l), while the contribution of NADPH oxidase to superoxide production was assessed using the NADPH oxidase inhibitor VAS2870, (10 µM). The temperature was controlled (37 °C) during the entire experiment and the measured light emission was expressed as relative light units (RLU) per mg protein per second. All samples were measured in duplicate and data averaged for each animal. Aditionally, myocardial NO production was evaluated in the same area, by measuring the production of NO metabolites NO_2^- and NO_3^- using the Griess reaction colorimetric assay kit (Cayman Chemical).

Endothelial nitric oxide synthase expression and phosphorylation

Protein expression of endothelial nitric oxide synthase (eNOS) and the phosphorylated eNOS were determined in homogenized, snap frozen LV sub-endocardial tissue samples. Furthermore, low temperature SDS-PAGE was performed for the detection of eNOS monomer and dimer fraction, as

previously described9. SDS-PAGE for phosphorylated eNOS, total eNOS protein content and housekeeping protein glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed at room temperature. Subsequently, proteins were transferred onto nitrocellulose membranes and the blots were probed with primary anti-phospho eNOS (1:1,000, Cell Signaling), anti-eNOS (1:500, Transduction Laboratory) and anti-GAPDH (1:10,000, Imgenex). All blots were analysed using the Odyssey system (LI-COR).

AMPK western blot analyses

Frozen LV samples were weighed and homogenized in 6 vol ice cold 62.5 mM Tris–HCl buffer (pH 6.8, with 1 µg/ml Leupeptin, 1 µg/ml Pepstatin, and 1 mM phenylmethanesulfonyl fluoride, Sigma-Aldrich, St. Louis, Missouri, USA) with Qiagen TissueLyser (85220, Hilden, Germany). The homogenates were centrifuged and the samples were then denatured in Laemmli solution at + 70 °C for 7 mins10. Protein concentration of samples was determined using BCA protein assay kit (Thermo Scientific, Waltham, Massachusetts, USA). The samples (10 µg of protein for antibodies AMPK and pAMPK) were run with SDS-PAGE gels. After separation, the proteins were transferred onto a Whatman Protran Nitrocellulose membrane (PerkinElmer, Boston, Massachusetts, USA). The immunoblot membranes were blocked with 5% bovine serum albumin (BSA, Sigma-Aldrich) in Tris buffered saline (TBS, Sigma-Aldrich and thereafter, the membranes where incubated overnight at + 4 °C with primary antibodies for AMPK and pAMPK, washed and then incubated for 30 min with horseradish peroxidase conjugated secondary anti-rabbit antibody (1:10,000, Abcam, ab6721). The band densities were analysed with Gel Doc XR System (BioRad). In order to avoid variation between membranes a control sample was loaded onto each gel and the band intensities were normalized against the control sample.

Enzyme assays

The samples were weighed and homogenized with 10 volumes of ice-cold homogenization solution (50 mM imidazole, 1 mM EDTA, pH 8.0) for HOAD (EC 1.1.1.35) or with 19 volumes homogenization buffer (50 mM Hepes, 1 mM, EDTA, 0.1% Triton X-100, pH 7.4) for CS (EC 2.3.3.1) and Lactate Dehydrogenase (LDH) (EC 1.1.1.27) with Qiagen TissueLyser. The HOAD samples were diluted 1:1 with homogenization solution, the LDH samples were diluted 1:1 with 50 mM Tris buffer, pH 7.4 and the CS samples were diluted 1:8 with 50 mM Tris buffer, pH 8.0. The protein concentrations of samples were determined with a BCA protein assay kit following the manufacturer´s protocol. The enzyme activity analyses were performed according to Dalziel et al.11 for CS and LDH and according to Lowry et al.12 for HOAD at 37 °C. The activities were read with PerkinElmer Enspire 2,300 Multilaber reader

(Turku, Finland), and the enzyme activities were calculated per mg protein with the background activity subtracted.

Histology and immunohistochemistry

After the heart was excised, LV myocardial tissue samples were fixated in 4% buffered formaldehyde, and embedded in paraffin. Thereafter, 4.5 µm thick slides were cut from the LV anterior wall, deparaffinized and stained for various histological analyses. For each analysis, 6–10 fields at × 20 magnification, were examined in the sub-endocardium of each slide. Collagen deposition was quantified using Picrosirius Red staining as follows: using a linear polarization filter, the area occupied by the collagen type I and type III fibers, was measured and expressed as percentage of the myocardial area. Myocyte size was quantified with a Gomori silver stain: cross-sectional areas of 300–350 round cells with clearly visible nuclei were measured in each slide. All measurements were performed using the Clemex Vision Image analysis system (Clemex Technologies, Quebec, Canada).

Passive cardiomyocyte stiffness and titin isoform composition

Passive stiffness of single cardiomyocytes was measured using membrane-permeabilized cardiomyocytes. In brief, single cardiomyocytes were isolated in cold relaxing solution containing (in mM) free Mg^{2+} 1, KCl 100, EGTA 2, Mg-ATP 4, imidazole 10 (pH 7.0, adjusted with KOH) and incubated for 5 min in relaxing solution containing Triton X-100 (0.5%) to remove all membranes as described previously13. Passive stiffness of the cardiomyocytes, was assesed by *F*pas measurements, performed in relaxing solution at 15 °C and at sarcomere lengths ranging from 1.8 to 2.2 µm. Cardiomyocyte diameters were measured microscopically, in two perpendicular directions, and cross-sectional area was calculated assuming an elliptical shape. Passive force data were normalized to cross-sectional area of cardiomyocytes. Titin isoforms were separated on 1% agarose gel and stained with SYPRO Ruby protein stain as described previously.[14,15]

Electron microscopy analysis of mitochondria

LV samples of 6 Control and 5 DMetD animals were prepared for electron microscopy as previously described.[16] In short, samples were fixed in 1.5% osmium tetroxide (10 min), dehydrated with acetone, and embedded in Epon812. Ultrathin sections were cut and placed on 300-mesh Formavar-coated nickel grids, and stained with uranyl acetate and lead citrate. Images from the intramyofibrillar region (longitudinal to the fiber orientation only) were taken at magnifications ranging from 4,500 × to

30,000 × with an electron microscope (Jeol-1200EX, Jeol Peabody, MA, USA). Glycogen content and the number of vacuoles were determined by scoring images on a scale for 1 (low) to 5 (extremely high) in a blinded fashion. Mitochondrial volume density was determined by overlaying a dense grid over images taken at 22,500 × and counting all mitochondria overlaying grid corners, as a percentage of all grid corners. Mitochondrial connectivity was assessed by visually inspecting whether mitochondria were touching other mitochondria, and expressed as a percentage of all mitochondria on the image. On average, 8 ± 1 images were analyzed per animal.

Mitochondrial respiration

Mitochondrial respiration was measured in fresh biopsies from the LV sub-endocardium as described before16. Thin strips were permeabilised with saponin (50 µg/ml) for 30 min at 4 °C in a solution consisting of 7.2 mM EGTA, 2.8 mMCaEGTA, 6.6 mM $MgCl_2$, 10 mM taurine, 5.8 mM ATP, 15 mM phosphocreatine, 20 mM imidazole, 50 mM MES and 0.5 mM DTT, and the pH was set to 7.1. The tissue was washed in respiration solution, consisting of 110 mM sucrose, 0.5 mM EGTA, 17 mM $MgCl_2$, 20 mM taurine, 60 mM K-lactobionate, 10 mM KH_2PO_4, 20 mM HEPES, 1 g/L BSA (fatty acid free), and pH set to 7.1 before being transferred to a respirometer (Oxygraph-2 k; Oroboros Instruments, Innsbruck, Austria). Oxygen concentration remained < 300 µM during the experiment and temperature was set to 37 °C. Leak respiration was measured during 10 mM sodium glutamate, 0.5 mM sodium malate and 5 mM sodium pyruvate. NADH-linked (through complex I) respiration was assessed using 2.5 mM ADP. Maximal NADH-linked respiration was measured after 10 µM cytochrome c, i.e. after correcting for possible outer-membrane damage. Maximal oxidative phosphorylation capacity was determined after 10 mM succinate. Maximal uncoupled respiration was assessed after titrations of 0.01 µM carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP). Succinate-driven (complex II) respiration was assessed by inhibiting mitochondrial complex I by 0.5 µM rotenone. Residual oxygen consumption was measured after 2.5 µM antimycin A and used for background correction. Experiments were performed in duplo. Respiration values were averaged and normalized to wet weight and expressed in pmol O_2/s/mg.

Mitochondrial complex protein determinations by Western immunoblotting

Protein content was determined using western immunoblotting. Tissue homogenates containing 7.5 or 12.5 µg (well within the linear range of detection) of protein were loaded onto a pre-cast 4–15% or 8–16% gradient criterion TGX SDS-PAGE gels. Proteins were transferred onto PVDF membranes and incubated with an antibody cocktail (Abcam, Cambridge, UK; dilution 1:1,000; 2 h) against complex I

subunit (NDUFB8), 30 kDa complex II subunit and complex IV subunit 1. Complex III subunit core 2 could not be determined due to species differences for the specificity of antibodies.

RNA extraction and gene expression analyses

From 20 mg snap-frozen sub-endocardial tissue samples of 3 Control and 3 DMetD pigs, total RNA was extracted using the Qiagen miRNeasy Mini kit protocol. RNA integrity was checked on an Agilent 2,100 Bioanalyser for a RIN score ≥ 8.0 and 20 µg RNA at 100 ng/µl was used for further analyses. At the Biomics center of Erasmus University Medical Center, RNA was prepared for sequencing with the Illumina TrueSeq RNA sample preparation kit. Sequencing was performed according to the Illumina TrueSeq v3 protocol on an Illumina HiSeq 2000 sequencing system, 43 bp single read, 7 bp index. Sequence data were mapped against the reference pig genome *Sus scrofa* sequence assembly version 10.2 by Illumina Tophat version 2.0.10. Gene expression values of the RNA-seq data were estimated using featureCounts17 using the gene annotation Sscrofa10.2. Statistical differences in gene expression between both conditions were estimated using edgeR18 where an absolute logFC > 1 with a P-value < 0.001 was considered statistically significant. Biological functions and molecular networks of the differentially expressed genes were determined using Ingenuity pathway analysis Pathway analysis (Ingenuity Systems, Redwood City, CA, USA). Interconnectivity of the genes was visualized by the molecular networks constructed by the program.

Data analysis

Data are presented as mean ± SEM. Comparison of variables between the DMetD and Control animals over time was performed by two-way ANOVA for repeated measures (fitness, echocardiography, PV-loop, myocyte force and blood variables) and Bonferroni post-hoc test or unpaired student t-test (variables measured only once at sacrifice) by GraphPad Prism 4.3 or SAS 9.2. $p < 0.05$ was considered statistically significant.

Results

Metabolic parameters and fitness

Several results are presented in the Supplementary Information file. Five months of DMetD resulted in hyperglycemia ($p < 0.05$), hypercholesterolemia ($p < 0.05$) and hypertriglyceridemia ($p < 0.05$), while no significant differences in body weight were observed between the groups (**Table 1**). DMetD

resulted in a significant reduction in physical fitness, as reflected in a ~ 30% decrease in total running time ($p < 0.05$ versus Control).

Table 1. Body weight and plasma metabolic parameters measured at baseline and 5-month time points.

		BASELINE	5-MONTH
BW (kg)	CON	32 ± 2	37 ± 1
	DMetD	31 ± 2	40 ± 3‡
Glucose (mmol/l)	CON	5.9 ± 0.4	6.8 ± 0.7
	DMetD	5.0 ± 0.3	13.9 ± 2.0*†‡
Cholesterol (mmol/l)	CON	1.01 ± 0.13	1.10 ± 0.04
	DMetD	0.98 ± 0.05	5.89 ± 1.03*†‡
LDL cholesterol (mmol/l)	CON	0.45 ± 0.09	0.39 ± 0.03
	DMetD	0.42 ± 0.03	3.68 ± 1.05*†‡
Triglycerides (mmol/l)	CON	0.30 ± 0.02	0.27 ± 0.03
	DMetD	0.26 ± 0.03	0.66 ± 0.19*†‡
Fitness test (min)	CON	39 ± 4	38 ± 4
	DMetD	43 ± 5	30 ± 4*‡

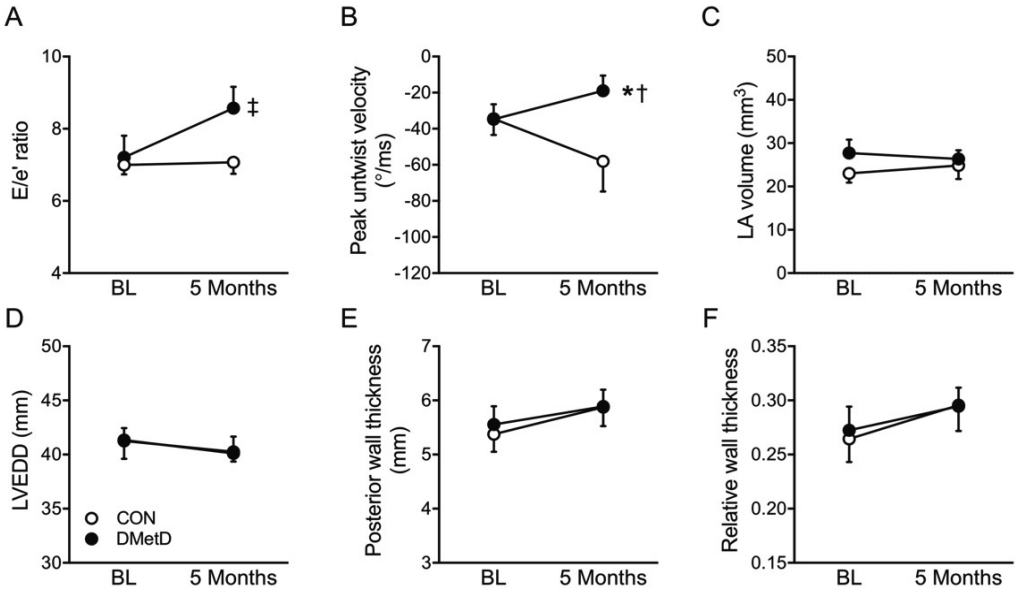
CON=healthy controls (n=8), DMetD=diabetic metabolic derangement animals (n=9). LDL = low density lipoproteins. *p<0.05 as time•diabetes interaction by two-way ANOVA, †p<0.05 versus corresponding CON by Bonferroni post-hoc analysis, ‡p<0.05 versus corresponding baseline by Bonferroni post-hoc analysis. Data are mean±SEM.

Cardiac function and remodeling

There were no differences in echocardiography variables between Control and DMetD at baseline (**Tables S1** and **S2**). Five months of DMetD resulted in an elevated E/e', while peak untwist velocity was significantly lower, in DMetD compared to Control (**Figure 1**), indicating impaired LV diastolic function in DMetD. In contrast, DMetD did not produce differences in left atrial (LA) volume, LV diameter, or absolute and relative LV wall thickness between DMetD and Control (**Figure 1**), while LV weight was also not affected (**Figure 2A**), indicating that 5 months of DMetD did not result in cardiac

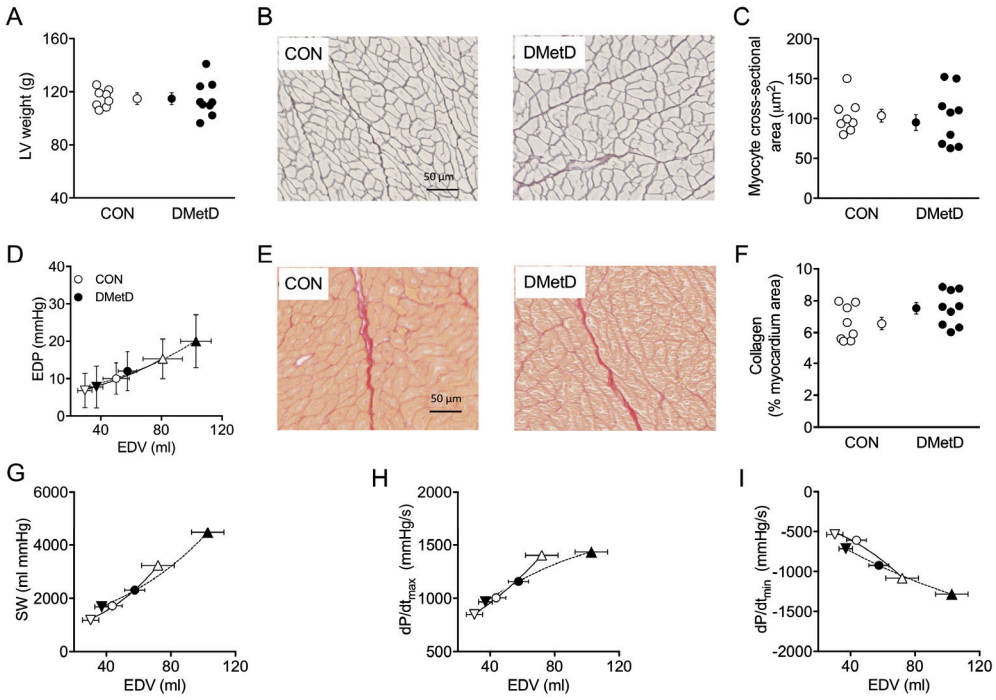
remodeling. Moreover, there were no significant differences in LV and aortic pressures, LV volumes, stroke volume, ejection fraction, or cardiac output between DMetD and Control (**Tables S3** and **S4**). Histological analysis showed that cardiomyocyte size (**Figure 2B, C**) and myocardial collagen content (**Figure 2E, F**) were not significantly different between DMetD and Control. Furthermore, there were no changes in type I or type III collagen or their ratio (data not shown). These histological findings correlated well with the nearly identical LV weights and LV end-diastolic pressure–volume relations, (**Figure 2D**), the preload recruitable stroke work (**Figure 2G**), and preload adjusted dP/dt_{max} (**Figure 2H**) and dP/dt_{min} (**Figure 2I**) in DMetD and Control.

Figure 1



Ratio of mitral peak velocity during early filling (E) to early diastolic mitral annular velocity (e', E/e' ratio, **A**), peak left ventricle untwist velocity (**B**), left atrial volume (LA, **C**), left ventricle end diastolic diameter (LVEDD, **D**), posterior wall thickness (PWd, **E**), and relative wall thickness ((2*PWd)/LVEDD, **F**) in the hearts of DMetD and Control (CON) swine at baseline (BL) and after 5 months. *$p < 0.05$ for interaction DMetD and time by two-way ANOVA, $^{\dagger}p < 0.05$ versus corresponding CON by Bonferroni post-hoc test, and $^{\ddagger}p < 0.05$ versus CON by unpaired t-test.

Figure 2

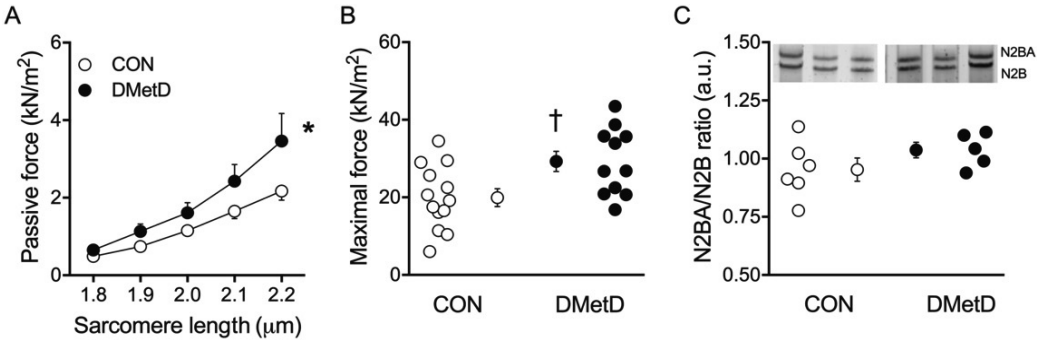


Left ventricular weight (LV weight, (**A**)), myocyte cross-sectional area (examples, (**B**), and data summary (**C**)), end-diastolic pressure (EDP)–volume (EDV) relation composed from measurements at preload reduction, (inverted triangle), baseline (open circle) and preload increase (open triangle) (**D**), myocardial collagen deposition (examples, (**E**) and data summary (**F**)), stroke work (SW, **G**), and dP/dt_{max} (**H**) and dP/dt_{min} (**I**), plotted as a function of end-diastolic volume, measured at sacrifice in CON and DMetD animals.

Isolated cardiomyocyte function

Single cardiomyocyte passive force was significantly higher in the DMetD group compared to Control (**Figure 3A**) as was the maximally developed force (**Figure 3B**). No differences were observed in titin isoform composition evidenced by an unaltered ratio between the compliant (N2BA) and stiff (N2B) isoform of titin between DMetD and Control (**Figure 3C**).

Figure 3

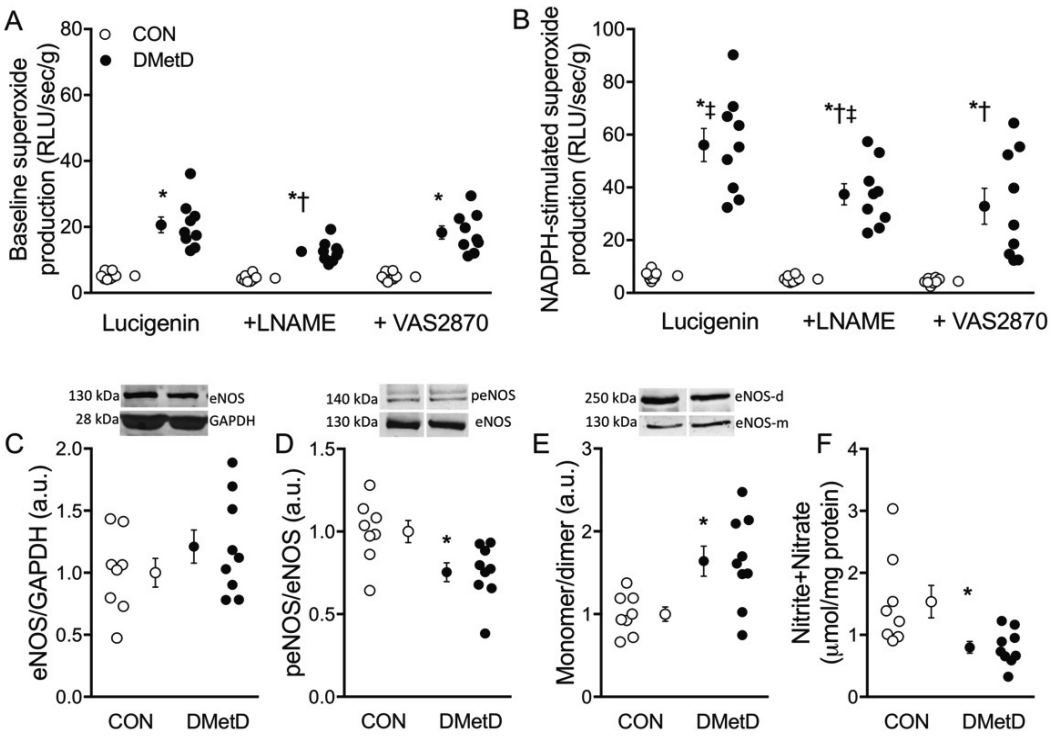


Single cardiomyocyte passive force (**A**) in response to increasing myocyte stretch and maximal forces (**B**). CON = controls (13 cardiomyocytes of 5 swine), DMetD, diabetic metabolic derangement (11 cardiomyocytes of 5 swine). Myocardial titin N2BA/N2B ratio of CON (n = 6) and DMetD (n = 5, C), and typical examples of the titin gels for 3 animals in each group . *$p < 0.05$ versus CON by two-way ANOVA, $^{\dagger}p < 0.05$ versus CON by unpaired t-test. Full-length gels are shown in Supplementary Figure S1.

Reactive oxygen species and nitric oxide

Superoxide production in the LV myocardium was markedly higher in DMetD compared to Control under baseline conditions as well as during inhibition of NOS and NADPH oxidase (**Figure 4A**). Furthermore, upon stimulation of NADPH oxidase (**Figure 4B**), superoxide production was further enhanced in the DMetD group, suggesting that NADPH oxidase is a significant source of superoxide. This increase was attenuated by L-NAME, both under basal conditions (**Figure 4A**), as well as under NADPH stimulation (**Figure 4B**), suggesting that a significant part of the superoxide production is NOS-dependent. eNOS expression in DMetD was comparable to Control (**Figure 4C**), but the ratio between phosphorylated and unphosphorylated eNOS was reduced in DMetD (**Figure 4D**). Moreover, monomer-to-dimer ratio was also significantly increased, suggestive of eNOS uncoupling (**Figure 4E**), and NO production was reduced in DMetD (**Figure 4F**). The decrease in eNOS phosphorylation and increase in eNOS uncoupling correlated with the superoxide production (both $p < 0.05$), further supporting the contribution of eNOS uncoupling to the oxidative stress in the hearts of DMetD animals.

Figure 4



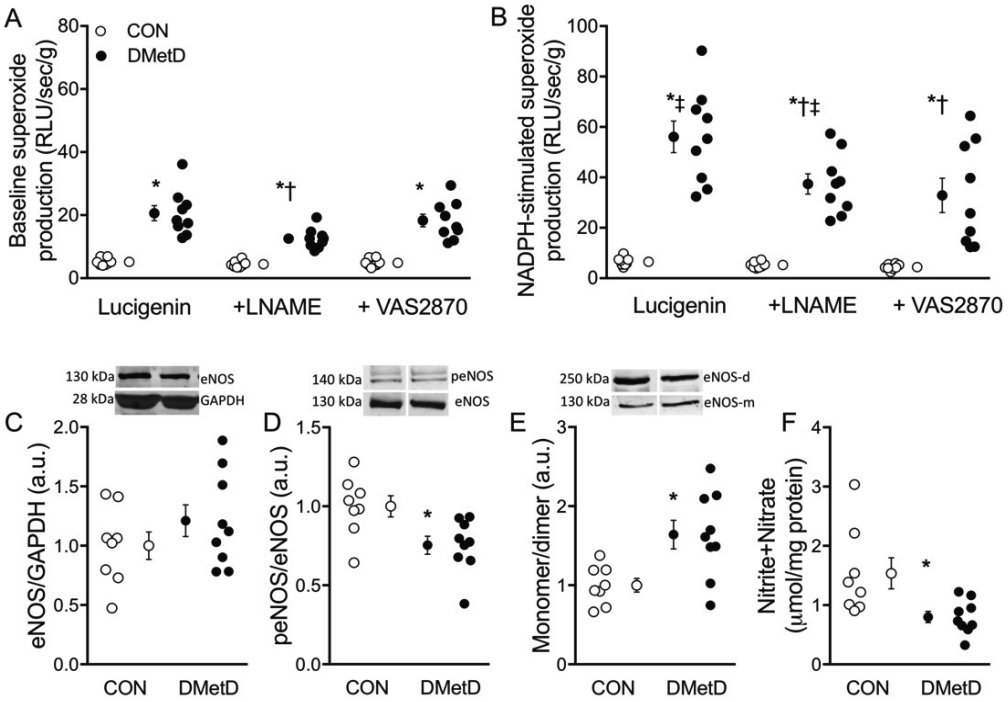
Basal (**A**) and NAPDH-stimulated (**B**) superoxide production in LV myocardium, myocardial eNOS expression (**C**), phosphorylation of eNOS (**D**), the monomer/dimer ratio (**E**), and nitric oxide (NO) production (**F**). CON = controls (n = 8), DMetD, diabetic metabolic derangement (n = 9). *$p < 0.05$ versus corresponding CON by two-way ANOVA and Bonferroni post hoc (panel **A** and **B**) or unpaired t-test (panel **C**–**F**), †$p < 0.05$ versus corresponding untreated by two-way ANOVA and Bonferroni post hoc, ‡$p < 0.05$ versus corresponding basal by two-way ANOVA and Bonferroni post hoc. Full-length gel blots are shown in Supplementary Figure S2.

Mitochondrial structure and function

Electron microscopy (EM) analysis of the cardiomyocytes (**Figure 5A, B**) demonstrated increased glycogen content in the cardiomyocytes of DMetD compared to Control (**Figure 5C**), indicating a clear diabetic phenotype. Additionally, more (and larger) vacuoles were observed in DMetD myocardium compared to Control (**Figure 5D**), indicative of either fatty acid accumulation or enlarged sarcoplasmic reticulum. Mitochondrial density (**Figure 5E**) and percentage of interconnected mitochondria (**Figure 5F**) were similar in both groups, suggesting that the network connectivity remained intact in DMetD. Total myocardial mitochondrial complex protein content was slightly higher in DMetD (**Figure 5G** and **S3**), but maximally uncoupled respiration (normalized to wet weight) was lower in

DMetD compared to Control (− 20%, $p < 0.05$; **Figure 5H**). This lower mitochondrial respiration could, at least in part, be explained by a lower maximal NADH (complex I)-stimulated respiration in DMetD compared to Control (− 31%, $p = 0.02$; **Supplementary Figure S4**), while maximal complex II-linked respiration was similar (**Figure S4**), indicative of a mitochondrial complex I dysfunction in DMetD.

Figure 5

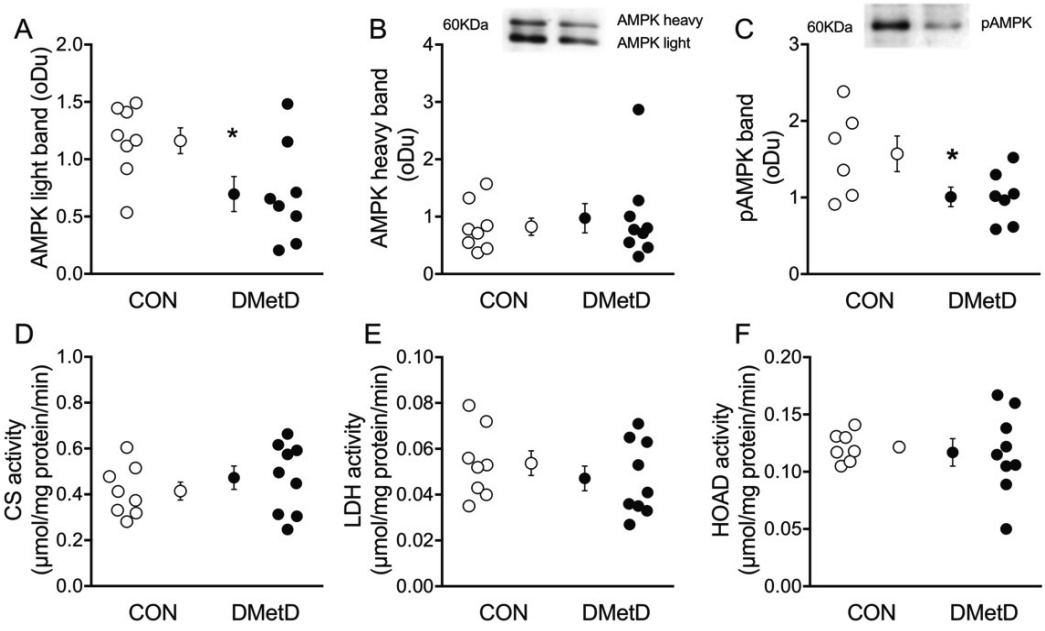


Examples of cardiomyocyte ultrastructure by electron microscopy in CON (**A**) and DMetD (**B**). M = mitochondrion, G = glycogen, V = vacuole. Scale bar represents 2 µm. The inserts highlight the altered glycogen content. Quantified glycogen content (**C**) and number of vacuoles (**D**) were significantly higher in DMetD compared to CON. Mitochondrial density (**E**) or percentage of connected mitochondria (**F**) were not different between groups. Total myocardial mitochondrial protein content (**G**) by Western blotting (see Supplementary Figure 1) was significantly higher in DMetD compared to CON. Electron transport (ET) capacity (**H**) however, was significantly lower in DMetD compared to CON, due to NADH-linked dysfunction (see Supplementary Figure 2), suggestive of an intrinsic mitochondrial dysfunction, independent of mitochondrial protein mass. *$p < 0.05$ *vs.* CON by unpaired t-test. Full-length gels and blots are shown in Supplementary Figure S3.

AMPK and metabolic enzymes

The analysis of AMPK light and heavy bands showed lower protein levels of the AMPK light bands in the DMetD animals (**Figure 6A**), while no differences were observed in the heavy bands (**Figure 6B**). The levels of pAMPK were also lower in the DMetD group (**Figure 6C**). Enzyme activities of citrate synthase (CS), lactate dehydrogenase (LDH) and 3-OH acyl CoA dehydrogenase (HOAD) were similar between groups (**Figure 6D–F**).

Figure 6

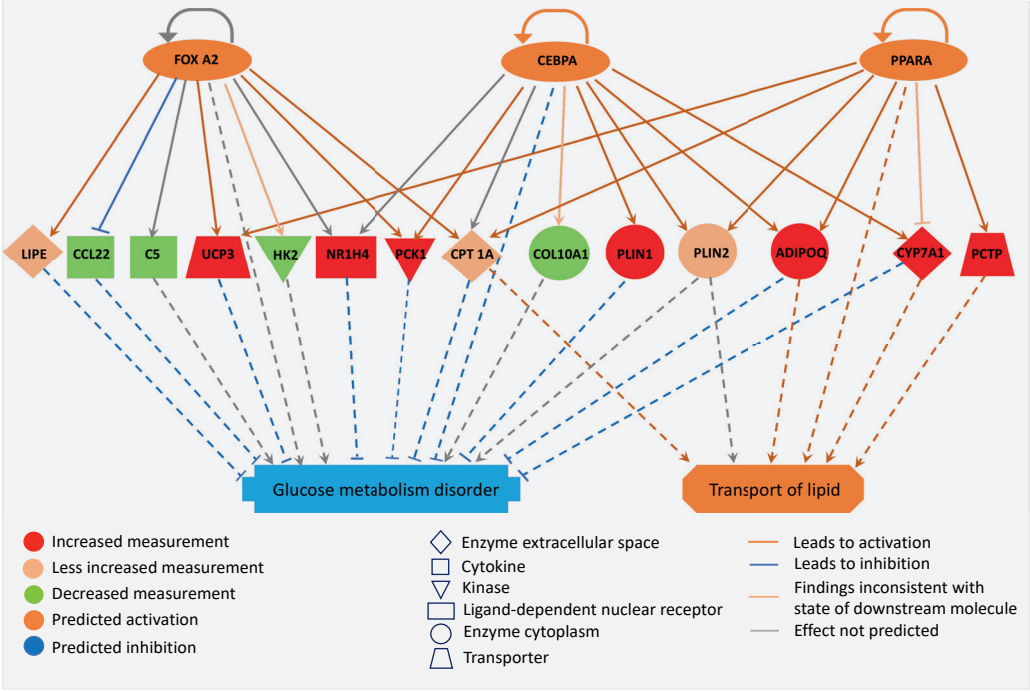


Protein levels of the AMPK light (**A**) and heavy bands (**B**), pAMPK (**C**) and enzyme activity of CS (**D**), LDH (**E**) and HOAD (**F**), in CON and DMetD animals. *p < 0.05 versus CON by unpaired t-test. *AMPK* AMP-activated protein kinase, *pAMPK* phosphorylated AMPK, *CS* citrate synthase, *LDH* lactate dehydrogenase, *HOAD* 3-hydroxyacyl-CoA dehydrogenase. Full-length gel blots are shown in Supplementary Figure S5.

Genome-wide gene expression

Genome-wide gene expression analyses indicated that transcription of 63 different genes were statistically significantly differentially expressed, with 29 genes up-regulated and 34 genes down-regulated by DMetD, of which many were related to alterations in glucose and fatty acid metabolism (Supplementary Table S6). The most significantly affected networks were 'glucose metabolism disorder' and 'transport of lipids' and within these networks we identified three transcriptional factors being involved, i.e. FOX A2, CEBPA and PPARA (Figure 7).

Figure 7



Myocardial genes and their networks in DMetD are connected to two major pathways associated with glucose and lipid metabolism disorders.

Discussion

The present study was designed to investigate the early cardiac functional and structural abnormalities produced by DMetD in a human-like, translational large animal model, at the level of gene and protein expression, cellular and tissue composition as well as organ function. The main findings were that: (*i*) After five months of DMetD, no changes in LV weight, LV end-diastolic-pressure, -volume, and -pressure–volume relation, and LA volume were observed. Similarly, no LV myocardial fibrosis or cardiomyocyte hypertrophy was observed, indicating the absence of overt remodeling at the LV chamber or myocardial tissue level. (*ii*) In contrast, cardiomyocyte resting tension was increased, which was accompanied by an elevated E/e' and a lower LV peak untwist velocity in DMetD, reflecting early impairments in LV diastolic function. (*iii*) Mitochondrial dysfunction in DMetD swine was also present with lower mitochondrial complex I function and lower maximal respiration, despite minor changes in mitochondrial protein content or mitochondrial density. (*iv*) DMetD also resulted in marked perturbations in LV myocardial nitroso-redox balance, due to increased superoxide and reduced nitric oxide production. (*v*) These abnormalities were accompanied by differential expression of a significant number of genes, many of which related to perturbations in glucose and fatty acid metabolism. The implications of these findings will be discussed.

DMetD-associated LV diastolic dysfunction in the absence of LV remodeling

DMetD is a major risk factor for heart failure, in particular heart failure with preserved ejection fraction (HFpEF).[19,20] HFpEF is characterized by an increased LV diastolic stiffness and LV concentric remodeling, while at the myocardial ultrastructural level fibrosis and concentric myocyte hypertrophy are typically observed.[21,22] In the present study, 5 months of DMetD did result in robust hyperglycemia and dyslipidemia, with hypercholesterolemia and increased plasma triglycerides, in the absence of liver or kidney dysfunction (see Supplementary Table S5), consistent with our and others' findings in similar animal models.[7,23,24] However, despite these clear metabolic alterations DMetD did not—yet—result in LV or cardiomyocyte hypertrophy or increased LV end-diastolic stiffness or produce myocardial fibrosis, suggesting that overt LV structural changes were still absent. Additionally, no macroscopic atherosclerosis development could be observed at this point in any of the large epicardial coronary arteries. In contrast, we observed several functional changes both in LV chamber and cardiomyocyte function. Thus, peak untwist velocity—a novel and early functional marker of LV diastolic dysfunction—was lower, while E/e'—an established marker of diastolic dysfunction—was elevated in DMetD versus Control swine at 5 months of DMetD. Interestingly, we recently found that

a reduction of peak untwist velocity was already observed after 3 months of DMetD, at a time when E/e' was still maintained normal.[25] The observation in the present study, that 5 months of DMetD produced a further reduction in peak untwist velocity and resulted in an elevated E/e', suggests that diastolic dysfunction progresses over time. The described increase in E/e' ratio and peak untwist velocity may be a reflection of the dysfunctional (subendocardial) cardiomyocytes as an early manifestation of diastolic dysfunction, before an overt increase in collagen deposition increases chamber stiffness more profoundly.[26] This may explain why LVEDP and LV end-diastolic elastance, as assessed with LV pressure–volume measurements, were still maintained at this stage of the disease. However, the lack of difference between DMetD and Control animals with respect to other early diastolic function variables, including Tau and LV dP/dtmin is not readily explained. One potential explanation is that echocardiography was performed under light sedation, while LV pressure–volume measurements were obtained under deep general anaesthesia with pentobarbital; the latter may have obscured subtle differences in early diastolic function parameters between DMetD and Control animals.

Indeed, the changes in LV diastolic function were accompanied by an increased passive force of single cardiomyocytes, suggesting that altered cardiomyocyte function, rather than structural changes, were responsible for the observed LV diastolic dysfunction. A higher resting cardiomyocyte tension has been shown in patients with HFpEF[21,27] as well as in DMetD patients[28] and animal models[7,29,30]. The increased resting tension has initially been ascribed to changes in titin isoform expression.[31] However, in line with more recent studies suggesting that changes in titin phosphorylation (rather than the isoform changes) are principally responsible for the increased resting tension[32,33], we also failed to observe differences in titin isoform expression. There is increasing evidence that hypo-phosphorylation of titin is the result of reduced NO-cGMP-PKG signaling.[27,33] Interestingly, we observed a reduced nitric oxide production in DMetD swine, warranting further investigation of changes in titin phosphorylation in DMetD animals in future studies.

Oxidative stress and impaired nitric oxide formation

Five months of DMetD resulted in substantially higher superoxide production in LV myocardium compared to Control swine. Superoxide production was already significantly higher at basal state, which was principally NOS mediated. In addition, superoxide production was further aggravated in DMetD myocardium upon exposure to NADPH, which appeared to be mediated by both NOS and

NADPH oxidase. These findings are in line with other studies demonstrating oxidative stress in DMetD and suggest that a variety of mechanisms can contribute to oxidative stress in DMetD.[34,35] Moreover, the increased superoxide production was likely directly related to abnormalities in NO bio-availability. An increased NOS dependent superoxide production is consistent with our finding that monomer/dimer ratio of eNOS was significantly higher in DMetD myocardium. This suggests substantial eNOS uncoupling, which resulted in superoxide— rather than NO-production by eNOS. Moreover, although total eNOS was not different between the groups, the ratio of phosphorylated and unphosphorylated eNOS was reduced in DMetD, which may have been the result of the observed lower levels of AMPK and pAMPK.[36,37] In line with these findings, reduced NO levels, as indicated by the NO metabolites nitrite-and nitrate, were present in the DMetD myocardium. Reduced NO levels are not only detrimental for coronary vascular function[38], but also for myocardial function[36,39], and the loss of NO, and consequently NO-cGMP-PKG signaling, likely explains the observed increase in cardiomyocyte resting tension[27].

AMPK and enzymatic activities in the LV

We observed an inhibition or deactivation of the AMPK system in DMetD, as we measured lower AMPK phosphorylation and AMPK light band levels. AMPK is an important regulator of cellular energy pathways and is activated by stressful situations such as prolonged exercise when AMP/ADP ratio is elevated.[37,40] Unlike in rodents, in fish and swine two AMPK bands can be detected. The physiological meaning of these two bands has not been fully elucidated, but they may represent different isoforms of AMPK. The downregulation of AMPK was likely the result of the increased circulating glucose and lipid levels in conjunction with the higher tissue glycogen levels in DMetD swine, which was previously observed also in mouse models of DMetD.[41,42] A decrease in AMPK activity explains, at least in part, the increased NADPH oxidase activity as well as the reduced peNOS levels in DMetD swine, and thus likely contributed to the perturbations in nitroso-redox signaling and the consequent increase in cardiomyocyte resting tension.[37]

Metabolic and mitochondrial function

Myocardial glycogen content was higher in DMetD, which is consistent with findings in previous studies.[43,44] The higher number of vacuoles in the DMetD LV myocardium likely reflects increased fat deposits, but could also be part of enlarged peroxisomes (for fatty acid oxidation) or sarcoplasmic

reticulum. Unfortunately, delineation between these possibilities cannot be derived from our electron microscopy images. However, the lack of functional alterations in systolic cardiac function in DMetD suggests that the sarcoplasmic reticulum remains intact in this animal model and these enlarged vacuoles are likely related to lipid overload, and a state of lipotoxicity.[6]

Additionally, mitochondrial density and connectivity were not affected in DMetD, while total mitochondrial complex protein content was slightly higher. No significant alterations were detected in the maximal activity of myocardial enzymes essentially involved in cardiomyocyte energy generation, including CS, LDH and HOAD. Maximally stimulated mitochondrial respiration was lower in DMetD, due to a lower NADH-linked (complex I) respiration. Mitochondrial complex I is the most vulnerable complex for mitochondrial (supercomplex) damage, and dysfunction has been seen in other models of heart failure[16], and type 2 diabetes mellitus[45]. Likely, the mitochondrial complex I dysfunction causes bioenergetic dysfunction and ADP insensitivity of the heart, contributing to ADP-induced stiffening of the heart.[46] The cause of this impaired mitochondrial complex I function is currently unknown, but could relate to local inflammation and/or lipotoxicity,[6,45] or oxidative stress-induced alterations in supercomplex formation[6, 16] that in turn can cause an increase in mitochondrial superoxide formation[47]. Further studies are required to understand the contribution of mitochondrial dysfunction and the role of oxidative stress in the development of diastolic dysfunction in DMetD.

Gene expression profiles

Our genome-wide gene expression data analysis indicated that after 5 months of DMetD transcription of 364 different genes was either up-or down-regulated by DMetD by at least twofold. From these 364 genes two highly significant networks emerged that were related to abnormalities in glucose metabolism (glucose metabolism disorder) and fatty acid metabolism (transport of lipid). After p-value correction (FDR < 0.1), 63 genes remained statistically significant, of which several genes are particularly relevant in relation to the diabetic cardiomyopathy.

First, the upregulation of UCP3 (uncoupling protein 3) which results in dissipation of energy as heat, can protect mitochondria against lipotoxicity and lipid-induced oxidative stress, observed here. Expression levels of UCP3 increase when fatty acid supply to mitochondria exceeds their beta-oxidation capacity and the protein enables the export of excess fatty acid load from mitochondria.[48] Another upregulated gene, ANGPTL4, encodes for the protein Angiopoietin-like 4, of which expression is induced by low oxygen levels and is also directly involved in regulating lipid metabolism. In diabetes, increased expression leads to reduced triglyceride clearance in blood, and explains, at least in part,

the observed hypertriglyceridemia in DMetD swine. Additionally, we observed upregulated CPT1A, carnitine palmitoyltransferase 1A, which has a rate-limiting role for long chain fatty acid oxidation in cardiac mitochondria. Its expression has been shown to be increased in hypertrophied rat cardiomyocytes[49], resulting in impaired fatty acid oxidation[50]. Although the mechanisms responsible for such increase in the present study are unclear, and may be related to an impaired inhibition of malonyl-CoA by glucagon in the DMetD hearts, it may contribute to accumulation of fatty acid metabolites in the myocardium, contributing to the increased lipotoxicity.[50] Lipase E was also up-regulated in DMetD hearts, and is known to regulate hydrolysis of stored triglycerides to free fatty acids. Perilipin 2 (PLIN2) coats intracellular lipid storage droplets, and its up-regulation together with lipase E indicates enhanced free fatty acid traffic in the DMetD myocardium, and suggests that the higher number of vacuoles in DMetD indeed represent lipid accumulation. The protein encoded by the CREB3L3 is a transcription factor that may act during endoplasmic reticulum stress by activating unfolded protein response target genes, suggesting that endoplasmic reticulum stress is present in the DMetD myocardium.[51]

Out of the most down-regulated genes (largest fold-change), the physiological meaning of the protein encoded by GNMT gene (an enzyme that catalyzes the conversion of S-adenosyl-L-methionine to S-adenosyl-L-homocysteine and sarcosine) is currently unclear in the diabetic heart. The observed down-regulation of SLC2A1 gene, which normally provides instructions for producing glucose transporter protein type 1 (GLUT1) is consistent with reduced glucose uptake in diabetic myocardium.[52] Furthermore, HAPLN3 (Hyaluronan and proteoglycan link protein 3) has previously been associated with diabetes in epidemiological studies. The ALDH4A1 gene codes for the enzyme delta-1-pyrroline-5-carboxylate dehydrogenase, which is a mitochondrial matrix NAD-dependent dehydrogenase producing glutamate; its down-regulation confirms early impairments in NADH-linked respiration. Down-regulation of CYP4F55 (cytochrome P450) also suggests overall alterations in mitochondrial substrate oxidation, but the role of the cytochrome P450 system in the heart is largely unknown.[53]

Although we did not investigate the causal relation between the observed alterations in gene-expression and the cardiac phenotype in DMetD animals, it could be speculated that some of these genes, expecially those related to mitochondrial function, could serve as potential drug targets to treat diabetic cardiomyopathy. Future studies are therefore needed to determine the therapeutic potential of interfering with (or enhancing) the DMetD-induced alterations in gene-expression.

Conclusions

The present study shows that diabetic metabolic derangement in a large animal model, with high resemblance to the human heart, resulted in myocardial oxidative stress, eNOS uncoupling and reduced NO production, together with an altered metabolic gene expression profile and mitochondrial dysfunction. These myocardial tissue alterations were associated with cardiomyocyte stiffening and early left ventricular diastolic dysfunction, before any overt structural cardiac remodeling occurs. Therapies should be directed to ameliorate these early DMetD-induced myocardial changes to prevent the development of overt cardiac failure.

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Contributions I.H., O.S. and D.D. designed the study. I.H., O.S., B.D., V.B., J.W., R.W., R.D., Y.O., Y..H., L.B., K.A., M.A., A.S., J.V., D.M. and D.D. I.H., O.S., B.D., J.W., Y..H., K.A., A.S., J.V., D.M. and D.D collected and analysed the data. I.H., O.S., B.D., V.B., J.W., R.W., R.D., Y.O., Y..H., L.B., K.A., M.A., A.S., J.V., D.M. and D.D. I.H., O.S., B.D., J.W., Y..H., K.A., A.S., J.V., D.M. and D.D.reviewed/edited and contributed to the discussion of the manuscript. I.H. wrote the first draft of the manuscript. D.J. Duncker is the guarantor of the study.

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Table S1 Body weight, left atrium and conventional ventricular echocardiography characteristics from baseline to 5-month follow-up.

		Baseline	5-MONTH
Body weight (kg)	CON	32 ± 2	37 ± 1
	DMetD	31 ± 2	40 ± 3
LA volume (mm^3)	CON	28 ± 3	26 ± 2
	DMetD	23 ± 2	25 ± 3
Relative LA volume (mm^3/kg)	CON	0.86 ± 0.07	0.71 ± 0.05
	DMetD	0.75 ± 0.06	0.63 ± 0.06
LVEDD (mm)	CON	41 ± 2	40 ± 1
	DMetD	41 ± 1	40 ± 1
Relative LVEDD (mm/kg)	CON	1.31 ± 0.26	1.10 ± 0.16
	DMetD	1.36 ± 0.02	1.05 ± 0.24
PWd (mm)	CON	5.4 ± 0.3	5.9 ± 0.4
	DMetD	5.6 ± 0.3	5.9 ± 0.3
IVSd (mm)	CON	5.9 ± 0.2	6.3 ± 0.4
	DMetD	5.9 ± 0.4	5.8 ± 0.3
E (cm/s)	CON	55 ± 3	51 ± 3
	DMetD	53 ± 3	53 ± 5
RWT (unitless)	CON	0.26 ± 0.02	0.30 ± 0.02
	DMetD	0.27 ± 0.02	0.29 ± 0.05
E/A ratio	CON	1.37 ± 0.12	1.47 ± 0.18
	DMetD	1.39 ± 0.12	1.46 ± 0.19
e' mean (cm/s)	CON	10.5 ± 0.2	10.4 ± 0.7
	DMetD	11.1 ± 0.5	8.9 ± 0.6
E/e' ratio	CON	7.0 ± 0.3	7.1 ± 0.3
	DMetD	7.2 ± 0.6	8.6 ± 0.6*
DET (ms)	CON	114 ± 5	132 ± 13
	DMetD	125 ± 12	124 ± 9

CON=healthy controls (n=8), DMetD=diabetic metabolic dysfunction (n=9). LA=left atrium, Relative=normalized for body weight, LVEDD=left ventricular end diastolic diameter, PWd=posterior wall diameter, IVSd=intraventricular septum thickness end-diastole, RWT=relative wall thickness (2*PWd)/LVEDD), E=early diastolic filling velocity, E/A ratio=ratio between early and late filling velocities, e' mean=early diastolic tissue relaxation velocity, mean of lateral and septal wall, DET=deceleration time of early diastolic filling. *$p<0.05$ vs CON by unpaired t-test at 5 months' time-point. Data are mean±SEM.

Table S2. Left ventricular speckle tracking echocardiography characteristics from baseline to 5-month time points.

		BASELINE	5-MONTH
Peak rotation basal (°)	CON	0.34 ± 0.68	0.82 ± 0.53
	DMetD	1.26 ± 0.54	1.17 ± 0.41
Peak rotation apical (°)	CON	3.8 ± 0.9	5.6 ± 1.5
	DMetD	5.1 ± 2.0	3.0 ± 0.9
Peak velocity basal rotation (°/ms)	CON	5.4 ± 1.0	5.4 ± 2.0
	DMetD	5.3 ± 1.2	7.4 ± 3.4
Peak velocity apical rotation (°/ms)	CON	-27.4 ± 9.5	-35.7 ± 14.2
	DMetD	-26.7 ± 8.6	-13.3 ± 3.5
Peak twist (°)	CON	4.6 ± 1.7	4.8 ± 1.7
	DMetD	4.9 ± 2.1	2.3 ± 0.8
Time to peak twist (s)	CON	0.43 ± 0.02	0.40 ± 0.01
	DMetD	0.47 ± 0.07	0.42 ± 0.03
Peak untwist velocity (°/ms)	CON	-34 ± 9	-58 ± 17
	DMetD	-35 ± 8	-22 ± 8*†
Time to peak untwist velocity (s)	CON	0.40 ± 0.02	0.48 ± 0.02
	DMetD	0.38 ± 0.02	0.44 ± 0.02

CON=healthy controls (n=8), DMetD=diabetic metabolic dysfunction animals (n=9). *p<0.05 as time•diabetes interaction by two-way ANOVA, †p<0.05 versus CON by Bonferroni post-hoc analysis. Data are mean±SEM.

Table S3. Hemodynamic characteristics of DMetD and CON animals at sacrifice measured by Millar or Swan Ganz catheter.

	CON	DMetD	t-test
HR (bpm)	100 ± 7	85 ± 9	0.20
MAP (mmHg)	74 ± 5	88 ± 7	0.13
SAP (mmHg)	82 ± 5	95 ± 7	0.15
DAP (mmHg)	66 ± 6	79 ± 7	0.16
LV SP (mmHg)	86 ± 3	95 ± 7	0.24
LV dP/dt_{max} (mmHg s^{-1})	1323 ± 54	1286 ± 153	0.83
LV dP/dt_{min} (mmHg s^{-1})	-1181 ± 106	-1441 ± 114	0.12
LV EDP (mmHg)	9.2 ± 1.1	8.7 ± 1.3	0.76
Tau (ms)	63 ± 6	68 ± 9	0.61
DTF	0.42 ± 0.02	0.49 ± 0.03	0.11
SV (ml)	24 ± 3	27 ± 2	0.44
CO (L min^{-1})	2.4 ± 0.2	2.2 ± 0.2	0.57
SVR (mmHg L^{-1} min^{-1})	33 ± 3	42 ± 4	0.11

CON=healthy controls (n=8), DMetD=diabetic metabolic derangement animals (n=9). HR=heart rate, MAP=mean arterial pressure, SAP=systolic arterial pressure, DAP = diastolic arterial pressure, LV SP = maximal left ventricular pressure, LV dP/dt_{max}= maximum rate of rise of left ventricular pressure, LV dP/dt_{min}=maximum rate of fall of left ventricular pressure, LV EDP=left ventricular end diastolic pressure, Tau=diastolic time constant, DTF=diastolic time fraction, SV stroke volume, CO cardiac output, SVR systemic vascular resistance. Data are mean±SEM.

Table S4. Left ventricular end diastolic and end systolic volumes and pressures and stroke volume and ejection fraction based on pressure-volume loop analyses.

		Preload reduction	Baseline	Preload increase
LV EDV (ml)	CON	30 ± 5	43 ± 6	71 ± 9*
	DMetD	37 ± 4*	58 ± 6	103 ± 10*†
LV ESV (ml)	CON	7 ± 3	15 ± 3	36 ± 7*
	DMetD	16 ± 4	28 ± 5	62 ± 11*†
LV EF (%)	CON	78 ± 8	65 ± 4	49 ± 5
	DMetD	62 ± 7	54 ± 4	42 ± 7
SV (ml)	CON	23 ± 5	28 ± 4	35 ± 6
	DMetD	21 ± 2	30 ± 2	40 ± 6
LV EDP (mmHg)	CON	6.8 ± 1.7	9.7 ± 1.8	14.3 ± 2.1*
	DMetD	7.6 ± 2.0*	12.0 ± 1.9	20.0 ± 2.5*
LV ESP (mmHg)	CON	56 ± 6	68 ± 7	94 ± 9*
	DMetD	73 ± 6*	91 ± 6	113 ± 11*

CON=healthy controls (n=6), DMetD=diabetic metabolic derangement animals (n=8). All measurements were obtained during inspirational breath hold. EDV=end diastolic volume, ESV=end systolic volume, EF=ejection fraction, SV = stroke volume, EDP=end diastolic pressure, ESP end systolic pressure. *$p < 0.05$ versus corresponding baseline, †$p<0.05$ versus corresponding control by two-way ANOVA and Bonferroni post-hoc analysis. Data are mean±SEM.

Table S5. Plasma metabolic parameters of CON and DMetD animals measured at sacrifice.

	CON	DMetD	t-test
Liver function			
ASAT (U L^{-1})	34 ± 2	30 ± 4	0.44
ALAT (U L^{-1})	55 ± 4	17 ± 2	<0.001
Renal function			
Urea (mmol L^{-1})	2.9 ± 0.3	1.7 ± 0.3	0.009
Creatinine (μmol L^{-1})	111 ± 6	92 ± 8	0.07
Albumin (g L^{-1})	44 ± 1	45 ± 2	0.59

CON=healthy controls (n=8), DMetD=diabetic metabolic derangement animals (n=9); ASAT aspartate aminotransferase; ALAT alanine aminotransferase. Data are mean±SEM.

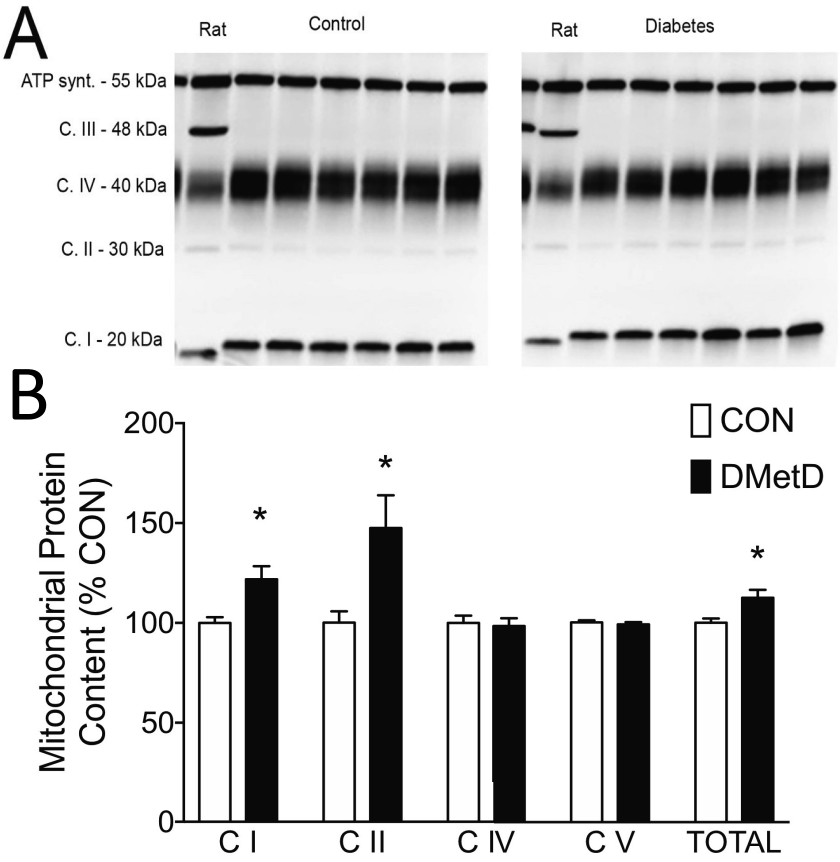
Table S6. Gene expression of significantly up- or down-regulated genes in DMetD compared to controls.

Gene-symbol	logFC	logCPM	LR	PValue	FDR
CREB3L3	4.53	1.07	16.96	3.818E-05	1.32E-02
UCP3	4.41	2.23	31.12	2.420E-08	3.24E-05
MARCO	3.93	0.12	20.40	6.274E-06	3.06E-03
ALAS2	3.54	3.76	11.78	5.973E-04	9.15E-02
CDH9	3.29	-0.65	13.52	2.359E-04	4.96E-02
AIRE	3.04	-0.13	17.97	2.239E-05	8.57E-03
MYO5B	2.98	2.52	31.74	1.767E-08	2.91E-05
HS3ST2	2.59	-0.76	12.67	3.724E-04	6.82E-02
FCN1	2.19	0.22	16.33	5.333E-05	1.68E-02
ANGPTL4	2.16	5.87	28.13	1.136E-07	1.11E-04
MYOZ1	2.16	1.35	12.71	3.637E-04	6.82E-02
PLIN2	2.15	6.59	30.91	2.709E-08	3.42E-05
BPIFC	1.96	0.12	11.96	5.428E-04	8.74E-02
SLCO6A1	1.87	0.09	12.59	3.879E-04	6.93E-02
LIPE	1.85	5.29	36.59	1.460E-09	3.91E-06
CPT1A	1.84	4.91	23.10	1.535E-06	9.97E-04
HTR4	1.71	1.65	19.03	1.288E-05	5.52E-03
RETSAT	1.56	7.95	25.37	4.739E-07	3.50E-04
PGLYRP2	1.46	0.00	12.11	5.020E-04	8.28E-02
CA4	1.44	5.40	18.13	2.063E-05	8.19E-03
RBP1	1.38	4.53	23.66	1.152E-06	7.96E-04
BTNL9	1.35	5.75	14.38	1.497E-04	3.56E-02
C7	1.25	5.95	12.97	3.163E-04	6.16E-02
LY96	1.18	2.18	11.93	5.514E-04	8.76E-02
SRPX	1.17	4.76	13.62	2.235E-04	4.74E-02
PDGFD	1.17	1.61	15.69	7.457E-05	2.16E-02
UCP2	1.04	3.37	18.89	1.388E-05	5.83E-03
CDC14A	1.01	1.00	11.62	6.512E-04	9.69E-02
ART4	1.00	3.92	16.49	4.886E-05	1.61E-02
OVOL3	-4.51	-1.98	13.44	2.461E-04	5.12E-02
CYP1A1	-2.99	3.04	12.63	3.791E-04	6.85E-02
SLC2A1	-2.25	2.99	28.70	8.437E-08	8.61E-05
STRC	-2.25	-0.35	15.83	6.917E-05	2.09E-02

GNMT	-2.19	3.45	29.78	4.850E-08	5.20E-05
SLC16A6	-2.15	0.99	20.59	5.675E-06	2.83E-03
ALDH4A1	-1.87	6.19	41.10	1.445E-10	1.03E-06
SLC26A6	-1.79	1.65	17.53	2.832E-05	1.03E-02
MCCC2	-1.78	4.35	27.56	1.525E-07	1.42E-04
AASS	-1.72	3.90	20.35	6.456E-06	3.08E-03
CYP4F55	-1.65	5.32	37.87	7.547E-10	2.70E-06
IZUMO4	-1.61	3.37	26.66	2.428E-07	2.08E-04
SQLE	-1.54	1.73	12.21	4.752E-04	7.96E-02
HAPLN3	-1.50	5.23	48.09	4.062E-12	8.71E-08
CYP4F2	-1.50	3.01	45.52	1.514E-11	1.62E-07
BDH1	-1.49	3.90	19.43	1.044E-05	4.66E-03
ADHFE1	-1.45	7.07	36.23	1.755E-09	4.18E-06
SYCE2	-1.42	4.12	18.45	1.740E-05	7.17E-03
BCKDHB	-1.40	5.60	29.83	4.722E-08	5.20E-05
WBSCR27	-1.38	0.75	17.01	3.711E-05	1.31E-02
MUT	-1.34	5.30	31.26	2.263E-08	3.23E-05
CBX8	-1.33	0.75	12.37	4.365E-04	7.49E-02
MPND	-1.33	5.11	20.64	5.532E-06	2.83E-03
CAMKV	-1.32	1.21	13.37	2.563E-04	5.28E-02
MCCC1	-1.31	5.64	31.38	2.124E-08	3.23E-05
MOCS1	-1.31	3.57	20.63	5.579E-06	2.83E-03
GCDH	-1.29	3.03	25.70	3.989E-07	3.17E-04
ALDH6A1	-1.27	6.98	36.99	1.189E-09	3.64E-06
CCT6B	-1.25	0.98	12.22	4.737E-04	7.96E-02
RANGRF	-1.24	5.50	25.07	5.516E-07	3.94E-04
SLC25A29	-1.24	5.65	34.75	3.752E-09	7.31E-06
PC	-1.19	3.17	12.30	4.519E-04	7.69E-02
GNB3	-1.02	4.04	14.86	1.160E-04	2.96E-02

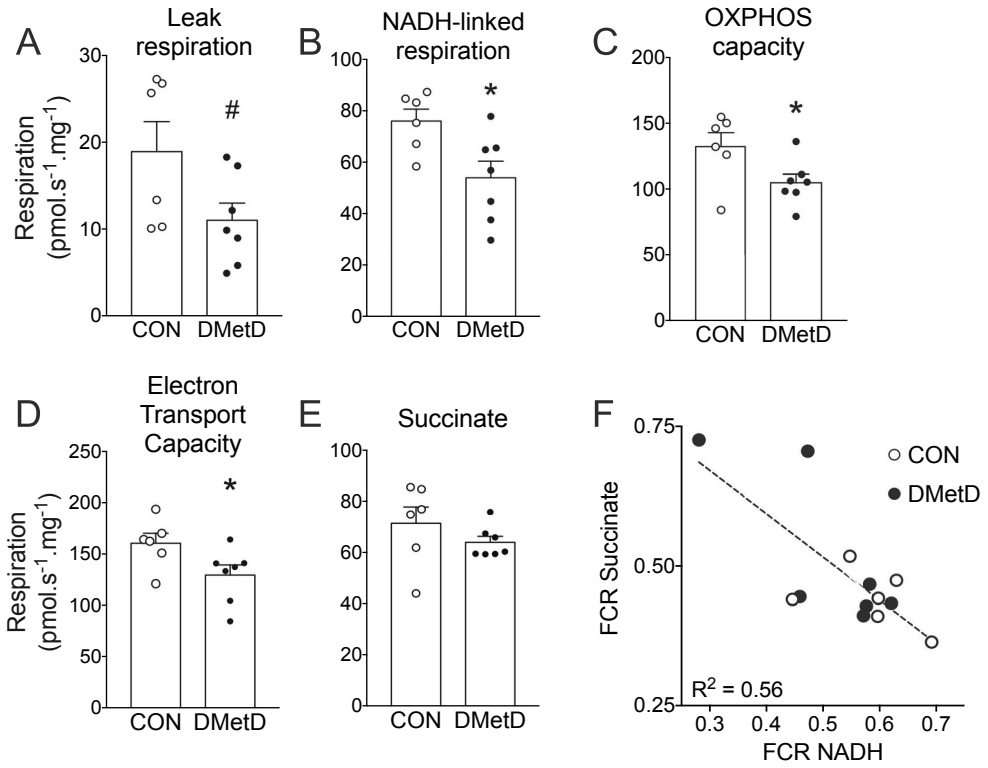
The genes differentially affected by diabetes. LogFC=log fold change, logCPM=log counts per million, LR=likelihood ratio, FDR=false discovery rate. Absolute logFC > 1 with a P-value < 0.001, indicating a minimal 2 fold change in gene expression; FDR<0.1.

Figure S3 (related to Figure 5G). Mitochondrial protein concentrations in the myocardium of CON and DMetD swine.

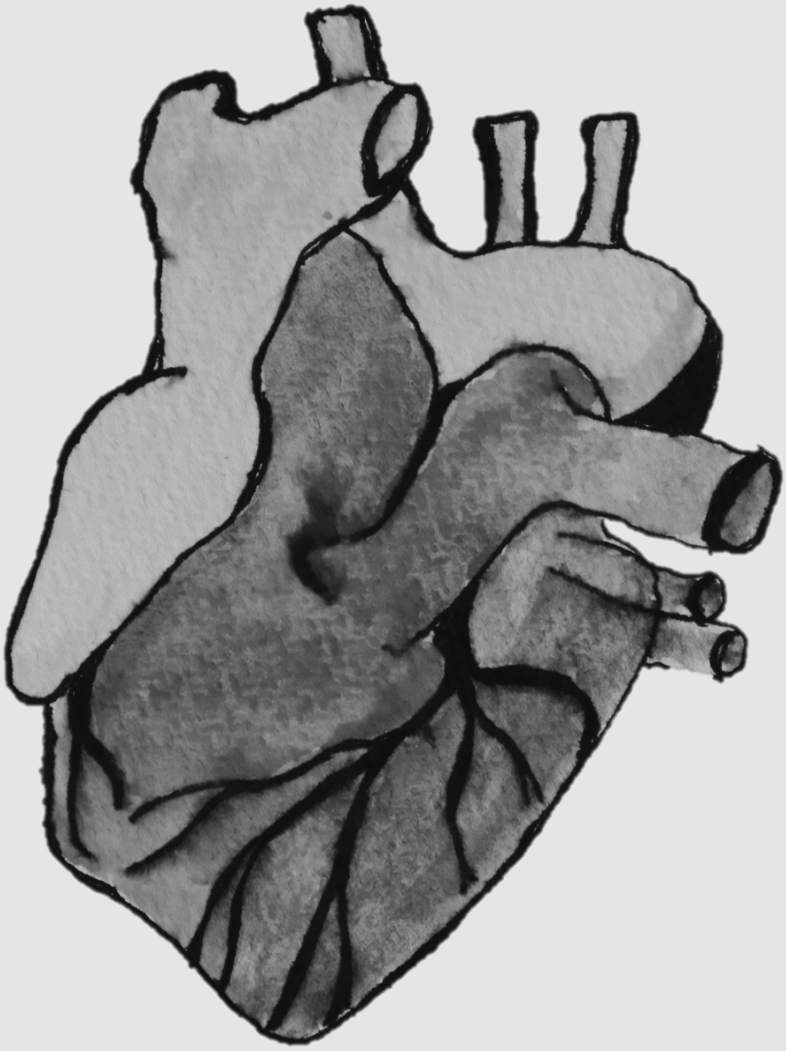


A. Typical example of a Western blot with an antibody cocktail against five complexes: complex I (C.I) subunit NDUFB8 at 20 kDa, complex II (C.II) subunit at 30kDa, complex III (C. III) core protein 2 at 48 kDa, complex IV (C.IV) subunit I at 40 kDa and ATP synthase (ATP synt) α-subunit. Note that C.III did not cross-react between rats and pigs. Samples from different blots were normalized based on a reoccurring rat sample and protein concentration. **B:** Total mitochondrial protein content was higher in DMetD, which was due to higher protein concentration of mitochondrial complex I and II subunits only. n=6 each group, in duplo. *: P<0.05 *vs.* CON.

Figure S4. (related to Figure 5H), Cardiac mitochondrial respiration is lower in DMetD compared to CON.



High-resolution respirometry was performed in permeabilized cardiac fibers from 6 CON and 7 DMetD hearts. **A**: Leak respiration with NADH-substrates (glutamate, pyruvate and malate) tended to be lower in DMetD compared to CON (#: P=0.06). NADH-linked respiration (**B**), OXPHOS capacity (with additional succinate, **C**) and electron transport capacity (**D**) were significantly lower in DMetD. Succinate-linked respiration via complex II, under the presence of the complex I blocker rotenone (**E**) was not different between groups (P=0.22), indicative that the lower maximal respiration was due to NADH-linked, complex I, dysfunction. The normalized flux (flux control ratio; FCR) for NADH-linked respiration correlated with the FCR for succinate, indicative that complex II partly compensates for the lower NADH-linked respiration in DMetD. Measurements performed in duplo and averaged. *: P<0.05 vs. CON



Chapter 5

Endothelial dysfunction and atherosclerosis increase von Willebrand factor and Factor VIII: a randomized controlled trial in swine

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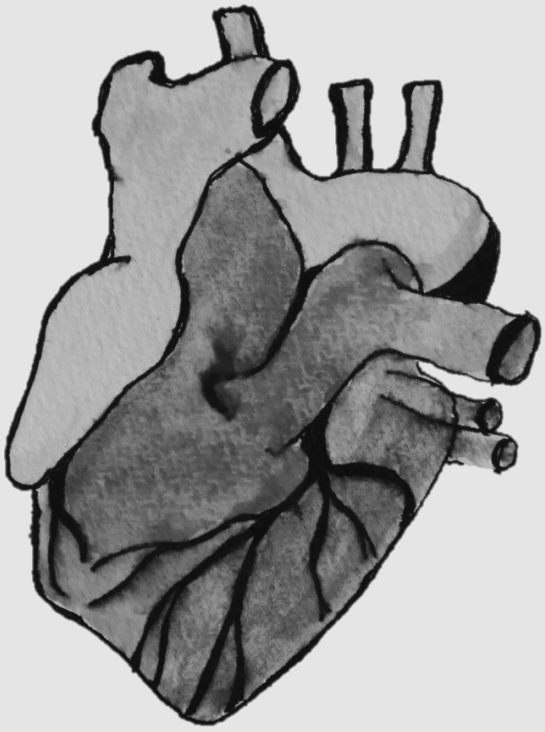
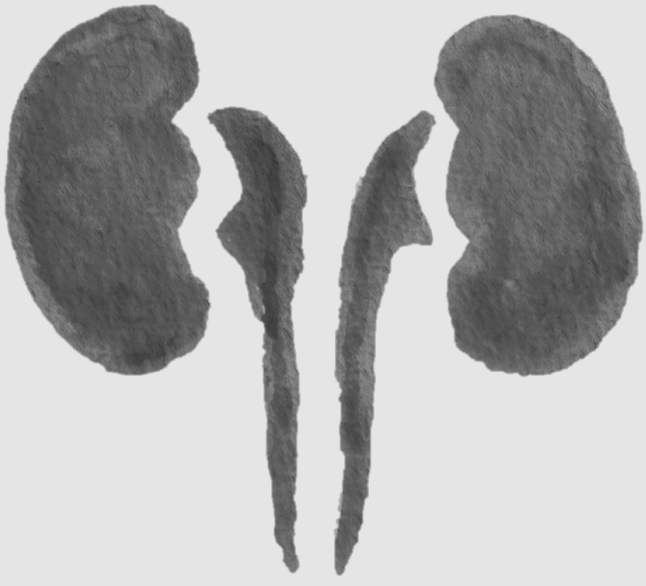
Thrombosis and Haemostasis (invited revision)

5

Part II

Microvascular and myocardial dysfunction in cardiovascular disease: an additional role for chronic kidney disease





Chapter 6

Chronic Kidney Disease as a Risk Factor for
Heart Failure With Preserved Ejection Fraction:
A Focus on Microcirculatory Factors and Therapeutic Targets

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6

Abstract

Heart failure (HF) and chronic kidney disease (CKD) co-exist, and it is estimated that about 50% of HF patients suffer from CKD. Although studies have been performed on the association between CKD and HF with reduced ejection fraction (HFrEF), less is known about the link between CKD and heart failure with preserved ejection fraction (HFpEF). Approximately, 50% of all patients with HF suffer from HFpEF, and this percentage is projected to rise in the coming years. Therapies for HFrEF are long established and considered quite successful. In contrast, clinical trials for treatment of HFpEF have all shown negative or disputable results. This is likely due to the multifactorial character and the lack of pathophysiological knowledge of HFpEF. The typical co-existence of HFpEF and CKD is partially due to common underlying comorbidities, such as hypertension, dyslipidemia and diabetes. Macrovascular changes accompanying CKD, such as hypertension and arterial stiffening, have been described to contribute to HFpEF development. Furthermore, several renal factors have a direct impact on the heart and/or coronary microvasculature and may underlie the association between CKD and HFpEF. These factors include: (1) activation of the renin-angiotensin-aldosterone system, (2) anemia, (3) hypercalcemia, hyperphosphatemia and increased levels of FGF-23, and (4) uremic toxins. This review critically discusses the above factors, focusing on their potential contribution to coronary dysfunction, left ventricular stiffening, and delayed left ventricular relaxation. We further summarize the directions of novel treatment options for HFpEF based on the contribution of these renal drivers.

Introduction

Heart failure with preserved ejection fraction (HFpEF) is characterized by impaired relaxation of the heart during diastole and accounts for over 50% of all patients with heart failure (HF).[1, 2] Both the proportion of HFpEF-patients and morbidity, mortality and healthcare costs associated with this disease are rising.[3-6] Multiple processes including cardiomyocyte hypertrophy, interstitial fibrosis, impaired calcium handling and increased passive cardiomyocyte stiffness contribute to the left ventricular stiffening characteristic for HFpEF.[7-9]

The current paradigm for HFpEF proposes that commonly present comorbidities such as diabetes mellitus (DM), obesity and hypertension lead to a systemic pro-inflammatory state. This pro-inflammatory state causes coronary microvascular dysfunction, evidenced by an imbalance between nitric oxide (NO) and reactive oxygen species (ROS) leading to stiffening of the left ventricle (LV).[9, 10] Excessive ROS-production in the endothelium of the coronary microvasculature lowers NO bioavailability through scavenging of NO. Loss of NO reduces soluble guanylate cyclase (sGC) activity in the cardiomyocytes, thereby lowering cGMP levels and decreasing PKG activity. The latter results in hypophosphorylation of titin and induces cardiomyocyte hypertrophy.[10, 11] Given the proposed central role for disruption of the NO pathway in pathogenesis of HFpEF, it is rather surprising that all large clinical trials which targeted the NO-cGMP-PKG pathway failed to date. Organic and inorganic nitrates are therapeutic agents that can be metabolized to NO systemically and thus act as NO-donors. However, the NEAT-HFPEF trial showed that isosorbide mononitrate, a long working organic nitrate, reduced physical activity and did not improve quality of life and exercise capacity.[12] Inhaled nebulized inorganic nitrate, did not improve exercise capacity, as recently shown in the INDIE-HFpEF trial.[13] The phase 2b SOCRATES-PRESERVED trial showed no reduction of NT-pro-BNP or left atrial dimensions at 12 weeks after treatment with the sGC stimulator Vericiguat. However, Vericiguat was well tolerated and increased quality of life, warranting further research.[14] Inhibition of the cGMP degrading enzyme phosphodiesterase 5 with Sildenafil did not improve clinical status rank score or exercise capacity[15], and failed to improve vascular and cardiac function.[16] Therefore, new therapeutic targets need to be identified that can interfere with the development and progression of HFpEF.

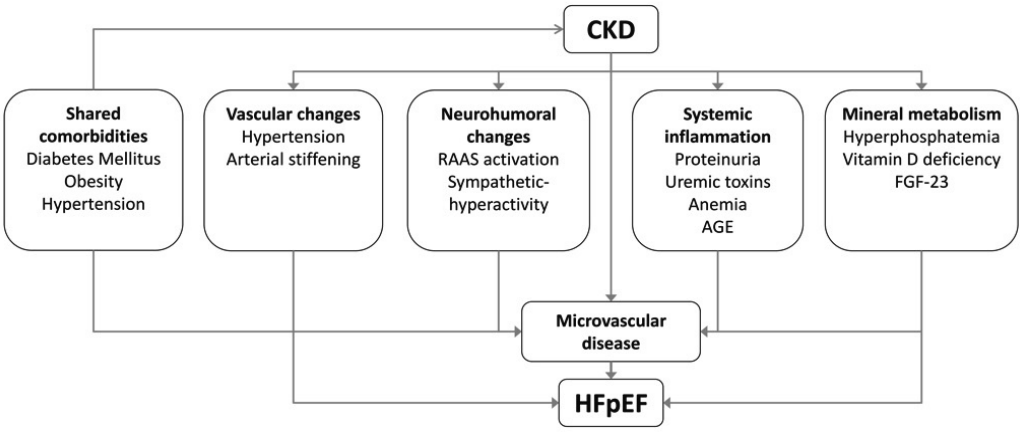
The so-called cardio-renal syndrome describes the co-existence of HF and chronic kidney disease (CKD). Approximately 50% of the patients with HFpEF also suffer from CKD.[17] Although this co-existence is partially due to shared risk factors, such as hypertension, DM and obesity, it has also been proposed that HF directly impacts kidney function, and, vice versa, CKD worsens cardiac function.[18] Interdependence of the heart and kidneys, similarities between their microvascular

networks, and the coexistence of CKD and HF further imply a role for microvascular dysfunction in development and progression of both diseases.[17]

It is important to note that the impact of microvascular dysfunction on cardiac structure and function is not limited to dysfunction of the NO-cGMP-PKG pathway. Indeed, upregulation of VCAM-1 and E-selectin on the coronary microvascular endothelium induces transendothelial leucocyte migration and activation, increased transforming growth factor β (TGF-β) levels, thereby promoting pro-fibrotic pathways and differentiation of fibroblast to myofibroblasts[10, 19] and increasing interstitial fibrosis.[8, 20] Secretion of autocrine and paracrine factors, such as apelin, TGF-β, and endothelin-1, by dysfunctional coronary microvascular endothelial cells can also directly induce left ventricular hypertrophy.[21] Finally, capillary rarefaction and inadequate angiogenesis could contribute to a decreased oxygen supply and subsequent left ventricular myocardial stiffening.[9]

Given the co-incidence of HFpEF and CKD, the present review aims to provide a mechanistic link between CKD and HFpEF, by describing potential pathways through which CKD can induce or aggravate coronary microvascular dysfunction and thereby contribute to the development and progression of left ventricular hypertrophy and diastolic dysfunction. These include mechanical effects, neurohumoral activation, systemic inflammation, anemia and changes in mineral metabolism as induced by CKD (**Figure 1**).

Figure 1 Schematic overview of the risk factors that can contribute to the development of heart failure with preserved ejection fraction (HFpEF) in patients with chronic kidney disease (CKD)



As some of these CKD-induced effects may induce HFpEF and contribute to cardiovascular disease in general, they may provide targets to intervene with development of diastolic dysfunction and/or its progression towards HFpEF. Hence, this review will also describe the (potential) druggable therapeutic targets within these pathways, and where applicable, clinical trials intervening with these pathways.

Clinical associations between CKD, coronary microvascular dysfunction and HFpEF

CKD is defined as a progressive decline of renal function and is associated with hypertension, proteinuria and the loss of nephron mass.[22] CKD is an independent risk factor for development of HF, with increasing cardiovascular risk and mortality as renal function declines.[23, 24] Additionally, HF is the major cause of death among patients with CKD.[25, 26] Although renal dysfunction is present in about half of the patients with HF in general[27, 28], and is an important prognostic marker for adverse outcomes[1, 29, 30], particularly the association between HFpEF and CKD is very strong. In a cohort comparing patients with HFrEF, HF with mid-range ejection fraction and HFpEF, renal dysfunction was most prevalent in HFpEF and was associated with increased mortality in all HF subtypes.[31] Gori *et al.* showed that 62% of the patients with HFpEF display abnormalities in at least one marker of renal insufficiency, with different markers correlating with different HFpEF phenotypes.[32] Further evidence for a causal relationship between CKD and HFpEF comes from a rat model, in which CKD was mimicked by nephrectomy of one whole kidney and 2/3 of the remaining kidney. Loss of nephron mass in these rats results in a cardiac HFpEF-like phenotype, with LV hypertrophy and diastolic dysfunction, but critical HFpEF features such as lung congestion and exercise intolerance were not reported.[33] In accordance with CKD as a causative factor for HFpEF, the majority of patients on hemodialysis display diastolic dysfunction and left ventricular hypertrophy, whereas systolic dysfunction and HFrEF are visible in only a minority of these patients.[34, 35] In a prospective cohort study, 74% of the patients admitted for dialysis displayed left ventricular hypertrophy. In contrast, systolic dysfunction and left ventricular dilatation were present in only 15% and 32% of the patients respectively.[36] Left ventricular hypertrophy is not restricted to end stage CKD, but already highly prevalent in the general CKD population.[37] Indeed, the first visible myocardial alteration in patients with CKD is left ventricular hypertrophy[38], developing early in the progression of kidney dysfunction[39, 40] and often co-occurring with myocardial fibrosis and diastolic dysfunction[41]. Hypertension is an important predictor for

development of left ventricular hypertrophy and HFpEF in patients with CKD[39, 42], while blood pressure reduction is associated with a lower cardiovascular risk[43].

Vascular consequences of CKD

Arterial remodeling in CKD patients is characterized by arterial stiffening, increasing pulse pressure, as a consequence of premature ageing and atherosclerosis of the arteries.[44, 45] Premature vascular ageing is common in both CKD and HFpEF. Increased aortic stiffness has been strongly associated with both left ventricular dysfunction, and markers of renal dysfunction[46, 47], which precede and increase cardiovascular risk in patients with CKD.[48, 49] Stiffer arteries result in an increased pulse pressure, as well as an increased pulse wave velocity, which cause the increased pulsatility to be transmitted into the microvasculature.[50] Renal and coronary microvascular networks are very vulnerable to pulsatile pressure and flow, thus failure in decreasing pulsatility can result in damage of the capillary networks[50, 51] and thereby contribute to coronary microvascular dysfunction. Fukushima *et al.* showed an impaired global myocardial flow reserve in CKD patients, even with a normal regional perfusion and function of the LV.[52] Furthermore, coronary microvascular dysfunction was shown to be present in patients with end stage CKD[53], and was associated with an increased risk of cardiac death in patients with renal failure.[54]

Hypertension in CKD is thought to be mainly a consequence of volume overload due to increased sodium reabsorption by the kidneys.[55, 56] Increased sodium loading might also contribute to HFpEF development independent of hypertension, through inducing a systemic pro-inflammatory state which is detrimental to the coronary microvasculature.[57] Indeed, empagliflozin, a sodium glucose co-transporter-2 (SGLT2) inhibitor, initially developed as an anti-diabetic drug, resulted in decreased cardiovascular mortality in an initial type 2 diabetes cohort.[58] Interestingly, these effects seem to, at least for some part, be specific for empagliflozin as canagliflozin protected less against cardiovascular death.[59] Although the mechanisms of action have not completely been elucidated yet, multiple pre-clinical studies are being conducted to investigate the myocardial effects of SGLT2-inhibitors.[60, 61] Currently three mechanisms have been proposed to contribute to reduced cardiovascular mortality in patients receiving SGLT2-inhibitors in general and/or empaglifozin in particular[62]; (*i*) osmotic diuresis and natriuresis lower blood pressure and subsequently reduce left ventricular afterload; (*ii*) empagliflozin may instigate a shift to cardiac ketone bodies oxidation, increasing respiratory efficiency and reducing ROS production; (*iii*) empagliflozin can lower intracellular Na^+ by inhibition of the cardiac Na^+/H^+ exchanger (NHE) and induce coronary

vasodilation.[60] This effect is especially promising as increased intracellular Na^+, as present in failing cardiomyocytes, results in altered mitochondrial Ca^{2+} handling and subsequent ROS production, which may therefore be ameliorated by SGLT2-inhibitors.[62] SGLT2-inhibitors therefore seem promising in the cardiorenal field as they are both cardio- and reno-protective.[63] The effect of empagliflozin on cardiovascular mortality in HFpEF specifically, regardless of diabetic status, is being investigated in the ongoing EMPEROR-Preserved trial (ClinicalTrials.gov NCT03057951).

Neurohumoral consequences of CKD

CKD is associated with RAAS hyperactivation in response to renal hypoxia resulting in volume overload[64], which may contribute to development and/or progression of HFpEF. RAAS activation can increase myocardial workload, by elevating systemic resistance and left ventricular afterload, through vasoconstriction of systemic blood vessels in response to angiotensin II or by causing volume expansion due to increased sodium and water reabsorption in response to increased aldosterone levels[65, 66]. It is not clear if angiotensin II can also induce myocardial cell hypertrophy and fibrosis independently of hypertension. Although *in vitro* studies have shown that there is a hypertension-independent effect of angiotensin-II on cardiomyocytes, multiple *in vivo* studies could not confirm these findings, suggesting that the effect of angiotensin II is blood pressure dependent.[67, 68] Furthermore, RAAS-activation induces coronary microvascular endothelial dysfunction, through NADP(H)-oxidase activation and subsequent ROS formation.[69, 70] Myocardial perfusion might also be impaired by the vasoconstrictor effects of angiotensin II. During prolonged exercise, vasoconstriction within metabolically less active tissues, mediated by angiotensin II and endothelin-1, is inhibited in metabolically active tissues by NO and prostanoids, resulting in an efficient distribution of blood.[71] In a state of systemic inflammation, locally decreased NO bioavailability in the coronary microvasculature might result in disinhibition of angiotensin II-mediated vasoconstriction, resulting in reduced blood delivery to the heart.

Downstream from angiotensin II in the RAAS, aldosterone regulates blood pressure and sodium/potassium homeostasis through the mineralocorticoid receptor in the kidneys, by enhancing sodium reabsorption in the kidneys, thereby contributing to hypertension and high plasma sodium levels. Besides the renal effects, aldosterone has been shown to directly promote myocardial fibrosis, left ventricular hypertrophy and coronary microvascular dysfunction, acting through endothelial and myocardial mineralocorticoid receptors, independently of angiotensin II.[65]

RAAS inhibition is the preferred therapeutic strategy to slow down progression of renal failure and reduce proteinuria in CKD.[72] Despite the fact that most data show RAAS overactivation in HFpEF, clinical trials in HFpEF with drugs acting on the RAAS-system, have failed to improve (all-cause) mortality so far.[73, 74] It is, however, important to note that AT1-blockade with Irbesartan reduced mortality and improved outcome on cardiovascular endpoints in patients with natriuretic peptides below the median, but not in patients with higher natriuretic peptide levels[75], suggesting that RAAS inhibition may be beneficial in early HFpEF. Furthermore, post hoc analysis of the TOPCAT trial demonstrated geographical different effects of the mineralocorticoid receptor blocker spironolactone, with small clinical benefits in patients from the Americas.[76] However, these patients were generally older, had a higher prevalence of atrial fibrillation and diabetes, were less likely to have experienced prior myocardial infarction, had a higher ejection fraction and had a worse renal function[76], suggesting that a benefit of spironolactone was associated with a more HFpEF-like phenotype. A more recent post-hoc analysis of this trial further showed that spironolactone did show an improvement in primary endpoints in patients with lower levels of natriuretic peptides and hence less advanced disease.[77] Consistent with this suggestion, a recent meta-analysis showed that mineralocorticoid receptor antagonists do improve indices of diastolic function and cardiac structure in HFpEF patients.[78] Interestingly, treatment of DM type 2 with mineralocorticoid receptor antagonists also improved coronary microvascular function.[79] Altogether, these data suggest that intervening with the RAAS is beneficial in patients with less advanced HFpEF, whereas beneficial effects are lost in patients with more advanced disease. Therefore, clinical studies investigating HFpEF progression and clinical trials focusing on reducing or preventing progression of early HFpEF into advanced HFpEF need to be conducted.

Another approach intervening with the RAAS system is the use of Entresto, an angiotensin receptor neprilysin inhibitor (ARNI) which is a combination of valsartan (AT_1 receptor blocker) and sacubitril (neprilysin inhibitor). Neprilysin inhibition exerts its beneficial effects through inhibition of the breakdown of natriuretic peptides. Entresto was superior to the standard therapy, enalapril, in patients with HFrEF in reducing mortality and number of hospitalizations for HF.[80] In hypertensive rats with diabetes, ARNI reduced proteinuria, glomerulosclerosis and heart weight more strongly than AT_1 receptor blockade, and this occurred independently of blood pressure.[81, 82] In a phase 2 double-blind randomized controlled trial in HFpEF patients, Entresto reduced NT-pro-BNP plasma levels and left atrial diameters to a greater extent than valsartan.[83] These findings led to the ongoing PARAGON-HF trial (ClinicalTrials.gov NCT01920711) which investigates the long-term effect (26 months) of Entresto compared to Valsartan in HFpEF.[84]

Both CKD and HFpEF are accompanied by autonomic dysregulation.[85] Sympathetic hyperactivity has a detrimental effect on both the heart and the kidney and aggravates hypertension and proteinuria. Furthermore, HFpEF patients show attenuated withdrawal of parasympathetic tone and excessive sympatho-excitation during exercise, that cause β-adrenergic desensitization, chronotropic incompetence and may thereby contribute to the limited exercise tolerance of these patients.[86] A critical role for CKD in this process was suggested by Klein *et al*[87], showing a clear correlation between CKD, decreased heart rate variability, chronotropic incompetence in HFpEF and decreased peak VO_2. Unfortunately, neither the SENIORS trial[88], nor the OPTIMIZE-HF registry[89] showed a beneficial effect of beta-adrenoceptor blockade on all-cause mortality or cardiovascular hospitalizations. Furthermore, beta-adrenoceptor blockade failed to improve LV systolic or diastolic function in patients with ejection fraction >35%, as measured in the SENIORS echocardiography sub-study.[90] It should be noted that in the SENIORS trial ejection fraction cutoff was at 35%, which is lower than current consensus about the cutoff of reduced and preserved ejection fraction. Additionally, in these studies, beta-adrenoceptor blockade was administered on top of existing medication, which often included RAAS-inhibitors. Conversely, in patients with treatment resistant hypertension, renal sympathetic denervation did improve diastolic function and reduce left ventricular hypertrophy, besides reducing blood pressure[91], suggesting that there is indeed an interaction between CKD, sympathetic hyperactivity and diastolic cardiac function.

Systemic inflammatory consequences of CKD

A pro-inflammatory state is already present in early stages of CKD[92], and is likely an important risk factor for cardiovascular morbidity and mortality on the long term[93, 94]. In HFpEF, a systemic pro-inflammatory state has been proposed to be a critical causal factor in coronary microvascular endothelial dysfunction as inflammatory cytokines can directly induce coronary microvascular endothelial cell dysfunction, cause upregulation of adhesion molecules on coronary microvascular endothelial cells, and reduce NO bioavailability, resulting in impaired vasodilation and pro-fibrotic signaling (**Figure 2**).[10, 95]

Targeting this proinflammatory state with 14 days of treatment with the recombinant human IL1 receptor antagonist Anakinra, increased peak VO_2 which correlated with a reduction in C-reactive protein (CRP) in the D-HART trial including 12 patients.[96] Unfortunately, prolonged treatment (12 weeks) in the follow-up D-HART2 trial in 28 patients, did not increase VO_2, despite small

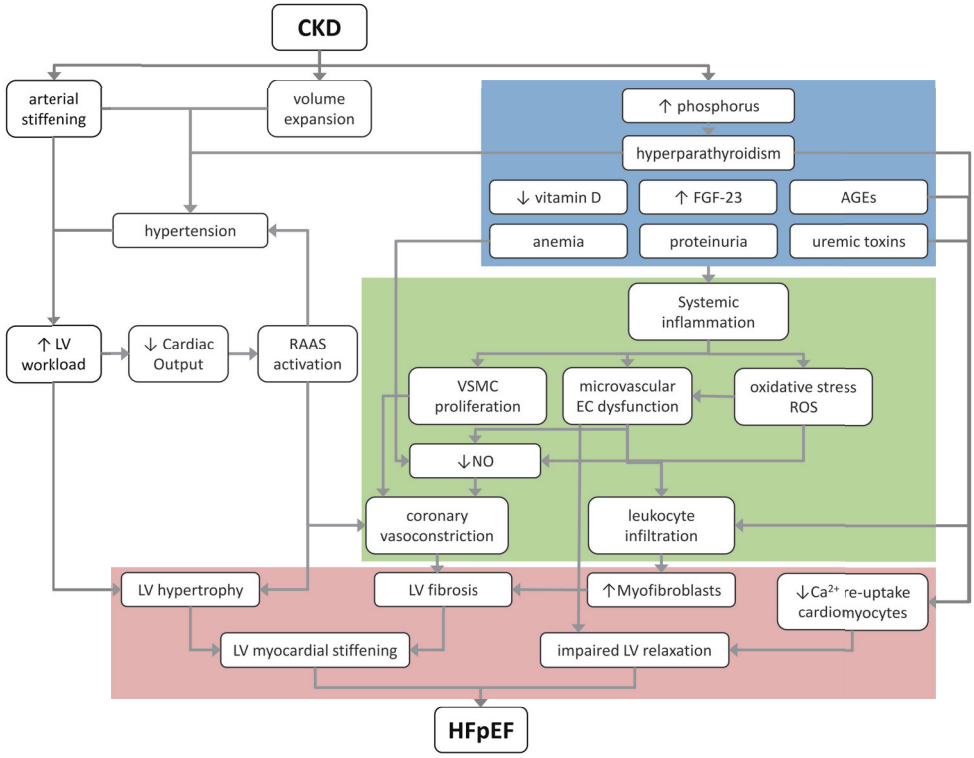
improvements in exercise duration and quality of life, as well as reductions in CRP and NT-pro-BNP compared to baseline values.[97]

It is possible that targeting systemic inflammation in general to ameliorate HFpEF is too broad to be successful. In the subsequent paragraphs, the contribution of the individual systemic factors: anemia, proteinuria and reduced excretion of so-called uremic toxins as consequences of renal dysfunction and possible contributors to systemic inflammation, development of microvascular dysfunction and HFpEF will be considered in more detail.

Anemia

Anemia is an independent risk factor for development of HFpEF[32, 36], and is strongly associated with CKD[42]. Although hemoglobin levels decreased with worsening of kidney function in both patients with HFpEF and HFrEF, hemoglobin levels were slightly lower in patients with HFpEF as compared to HFrEF.[98] The main causes for anemia are iron deficiency and deficient erythropoietin production in the renal tubular cells. In addition, urinary loss of red blood cells through enlarged fenestrations of endothelial cells in diseased glomeruli, hemolysis, vitamin B12 deficiency, hyperparathyroidism and hemodilution may contribute to anemia in CKD patients.[99, 100] Furthermore, the bone marrow erythropoietic response to erythropoietin is impaired in CKD patients.[99] Finally, the proinflammatory cytokine Il-6 can impair erythroid development, by inducing production of the iron regulatory peptide hepcidin by hepatocytes, increasing degradation of iron exporter ferroportin, and decreasing iron delivery to developing erythrocytes.[101] Hence, the systemic inflammatory state in CKD, but also HFpEF, can aggravate anemia.

Figure 2 A proposed schematic overview of the pathological mechanisms that underlie the progression of CKD to HFpEF



Blue box depicts renal factors; green box depicts coronary microvascular factors; and red box depicts myocardial changes contributing to HFpEF. AGEs, advanced glycation products; CKD, chronic kidney disease; EC, endothelial cell; FGF-23, fibroblast growth factor 23; HFpEF, heart failure with preserved ejection fraction; LV, left ventricle; NO, nitric oxide; RAAS, renin-angiotensin-aldosterone system; ROS, reactive oxygen species; VSMC, vascular smooth muscle cell.

It is unknown whether anemia, iron deficiency and/or reduced EPO are causal factors in the development of HFpEF or mere markers of CKD. The most obvious effect of anemia is a general reduction in O_2 transport. In 75% of the HFpEF patients, peripheral oxygen consumption was impaired due to impaired diffusive oxygen transport and utilization.[102] Hence, cardiac output needs to be increased to maintain systemic oxygen delivery. Both the consequent increase in myocardial work, and the reduced oxygen-carrying capacity of the blood may contribute to an impaired myocardial O_2 balance. Such a disbalance between myocardial oxygen demand and supply is also present in ischemia

with no obstructive coronary artery disease (INOCA), in which myocardial oxygen supply is limited by coronary microvascular dysfunction. Indeed, INOCA is recently being recognized as a risk factor for development of HFpEF.[103, 104]

Anemia can also directly affect microvascular function as red blood cells can modulate microvascular tone.[105, 106] Red blood cells release NO which is produced, particularly at low oxygen tensions, from deoxygenated hemoglobin and nitrite, to stimulate vasodilatation, cGMP formation in smooth muscle cells and cardiomyocytes, and to inhibit mitochondrial respiration.[107] Thus, low levels of red blood cells simulate a condition of coronary microvascular dysfunction, with increased ROS and reduced NO, thereby inducing true coronary microvascular dysfunction and cardiomyocyte damage, which eventually can contribute to progression of HFpEF (**Figure 2**).

CKD patients on erythropoietin therapy have shown signs of cardiovascular improvement and reversal of left ventricular hypertrophy[108, 109], suggesting that correction of anemia may prevent progression of HFpEF. In addition to promoting red blood cell formation and correction of anemia, erythropoietin can protect cardiomyocytes against ischemic injury and induce NO production by endothelial cells, thereby improving microvascular function.[99] Erythropoietin can induce tissue protective properties by activating the erythropoietin receptor and β common receptor, which are found on multiple peripheral tissues such as endothelial cells. EPO sensitivity can be increased by hypoxia but is decreased by a pro-inflammatory state which is considered a hallmark of HFpEF, therefore lower eNOS expression due to lower EPO or lower EPO receptors on the endothelium can contribute to the lower NO-bioavailability in coronary microcirculation.[110] Interestingly, in patients, EPO resistance is shown to be present in early CKD before EPO levels decrease, later stages of CKD show a decrease in EPO levels.[111] However, in a randomized controlled trial conducted in older adults with HFpEF, erythropoietin supplementation with epoetin alfa did not improve left ventricular geometry or exercise capacity despite increases in hemoglobin levels.[112] One potential explanation would be that the 1.5 g/dL increase in hemoglobin in the treatment group was insufficient, particularly since the placebo-treated patients also showed an 0.8 g/dL increase in hemoglobin. Alternatively, decreased endothelial and/or cardiomyocyte sensitivity to, rather than too low levels of erythropoietin and/or anemia are important in the progression of HFpEF.[99] If so, it would be more beneficial to restore erythropoietin sensitivity of specific cells rather than changing its levels. Reducing the pro-inflammatory phenotype of endothelial cells could potentially be beneficial in increasing endothelial erythropoietin sensitivity. Alternatively, although not specific an enhancer of erythropoietin sensitivity, targeting the protective tissue-specific effects of erythropoietin might

prove a viable therapeutic target, although to date, this is mostly evaluated in neurological disorders.[113]

Iron deficiency, even without anemia, was also shown to be detrimental to the functional capacity of advanced HFpEF patients[114], while diastolic dysfunction was not associated with functional iron deficiency.[115] Functional iron deficiency is detrimental to cardiomyocyte function as it reduces antioxidant capacity and limits oxidative phosphorylation thereby limiting energy production, potentially impairing energy-dependent Ca^{2+} reuptake during diastole.[116] Currently, iron supplementation with IV ferric carboxymaltose is being investigated in both anemic and non-anemic HFpEF patients in the FAIR-HFpEF trial (ClinicalTrial.org NCT03074591).

Proteinuria

Proteinuria, an abnormal high protein concentration in urine, is present in up to 26% of CKD patients with an eGFR below 30 mL/minute/1.73 m^2.[117, 118] Not only proteinuria, but also, more specifically, elevated urinary levels albumin, were associated with declining renal function.[119-121] Proteinuria is not just a mere marker of CKD, but also contributes to the exacerbation of CKD, by aggravating renal interstitial inflammatory cell influx resulting in interstitial fibrosis (**Figure 2**).[122, 123]

In 1989, Deckert *et al.* already introduced the Steno hypothesis, which implies that albuminuria is not just reflecting local renal disease, but indicating more general endothelial microvascular dysfunction.[124] Indeed, large population based studies have shown that microalbuminuria correlates with a decrease in flow mediated endothelium-dependent vasodilation in brachial arteries[125], as well as in coronary arteries of diabetic patients[126]. In patients with essential hypertension, microalbuminuria was shown to correlate with levels of circulating von Willebrand factor, a marker for endothelial damage.[127] Multiple studies have shown that (micro)albuminuria is highly prevalent in HFpEF, being associated with LV remodeling, and is a prognostic marker for further disease development.[18, 32, 128-130]

Currently, it is unclear, whether microalbuminuria just reflects a more generalized microvascular endothelial dysfunction or may act as a causal contributing factor to HFpEF development by inducing coronary microvascular endothelial damage.

Uremic toxins

Insufficient glomerular filtration results in the retention of a variety of biologically active compounds in the blood, called uremic toxins. The accumulation of uremic toxins can have a deleterious effect on multiple organs, of which the cardiovascular system is most severely affected.[131] Increased levels of uremic toxins are associated with an increased cardiovascular morbidity and mortality.[132] Moreover, blood urea nitrogen was shown to be an independent predictor for the progression from preclinical diastolic dysfunction to HFpEF, but not HFrEF.[133]

The mechanisms mediating the detrimental effects on the vascular system are multiple. The elevated uremia-associated pro-inflammatory cytokine levels, together with the associated chronic inflammatory state, can inhibit proliferation and enhance apoptosis of endothelial cells (**Figure 2**).[132] Furthermore, uremic toxins can increase von Willebrand factor levels, decrease NO bioavailability by inhibition of endothelial nitric oxide synthase (eNOS), and increase circulating endothelial microparticles.[134] Additionally, chronic low grade inflammation increases expression of adhesion molecules on endothelial cells and induces leukocyte activation with differentiation of fibroblasts to myofibroblasts, with subsequent production of collagen in the extracellular matrix, and migration and proliferation of vascular smooth muscle cells.[10, 135] Tryptophan-derived toxins can specifically activate the aryl hydrocarbon receptor pathway, and thereby induce endothelial dysfunction, and activate pro-fibrotic pathways in the myocardium, further enhancing inflammation and increasing vascular oxidative stress.[136] These processes all contribute to (coronary) microvascular dysfunction and remodeling. Uremic toxins might also directly affect the left ventricular relaxation. Exposure of cardiomyocytes to uremic serum of CKD patients elicited inhibition of Na^+/K^+-ATPase, increased contractile force, impaired calcium re-uptake, and delayed relaxation (**Figure 2**).[137]

Elevated circulating and cellular levels of advanced glycation end products (AGEs) have been measured in patients with CKD.[138] This is the result of impaired renal clearance of AGEs together with their increased formation resulting from oxidative stress and/ or diabetes mellitus. Elevated circulating AGEs are linked to development and progression of both HFpEF and HFrEF[139, 140] and correlated positively with increased diastolic dysfunction in patients with diabetes mellitus type 1[141]. In the LV, AGEs are particularly prominent in the coronary microvasculature, where their presence induces a pro-inflammatory phenotype[142], endothelial dysfunction by increasing oxidative stress and decreasing NO bioavailability and vascular stiffening by crosslinking of extracellular matrix (ECM) proteins[140, 143]. In the myocardium, AGE-induced crosslinking of ECM proteins increased myocardial stiffness.[140, 143] Furthermore, AGEs impair calcium handling in cardiomyocytes.[144] The latter is mediated by carbonylation of SERCA2a, which impairs its activity[145], as well as by enhancing calcium

leakage from the sarcoplasmic reticulum through the ryanodine receptor (RyR2), thereby promoting mitochondrial damage and oxidative stress[146]. Hence, reducing production and enhancing breakdown of AGEs could be a therapeutic option in HFpEF patients[147], particularly in patients with diabetes and CKD.

Besides glycemic control, there are 3 classes of drugs that can reduce AGEs: inhibitors of *de novo* AGE synthesis, drugs that break pre-existing AGE crosslinks and AGE receptor blockers.[148] Although, to our knowledge, none of these have been tested in HFpEF patients, aminoguanidine, a small hydrazine-like molecule capable of inhibiting AGE formation through interaction with and quenching of dicarbonyl compounds treatment resulted in a decrease of diabetes mellitus associated myocardial stiffening in rats, albeit without altering fibrosis.[149] Furthermore, in DM type 2 patients, benfotiamine, a transketolase activator that blocks several hyperglycemia-induced pathways, prevented microvascular endothelial dysfunction and oxidative stress after an AGE rich meal.[150] Similarly, treatment with the AGE crosslink breaker alagebrium improved endothelial function in patients with isolated systolic hypertension, which was associated with reduced vascular fibrosis and vascular inflammation.[151] For an overview of trials conducted with AGE-lowering therapies in CKD patients we refer to Stinghen *et al.*[138] Some of these therapies which reduced AGEs in CKD patients might also be a viable chronic treatment option, to prevent or reverse AGE-associated microvascular dysfunction and subsequent diastolic dysfunction in HFpEF.

Lowering uremic toxin levels in general might also provide a viable, but challenging treatment option for HFpEF. The main challenges are to identify the specific uremic toxins that play a role in the pathogenesis of HFpEF, and to target a large variety of uremic toxins with just one class of drugs. Clinical trials with allopurinol, a therapy to decrease uric acid levels, resulted in slower disease progression and a decreased cardiovascular risk in patients with CKD.[152, 153] Even asymptomatic hyperuricemic patients may benefit from allopurinol treatment, as they showed improvements in endothelial function and eGFR.[154]

Consequences of CKD on mineral metabolism

Vitamin D deficiency

Declining renal function results in a reduced capacity to perform 1α-hydroxylation and in progressive loss of active vitamin D.[155] Loss of active vitamin D subsequently leads to increased parathyroid hormone (PTH) production, so-called secondary hyperparathyroidism, eventually contributing to increased calcium, phosphate and FGF-23 levels. In patients on hemodialysis, an association was reported between low vitamin D levels, systemic inflammation, and myocardial hypertrophy[156] Furthermore, low levels of vitamin D in these patients were related to increased cardiovascular mortality.[156-158] In non-dialysis CKD patients, lower vitamin D levels were shown to be associated with decreased flow mediated dilatation in the brachial artery, reflecting systemic endothelial dysfunction.[159] Low vitamin D correlates with reduced coronary flow reserve in patients with atypical chest pain, suggesting that vitamin D also affects coronary microvascular function.[160] Recently, in a large cohort of patients with diastolic dysfunction or HFpEF, lower vitamin D levels were associated with increased cardiovascular hospitalizations but not with 5-year mortality.[161] Furthermore, in a univariate analysis, calcidiol, but not its active metabolite, calcitriol, was associated with new onset HFpEF in the PREVEND study, but the association disappeared after adjustment for confounding variables.[162] However, in patients with established HFpEF, vitamin D levels were lower as compared to healthy, sex–, race- and age-matched controls, and inversely correlated with exercise capacity.[163]

In a trial of vitamin D supplementation by cholecalciferol therapy, reductions were observed in the left ventricular mass, inflammatory markers and brain natriuretic peptide levels of CKD patients on hemodialysis.[164] In contrast, in the PRIMO-trial, 48 weeks of treatment with paricalcitol in a CKD cohort with preserved systolic function neither resulted in improved diastolic function nor reduced left ventricular mass.[165] However, cardiac MRI unveiled that just a minority of the included patients had left ventricular hypertrophy at baseline, possibly explaining lack of a beneficial effect. Although the administration of vitamin D has positive effects through inhibition of PTH secretion, it also results in increased serum phosphate levels, with opposing effects (see next paragraph for details). When modulating vitamin D status, one should consider the use of vitamin D analogues, such as paricalcitol, which inhibit PTH synthesis, without substantially inducing hyperphosphatemia, providing promising therapies for restoration of vitamin D levels.[166]

Phosphate and Parathyroid Hormone

In large cohorts of patients on hemodialysis, strong associations were found between serum phosphate, calcium, hyperparathyroidism, and an increased risk for overall cardiac mortality, elevated levels of cardiac injury markers and a worse systolic and diastolic cardiac function.[167, 168] Additionally, in a cohort of hospitalized patients with CKD, serum phosphate was related to elevated left ventricular concentric remodeling and diastolic dysfunction.[169] Furthermore, in late stage CKD patients—on peritoneal dialysis—phosphate was independently associated to impairment of left ventricular diastolic function.[170] At the structural level, elevated levels of phosphate (hyperphosphatemia) and PTH have been associated with the presence of hypertrophy and fibrosis of the LV specifically.[167, 171] In addition, in a small cohort of patients on chronic hemodialysis, higher levels of calcium phosphate product were associated with higher CRP levels, and thus with a pro-inflammatory state. In this cohort, intensive lowering of phosphate levels resulted in lower CRP levels, and a significantly improved inflammatory status.[172]

Hyperphosphatemia can also directly induce coronary endothelial dysfunction[173], and also acts directly on human vascular smooth muscle cells (VSMC), resulting in VSMC calcification.[174] Furthermore, hyperphosphatemia can contribute to microvascular dysfunction and HFpEF pathogenesis by reducing prostaglandin synthesis.[17] Prostaglandins synthesized in the blood vessel wall act as autocrine or paracrine factors and play a pivotal role in regulation of coronary microvascular function by exerting strong vasodilator effects and by inhibiting platelet aggregation. In clinical practice, supplementation of prostanoids is mostly used in patients with pulmonary hypertension. Prostacyclin analogues are available, such as Selexipag, an oral prostacyclin receptor agonist which has vasodilator, antiproliferative and antifibrotic effects. Currently there is one trial ongoing, which investigates oral Treprostinil, a prostacyclin analogue, in pulmonary hypertension caused by HFpEF (ClinicalTrials.org NCT03037580).

PTH can cause left ventricular interstitial fibrosis and coronary microvascular dysfunction, via its inflammatory effects on monocytes and interstitial fibroblasts.[175] Interestingly, primary hyperparathyroidism resulted in coronary microvascular dysfunction which was restored after parathyroidectomy, underlining the effect of PTH on coronary microvascular function.[176] In hemodialysis patients with secondary hyperparathyroidism, 20 weeks of treatment with cinacalcet ameliorated endothelial dysfunction, diastolic dysfunction and cardiac hypertrophy by decreasing oxidative stress and increasing nitric oxide production (**Figure 2**).[177]

Fibroblast growth factor 23 (FGF-23)

Fibroblast growth factor-23 (FGF-23) is a hormone produced by osteoblasts and osteocytes, which inhibits phosphate reabsorption in the kidneys and suppresses circulating calcitriol, effectively lowering plasma phosphate levels in physiological conditions.[178] In CKD, FGF-23 is no longer is able to reduce phosphate levels due to loss of renal Klotho-FGF receptor 1 complex, resulting in both high phosphate and high FGF-23 levels.[179] Elevated levels of FGF-23 are associated with an increased cardiovascular risk in patients with CKD[180], and with left ventricular hypertrophy in a cohort of CKD patients[181]. These findings were confirmed in rats, where FGF-23 could directly induce left ventricular hypertrophy while ejection fraction was preserved.[182] Furthermore, FGF-23 is associated with new-onset HFpEF in a large cohort study of people who were free of cardiovascular disease at baseline.[183] Interestingly, in a cohort of HFpEF patients, FGF-23 was not associated with increased mortality, while this was the case for a cohort of HFrEF patients[184], suggesting that FGF-23 may be linked to disease onset rather than progression.

Mechanistically, FGF-23 induces chronic inflammation by stimulating cytokine secretion from the liver, but is also locally produced by M1 macrophages, and can thereby further modulate inflammation in the heart (**Figure 2**).[185] FGF-23 inhibits ACE2, resulting in reduced degradation of angiotensin I and II into their vasodilator metabolites angiotensin-(1-9) and angiotensin-(1-7)[185], and consequently increased stimulation of AT_1 receptors by angiotensin II. High levels of FGF-23 were further shown to cause endothelial dysfunction, increase superoxide formation, and decrease NO bioavailability in mouse aortas.[186] Finally, FGF-23 causes inhibition of 1α-hydroxylase, and can thereby contribute to microvascular damage and cardiac dysfunction due to vitamin D deficiency.[185] Hence, elevated FGF-23 levels can contribute to development of HFpEF by attenuating coronary microvascular function and by enhancing angiotensin II induced vascular and myocardial fibrosis. Indeed, preliminary data of Roy et al, suggest that FGF-23 levels correlated with interstitial fibrosis in HFpEF.[187] Furthermore, FGF-23 counteracted the beneficial effect of paricalcitol on left ventricular hypertrophy, by modulation of the calcineurin/nuclear factor of activated T cell (NFAT) pathway in a rat model of CKD.[188] FGF-23 inhibition with KRN23, an anti-FGF antibody, is a viable treatment option as open label phase 1/2 studies for X-linked hypophosphatemia, and showed an increase in serum inorganic phosphate and active vitamin D in all subjects.[189] Further research into a potential causal role of FGF-23 in HFpEF development is required, prior to embarking on therapeutic interventions.

Conclusion

The kidneys and heart are interdependent organs, that are highly connected through multiple systems on both macrovascular and microvascular level. Unfortunately, many studies on the cardiorenal connection have not been conducted in specific HFpEF populations. Pathological processes which are present in CKD, such as vascular changes, deficiencies in kidney produced factors, and impairments in renal filtration can cause and/or contribute to development of HFpEF via several processes, as summarized in **Figure 2**. Elevated levels of phosphate, PTH, FGF-23, AGEs and uremic toxins, but also anemia and proteinuria can induce a systemic proinflammatory state. This state can lead to left ventricular stiffening and coronary microvascular dysfunction by initiating endothelial cell dysfunction, oxidative stress, and vascular smooth muscle cell proliferation. Arterial stiffening, volume expansion, hypertension and RAAS activation, as consequences of CKD, increase left ventricular workload and hypertrophy.

The complexity, and multitude of connections between the heart and kidney, make it unlikely that there is a single causal contributor for progression from CKD to HFpEF. Multiple large trials have been conducted with treatments for HFpEF, targeting different pathophysiological processes but unfortunately failed to show clinical benefit. Therefore, current guidelines on treatment of HFpEF focus on lifestyle interventions and the management of comorbidities such as diabetes mellitus, hypertension, obesity and CKD. In addition, it has been proposed that different HFpEF phenotypes exist that should be targeted with different therapeutic strategies. CKD patients are likely an interesting, identifiable subgroup of HFpEF patients, whom warrant further investigation both in pathogenesis as in clinical trials to further investigate cardiorenal connection in HFpEF specifically and to identify the unique mechanistic pathways involved in various phases of the disease.

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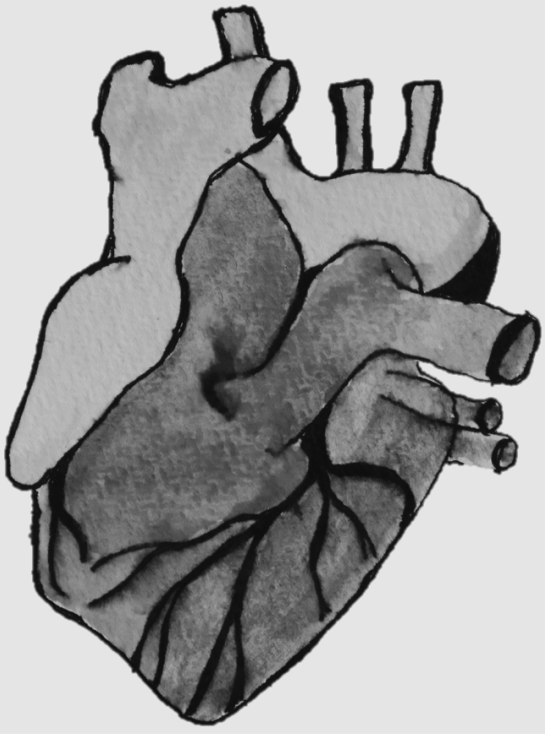

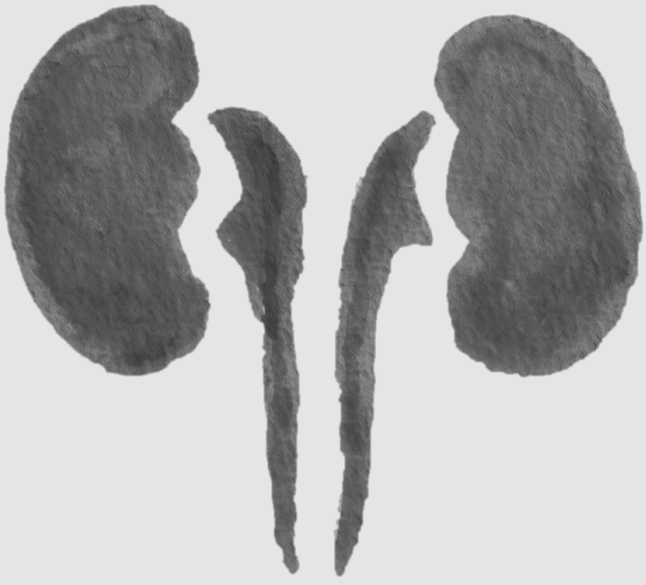

Chapter 7

Multiple common comorbidities produce left ventricular diastolic dysfunction associated with coronary microvascular dysfunction, oxidative stress, and myocardial stiffening

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Abstract

Aims More than 50% of patients with heart failure have preserved ejection fraction characterized by diastolic dysfunction. The prevalence of diastolic dysfunction is higher in females and associates with multiple comorbidities such as hypertension (HT), obesity, hypercholesterolemia (HC), and diabetes mellitus (DM). Although its pathophysiology remains incompletely understood, it has been proposed that these comorbidities induce systemic inflammation, coronary microvascular dysfunction, and oxidative stress, leading to myocardial fibrosis, myocyte stiffening and, ultimately, diastolic dysfunction. Here, we tested this hypothesis in a swine model chronically exposed to three common comorbidities.

Methods and results DM (induced by streptozotocin), HC (produced by high fat diet), and HT (resulting from renal artery embolization), were produced in 10 female swine, which were followed for 6 months. Eight female healthy swine on normal pig-chow served as controls. The DM + HC + HT group showed hyperglycemia, HC, hypertriglyceridemia, renal dysfunction and HT, which were associated with systemic inflammation. Myocardial superoxide production was markedly increased, due to increased NOX activity and eNOS uncoupling, and associated with reduced NO production, and impaired coronary small artery endothelium-dependent vasodilation. These abnormalities were accompanied by increased myocardial collagen content, reduced capillary/fiber ratio, and elevated passive cardiomyocyte stiffness, resulting in an increased left ventricular end-diastolic stiffness (measured by pressure–volume catheter) and a trend towards a reduced E/A ratio (measured by cardiac MRI), while ejection fraction was maintained.

Conclusions The combination of three common comorbidities leads to systemic inflammation, myocardial oxidative stress, and coronary microvascular dysfunction, which associate with myocardial stiffening and LV diastolic dysfunction with preserved ejection fraction.

Introduction

More than 50% of patients with heart failure present with heart failure with preserved ejection fraction (HFpEF), characterized by diastolic dysfunction.[1] Hospitalized patients with HFpEF have high mortality and rehospitalization rates, and there is currently no effective treatment available for these patients.[2–4] Common metabolic and cardiovascular risk factors appear to be critical in the onset of diastolic dysfunction and its progression towards HFpEF, as the incidence of HFpEF increases with rising prevalence of obesity, hypertension (HT), chronic kidney disease, female sex, and type 2 diabetes mellitus (DM).[5–7] Furthermore, studies in HFpEF patients have shown alterations in myocardial structure, function, and cell signaling that are unique to this form of heart failure.[8–11] However, the pathophysiology of HFpEF is still not fully understood, particularly at the myocardial tissue level. Findings from these earlier studies have led to the proposition of a novel paradigm, in which multiple comorbidities, including obesity, HT, hypercholesterolemia (HC), and DM induce a systemic pro-inflammatory state that leads to coronary microvascular dysfunction and oxidative stress. In turn, these disease mechanisms result in myocardial stiffening and ultimately left ventricular diastolic dysfunction.[12] Nevertheless, direct experimental evidence for this unifying hypothesis is still lacking. We set out to investigate the chain of events as proposed in this novel paradigm, using a large animal model chronically exposed to three common comorbidities that associate with diastolic dysfunction, i.e. hyperglycemia, HC, and HT. Since the prevalence of this disease is predicted to increase in our aging Western societies,[13] such unique large animal model that mimics the complex disease mechanisms of diastolic dysfunction would offer a much-needed platform for testing novel drug and lifestyle therapies.

2. Methods

2.1 Animals

Experiments were performed in accordance with the 'Guiding Principles in the Care and Use of Laboratory Animals' as approved by the Council of the American Physiological Society, and with approval of the Animal Care Committee at Erasmus University Medical Center, Rotterdam. Fourteen female (21.7 ± 0.3 kg at 2–3 months of age) Yorkshire x Landrace swine were included to study the effects of DM, HC, and HT (DM + HC + HT), while 12 healthy female swine of similar age were studied as controls (Control). Finally, six fresh control hearts from slaughterhouse female swine of similar body

weight as the control animals (~100 kg at sacrifice) were included to additionally study vascular function characteristics.

2.2 Induction of risk factors

The induction of risk factors in the DM + HC + HT group is described in detail in the Supplementary material online, *Supplementary Methods*. Briefly, DM was produced by injection of streptozotocin (50 mg/kg/day i.v. for 3 days, Bio-connect B. V., Huissen, The Netherlands). 9- to 11 days later, animals were sedated with intramuscular Zoletil (tiletamine/zolazepam; 5 mg/kg), Rompun (xylazine; 2.25 mg/kg) and atropine (1 mg), and artificially ventilated (O_2 and N_2 [1:2]), to which 1–2% (vol/vol) isoflurane was added. HT was produced by micro-embolization of the global right kidney as well as the lower pole of the left kidney using 75 mg of polyethylene microspheres (38–42 µm diameter, Cospheric, Santa Barbara, CA, USA) per kidney. One week after HT induction, a high fat diet (see Supplementary material online, Table S1), supplemented with 10 g NaCl/day was gradually introduced to produce HC.

2.3 Hemodynamic assessment

At 6 months follow-up, extensive *in vivo* hemodynamic assesment was performed with the animals under anesthesia (pentobarbital, 20 mg/kg i.v.) and in the awake state. All procedures are described in detail in the Supplementary material online, *Supplementary Methods*. Briefly, LV function was assessed using MRI (Discovery MR450, GE Medical System, Milwaukee, Wisconsin, USA), including end-diastolic volume (EDV), end-systolic volume (ESV), E/A ratio, stroke volume (SV), and ejection fraction (EF), and using a pressure–volume catheter (CD Leycom, The Netherlands), including EDV, ESV, SV, EF, end-diastolic (EDPVR), and end-systolic (ESPVR) pressure–volume relationships. Eight animals (4 DM + HC + HT, and 4 Control) were instrumented between 5- and 6-month follow-up with a Transonic flow probe around the ascending aorta and fluid filled catheters in the left atrium and aorta for hemodynamic measurements at rest and during exercise and the evaluation of kidney function.

2.4 Coronary small artery function in vitro

In order to assess coronary vascular endothelial function, coronary small arteries (~300 µm diameter) were isolated from the epicardial surface of the LV apex and studied *in vitro* using a Mulvany wire myograph as presented in the Supplementary material online, *Supplementary Methods*. In short, the concentration–response curves (CRC) were measured for the endothelium-dependent vasodilator bradykinin (BK, 10^{-10} to 10^{-6} mol/l, Sigma–Aldrich, Zwijndrecht, The Netherlands) and the exogenous NO-donor, S-nitroso-N-acetylpenicillamine (SNAP, 10^{-10} to 10^{-5} mol/l, Sigma–Aldrich, Zwijndrecht, The Netherlands) following preconstriction with 10^{-6} mol/l thromboxane-A_2 analogue U46619 (Sigma–Aldrich, Zwijndrecht, The Netherlands).

2.5 Tissue analysis

All analyses are described in detail in the Supplementary material online, *Supplementary Methods*. Briefly, snap frozen samples of the subendocardium of the LV anterior free wall were analysed for mRNA expression levels of various genes (see Supplementary material online, Supplementary Methods Table S2), involved in different phases of development of diastolic dysfunction, reactive oxygen species (ROS), and NO production for NO-ROS balance, eNOS expression, and phosphorylation in order to asess eNOS uncoupling and activity. Myocardial levels of cyclic guanosine monophosphate (cGMP), and activity of phosphodiesterase 5 (PDE5) and protein kinase G (PKG) were measured using ELISA kits, to assess alterations in the downstream signalling pathway of NO. Calcium-force relations of single cardiomyocytes were performed for cardiomyocyte stiffness measurements. In addition, titin isoform- (N2BA and N2B) expression and phosphorylation were measured as previously described.[14] Furthermore, histological analyses of myocardial collagen deposition, capillary density, and myocyte size were performed for myocardial structure characterization. Finally, the upper pole of the left kidney was used for analysis of tubulo-interstitial (TI) damage. Scored variables were the amount of inflammatory infiltrate between tubuli, interstitial fibrosis, tubular atrophy, and dilatation. A total TI damage score was calculated by summing the scores for the four variables. Fat deposition in the liver was also analysed for liver steatosis.

2.6 Data analysis

Data are presented as mean ± SEM. Comparison of variables between the two groups was performed by unpaired Student's *t*-test (StatView 5.0 SAS Institute Inc.). Vasodilator responses to BK and SNAP were expressed as percentage of the preconstriction to U46619. Vasoconstrictor responses to U46619 were normalized to 10^{-1} mol/l KCl. Statistical analysis of CRCs, changes in mean aortic pressure over time and the measurements of F_{pas} were performed using two-way ANOVA and the analysis of the LAP measurements during exercise using regression analysis. $P < 0.05$ was considered statistically significant.

3. Results

3.1 Model characteristics

At 6-month follow-up, DM + HC + HT animals had lower body weights (79±3 kg) than their age-matched controls (102±4 kg, $P < 0.05$). No significant group differences were detected in LV-, left atrial-, or right ventricular weights, when normalized to body weight (see Supplementary material online, Table S3).

A significant decrease in insulin as well as significant increases in glucose, total cholesterol, LDL-, and HDL-cholesterol values, and the LDL/HDL ratio and to a lesser extent in triglycerides ($P = 0.07$), were observed in DM + HC + HT compared to controls (*Table 1*). Metabolic dysregulation was also accompanied by increased mRNA-expression of pyruvate dehydrogenase lipoamide kinase isozyme 4 (PDK4, a regulator of glucose metabolism and a marker of diastolic dysfunction[15]), in DM + HC + HT as compared to controls (1.91±0.24 vs. 1.00±0.22 AU, $P < 0.05$, see Supplementary material online, Table S4), which correlated with plasma glucose levels ($P < 0.05$). TNF-α plasma levels were significantly higher than those of healthy controls, consistent with a chronic inflammatory status in these animals, which correlated with the levels of glucose (see Supplementary material online, Figure S1A, $P < 0.05$), but not with plasma lipids (see Supplementary material online, Figure S1B). Plasma levels of ASAT were similar between groups (42±11 in DM + HC + HT vs. 40±2 U/l in Control), but ALAT was significantly lower in DM + HC + HT as compared to controls (24±4 vs. 51±2 U/l, $P < 0.05$), despite a significant increase in fat deposition in the liver of DM + HC + HT animals (0.86 ± 0.31% vs. 0.03 ± 0.01%; $P < 0.05$). These data are consistent with findings in other pig models of metabolic dysfunction and might be related to the high fructose and high sucrose content of the diet.[16,17]

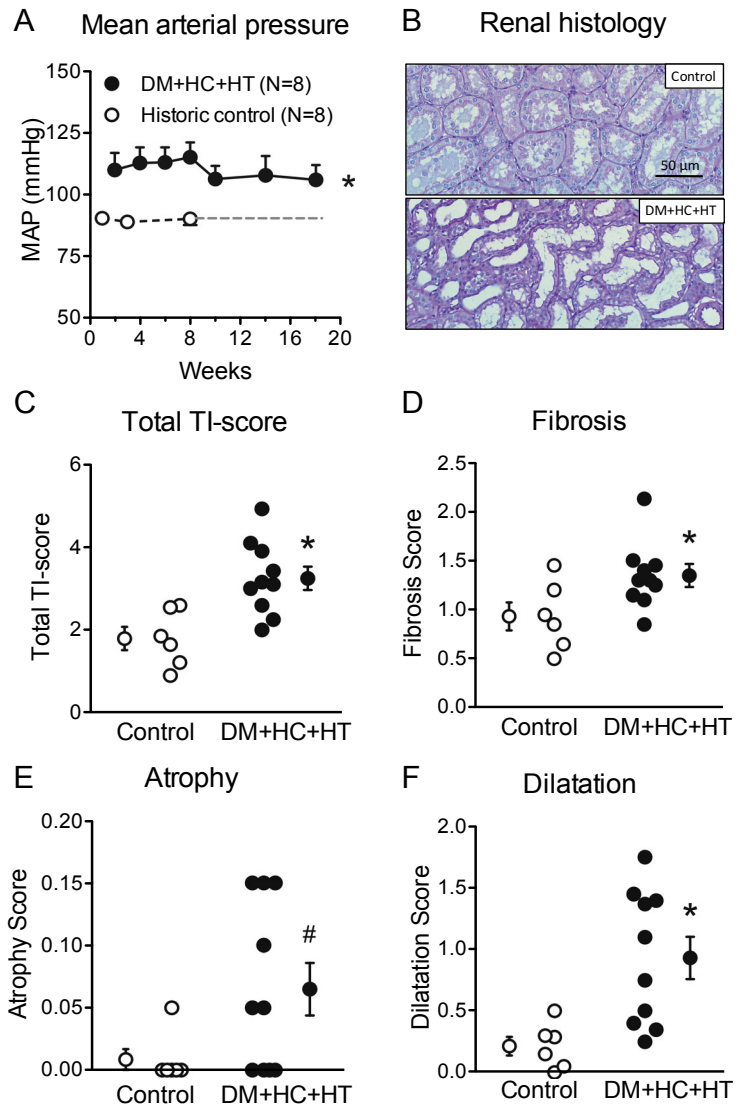
Table 1. Arterial blood characteristics in DM+HC+HT swine group versus Control, obtained at fasting state under anesthesia. GFR was measured in chronically instrumented swine in the awake state.

Parameter	Control (N=8)	DM+HC+HT (N=10)
Metabolic function		
Glucose (mmol/l)	6.1 ± 0.7	22.7 ± 0.9*
Insulin (ng/l)	39 ± 14	12 ± 1*
Cholesterol (mml/l)	2.2 ± 0.12	16.8 ± 3.4*
LDL-cholesterol (mmol/l)	1.1 ± 0.1	14.0 ± 3.2*
HDL-cholesterol (mmol/l)	1.1 ± 0.1	5.1 ± 0.7*
LDL/HDL-Cholesterol	1.1 ± 0.1	2.7 ± 0.4*
Triglycerides (mmol/l)	0.35 ± 0.05	1.16 ± 0.36[#]
Renal function		
Urea (mmol/l)	4.2 ± 0.5	3.8 ± 0.4
Creatinine (μmol/l)	130 ± 6	129 ± 11
Cystatin C (mg/l)	0.42 ± 0.01	0.51 ± 0.03*
Aldosterone (pg/ml)	1.4 ± 0.1	10.2 ± 4.1[#]
GFR[§] (ml/min)	202 ± 7	123 ± 12*
Inflammation		
TNF-α (pg/ml)	74 ± 24	231 ± 64*
IL-6 (pg/ml)	21 ± 8	67 ± 32

LDL=low density lipoprotein, HDL=high density lipoprotein, GFR=glomerular filtration rate, TNF-α=tumor necrosis factor alpha, IL-6=interleukin-6; [§]N=4 DM+HC+HT and 4 Controls *P≤0.05, [#]P≤0.07, DM+HC+HT vs Control

Mean aortic pressure, measured under general anesthesia, rose from 62±3 mmHg immediately prior to injection to 87±4 mmHg following infusion of the polyethylene beads in the kidneys ($P < 0.05$). The increase in aortic pressure was well maintained over time, as indicated by the biweekly measurements in the awake state, with animals standing quietly in their cage (*Figure 1A*).

Figure 1



Mean arterial pressure (MAP), measured in resting awake state, was increased over time in DM + HC + HT as compared to healthy control swine from our laboratory (unpublished data) performed in the same time period (*A*). Representative PAS stained sections of the top part of the left kidney of control and DM + HC + HT swine at a magnification of 200×. Scale bar = 50 µm (*B*). Increased tubulo-interstitial damage in kidney sections of DM + HC + HT swine compared to control healthy swine (*C*). Total tubulo-interstitial damage score was calculated by summing the scores for peritubular inflammatory infiltrate (not shown), interstitial fibrosis (*D*), atrophy (*E*) and dilatation (*F*) (Control $N = 8$, DM + HC + HT $N = 11$). $^{*}P < 0.05$, $^{\#}P = 0.06$.

Kidneys of the DM + HC + HT animals were smaller than kidneys of controls; however, when corrected for body weight, these differences were no longer apparent (see Supplementary material online, Table S3). Representative histology is shown for control and DM + HC + HT kidney cortex (*Figure 1B*). Total tubulo-interstitial (TI) damage score was higher in the DM + HC + HT group ($P < 0.001$, *Figure 1C*). The DM + HC + HT group showed no increase in peritubular infiltrate (data not shown), but showed more interstitial fibrosis (*Figure 1D*), a trend towards atrophy (*Figure 1E*, $P = 0.06$) and increased dilatation (*Figure 1F*) of the tubuli compared to controls. Consequently, GFR, measured by inulin clearance, was significantly reduced in DM + HC + HT as compared to healthy controls, indicative of kidney dysfunction ($P < 0.05$, *Table 1*). Although no differences in plasma creatinine or urea values were observed between the groups, cystatin C levels were significantly higher in the DM + HC + HT group ($P < 0.05$). Cystatin C strongly correlated with the levels of TNF-α ($P = 0.005$, see Supplementary material online, Figure S1C). There was a trend towards an increase in plasma aldosterone (10.4±4.1 vs. 1.4±0.01 pg/ml, $P = 0.06$).

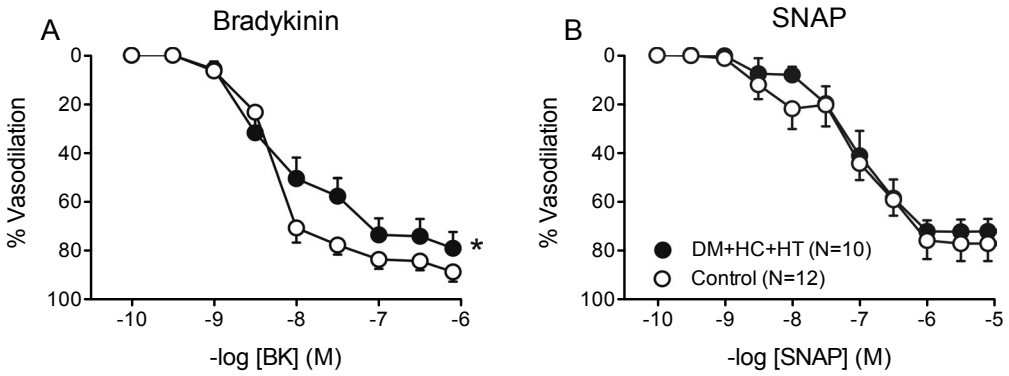
3.2 Coronary small artery function

Small arteries from DM + HC + HT showed similar preconstriction to 10^{-6} M U46619 as control vessels, i.e. 65±6% vs. 70±16% of the response to 10^{-1} mol/l KCl in the BK experiments, and 62±8% vs. 64±8% in the SNAP experiments (both P = NS). The vasorelaxation to the endothelium-dependent vasodilator BK was significantly blunted in DM + HC + HT (*Figure 2A*), whereas the vasorelaxation to the endothelium-independent vasodilator SNAP was maintained (*Figure 2B*), indicative of endothelial dysfunction. Interestingly, in isolated small arteries obtained from five additional DM + HC + HT swine, pretreatment with the ROS scavenger N-2-mercaptopropionyl glycine (MPG) restored the vasodilator response to bradykinin (see Supplementary material online, Figure S2).

3.3 Myocardial ROS measurements

Myocardial production of the NO metabolites NO_2^- and NO_3^- was significantly lower in the DM + HC + HT compared to control suggesting reduced NO production (*Figure 3A*). However, neither myocardial cGMP levels (DM + HC + HT: 5.74 ± 2.14 pmol/mg protein, control swine: 8.12 ± 2.16 pmol/mg protein, $P = 0.4$), nor PDE5 activity (1.03 ± 0.49 vs. 1.40 ± 1.04 AU/µg protein, $P = 0.6$), were significantly altered, resulting in preserved PKG activity (0.49 ± 0.01 vs.

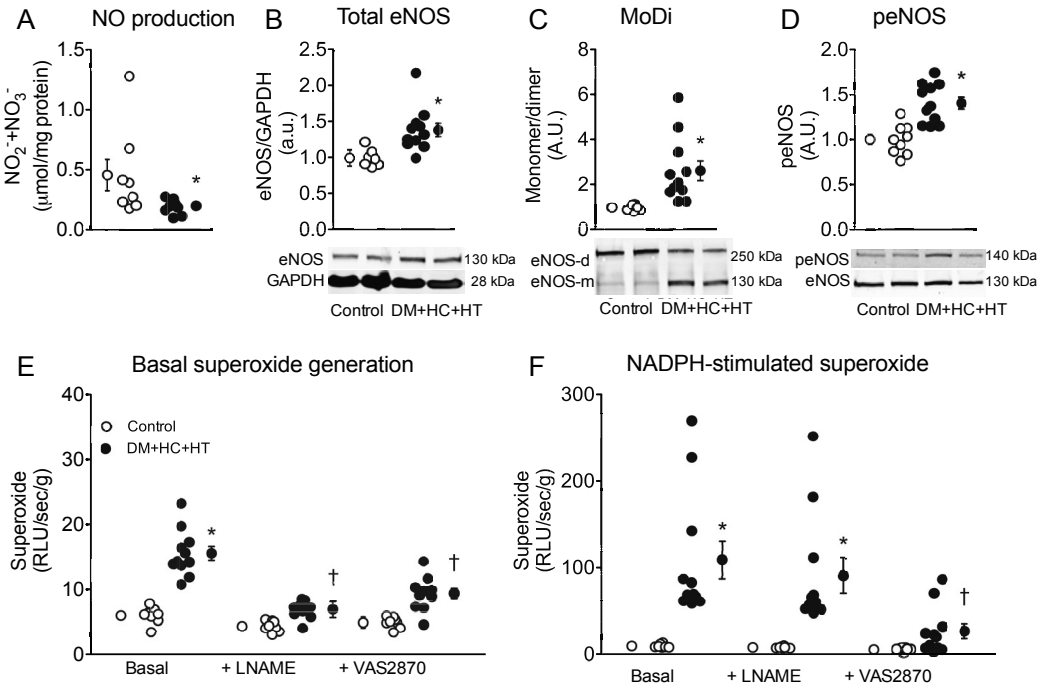
Figure 2



Concentration response curves to bradykinin (BK, *A*) and the NO-donor S-nitroso-N-acetylpenicillamine (SNAP, *B*) in small arteries isolated from DM + HC + HT and healthy control hearts. **P* < 0.05 DM + HC + HT vs. Control by 2-way ANOVA.

0.50 ± 0.02 AU/μg protein, *P* = 0.7). Although total eNOS expression was increased in DM + HC + HT as compared to control (*Figure 3B*), this increase was principally due to an increase in eNOS monomer, as the monomer/dimer ratio was markedly higher than in controls (*Figure 3C*). In addition, eNOS phosphorylation of residue Ser1177 was higher (possibly reflecting phosphorylation of the eNOS monomer[18]), suggesting that not only eNOS expression but also eNOS activity was increased (*Figure 3D*). The increase in monomer/dimer ratio in DM + HC + HT reflects uncoupling of eNOS. Accordingly, basal superoxide production was 3-fold higher in DM + HC + HT (*Figure 3E*) with the increased superoxide values correlating with inflammation (see Supplementary material online, Figure S1D) and the decrease in cardiac NO production (*P* = 0.008, see Supplementary material online, Figure S1E). This increase was suppressed by both L-NAME and VAS2870 (both *P* < 0.05), indicating that both NOS and NADPH oxidase contributed to superoxide production (*Figure 3E*). NADPH resulted in exaggerated—and VAS2870-inhibitable, but not L-NAME-inhibitable—superoxide production in DM + HC + HT as compared to controls (*Figure 3F*), confirming NADPH oxidase as a major source of superoxide in DM + HC + HT animals in addition to the uncoupled NOS-dependent superoxide production. NOX2 and 4 expression in the myocardium of DM + HC + HT did not differ from controls (see Supplementary material online, Table S4), suggesting that enzyme activity rather than transcriptional activation was higher in these animals. In contrast, gene expression of SOD-1 and

Figure 3



NO production was decreased in the LV subendocardium of DM + HC + HT ($N = 10$) vs. Controls ($N = 8$) (*A*). However, myocardial eNOS expression was increased in DM + HC + HT (*B*), as was the monomer/dimer (MoDi) ratio (*C*), suggestive of eNOS uncoupling. Phosphorylation of eNOS (peNOS) was also significantly increased in DM + HC + HT (*D*) ($N = 10$) vs. Control ($N = 8$). Superoxide generation was increased in the LV subendocardium of DM + HC + HT vs. Controls and was suppressed by L-NAME and VAS2870 (*E*). Upon NADPH oxidase stimulation, the superoxide anion production was dramatically increased (*F*), which was inhibited by VAS2870 but not by L-NAME treatment. $^*P < 0.05$ DM + HC + HT vs. Control; $^{\dagger}P < 0.05$ vs. corresponding basal.

catalase were higher in the DM + HC + HT as compared to controls (SOD-1 1.30 ± 0.09 vs. 1.00 ± 0.08, $P < 0.05$; catalase 2.44 ± 0.21 vs. 1.00 ± 0.22 AU, $P < 0.05$), likely representing a feedback mechanism to compensate for the increased oxidative stress. In accordance with the expression data, myocardial catalase activity was also increased in DM + HC + HT swine as compared to the healthy controls (22.3 ± 1.4 vs. 12.1 ± 0.3 nmol/min/mg protein, $P = 0.0001$). Both catalase ($P = 0.0007$) and SOD-1 ($P = 0.02$) expression correlated with superoxide production (see Supplementary material online, Figure S1F and G).

3.4 Myocardial structure: collagen content, myocardial hypertrophy, and capillary density

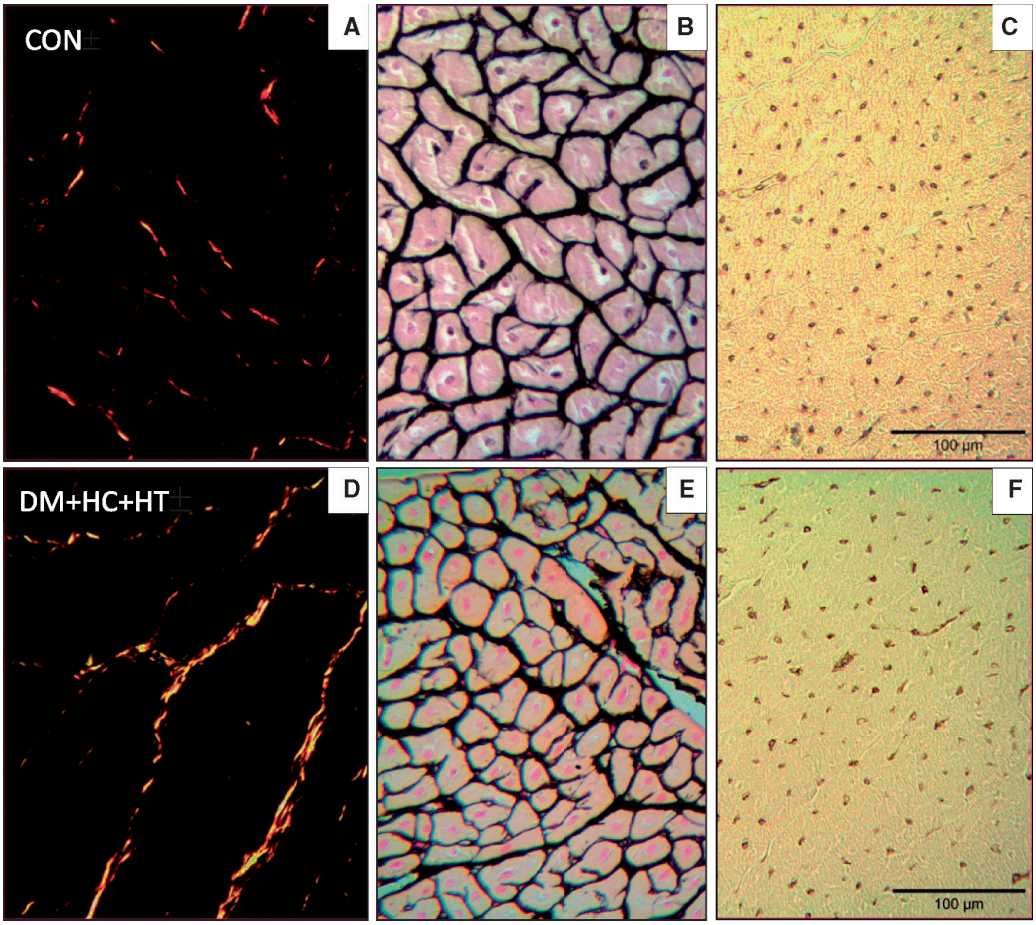
The histological findings in the subendocardial layer of the LV anterior wall of animals from both groups are shown in *Figure 4* and summarized in *Figure 5*. Total collagen deposition (collagen type I + III, *Figure 4A* and *D*) was assessed in the subendocardial and subepicardial layers of the LV. Collagen deposition was significantly increased in the subendocardium of DM + HC + HT as compared to control swine (*Figure 5A*), which correlated with the superoxide production ($P = 0.0001$, see Supplementary material online, Figure S1H). No difference was found between the subendocardium and the subepicardium in each group (data not shown). Moreover, molecular data suggest reduced matrix turnover in the subendocardium of DM + HC + HT animals, since expression of both MMP-2 and TIMP-2 was lower, while expression of MMP-9 and TIMP-1 was unaltered in DM + HC + HT animals as compared to controls (see Supplementary material online, Table S4). The MMP-2/TIMP-2 ratio was also significantly lower in the DM + HC + HT groups compared to control animals (0.72 ± 0.05 vs. 0.89 ± 0.03, $P = 0.01$), while the ratio MMP-9/TIMP-1 was similar (1.17 ± 0.21 vs. 1.73 ± 0.51, P = NS).

The measurement of the cardiomyocyte cross-sectional area (*Figure 4B* and *E*), revealed that cardiomyocytes from the DM + HC + HT were significantly smaller than those of control swine, which was observed in both subendocardium (413±42 μm^2 vs. 598±23 μm^2, $P < 0.05$; *Figure 5B*) and subepicardium (379±29 μm^2 vs. 676±31 μm^2, $P < 0.05$; data not shown in *Figure 5B*). Cardiomyocyte size was inversely correlated with the levels of cystatin C ($P = 0.02$), a potent risk factor for cardiovascular disease associated mortality and a strong marker for renal dysfunction. Interestingly, when normalized to body weight, the difference in myocyte area between Control and DM + HC + HT was no longer observed. In line with the observed reduction in cardiomyocyte size in DM + HC + HT, myocardial ATF4 expression was significantly higher in the DM + HC + HT as compared to controls (1.51 ± 0.12 vs. 1.00 ± 0.08 AU, $P < 0.05$), which may suggest increased ER stress in the cardiomyocytes as a result of the comorbidities. The gene expressions of ANF/NPPA, UBE2H, and ACTA1 and 2 in the myocardium were not different between groups (see Supplementary material online, Table S4), in line with the lack of myocyte hypertrophy in DM + HC + HT.

Capillary density (*Figure 4C* and *F*) was similar between the groups (*Figure 5C*) and also between the subendocardium and subepicardium in each group (data not shown). However, the capillary-to-fiber ratio was significantly lower in DM + HC + HT as compared to controls, suggestive of capillary rarefaction (*Figure 5D*). No differences were observed between the subendocardium and subepicardium of either group (not shown). Unpublished data from our laboratory obtained in weight-matched DM + HC + HT animals showed no difference in myocyte size vs. control animals, while

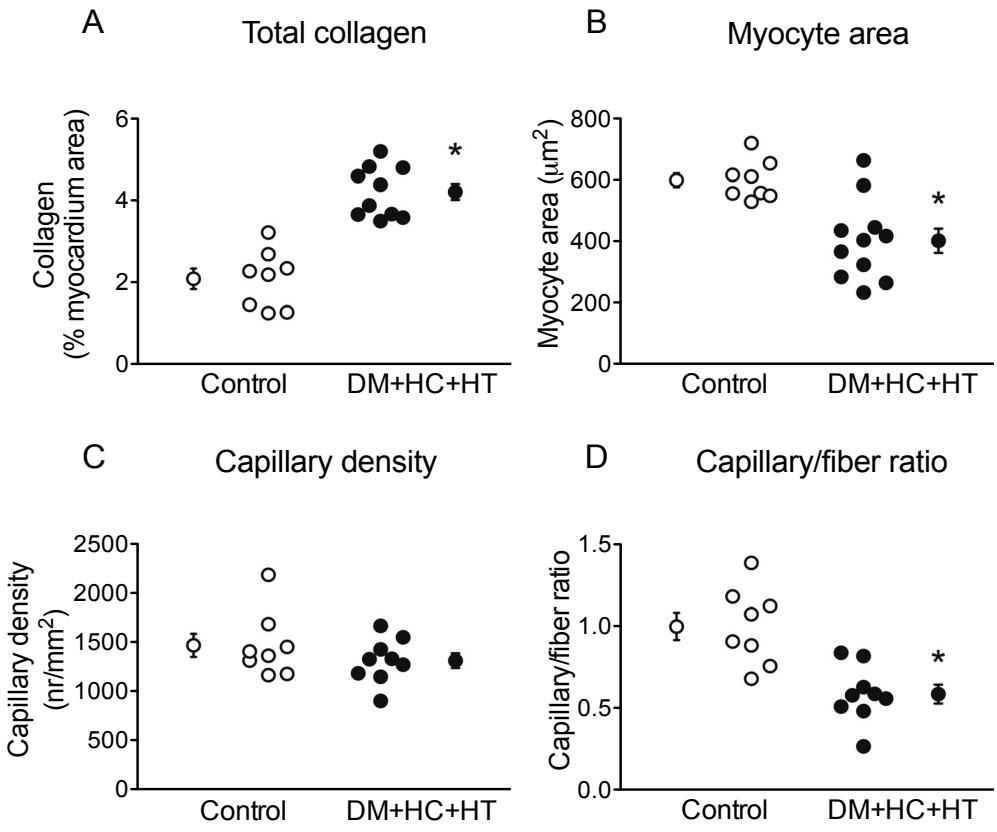
showing a significant reduction in capillary density (1237 ± 81 in DM + HC + HT vs. 1548 ± 88 capillaries/mm^2 in control, $P = 0.03$).

Figure 4



Examples of histological staining for quantification of collagen deposition (Picrosirius Red *A*, *D*), myocyte size (Gomori *B*, *E*), and capillary density (Lectin *C*, *F*) in the LV subendocardium in Control (CON), and DM + HC + HT animals

Figure 5

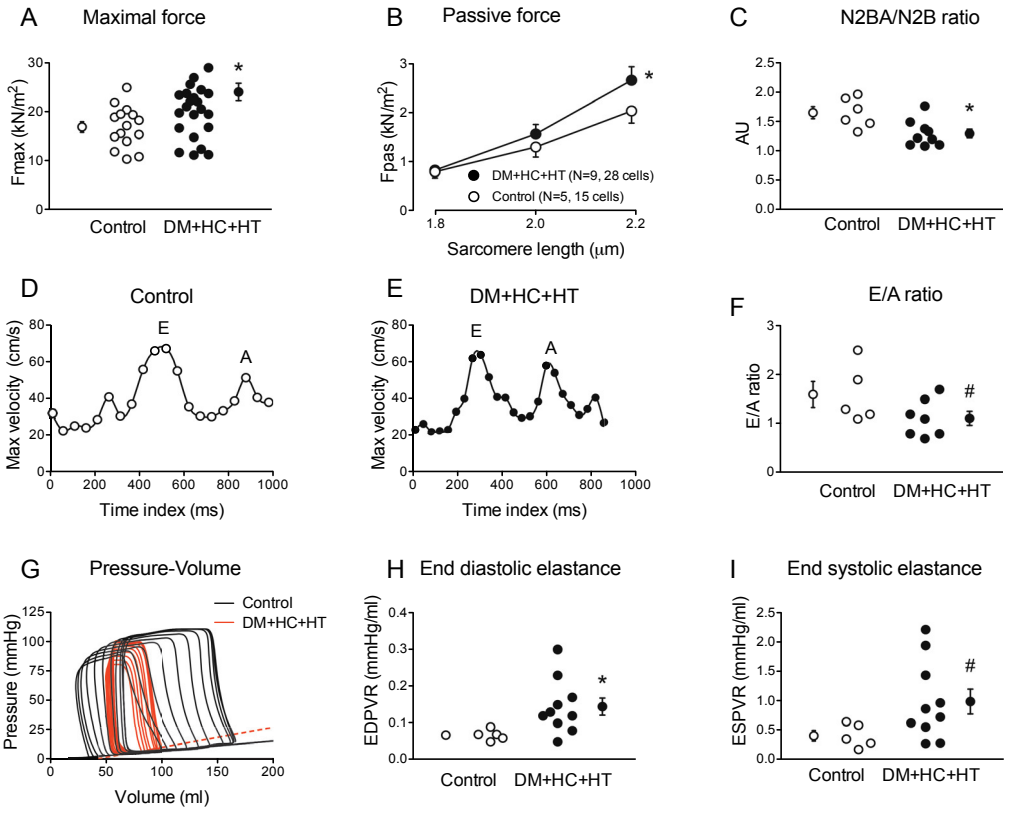


Increased collagen deposition (*A*) but no myocyte hypertrophy (*B*) was recorded in LV subendocardium of the DM + HC + HT animals. Capillary density was similar between groups (*C*), however the capillary-to-fiber ratio was significantly reduced in DM + HC + HT (*D*). **P* < 0.05 DM + HC + HT (*N* = 10) vs. Control (*N* = 8).

3.5 Single myocyte force measurements

Increased maximal force (F_{max}, *Figure 6A*) as well as increased passive stiffness (F_{pas}, *Figure 6B*) of the cardiomyocytes isolated from the subendocardium of the LV anterior wall was observed in DM + HC + HT as compared to controls. Myocardial N2BA/N2B titin expression showed a shift towards the stiff N2B titin isoform (*Figure 6C*, *P* = 0.01), consistent with the increased passive stiffness of the cardiomyocytes, while no significant change in total phosphorylation of either isoform was observed (N2BA: 0.76 ± 0.06 AU in Control vs. 0.74 ± 0.05 AU in DM + HC + HT; N2B: 0.98 ± 0.11 AU in Control vs. 0.92 ± 0.07 AU in DM + HC + HT).

Figure 6



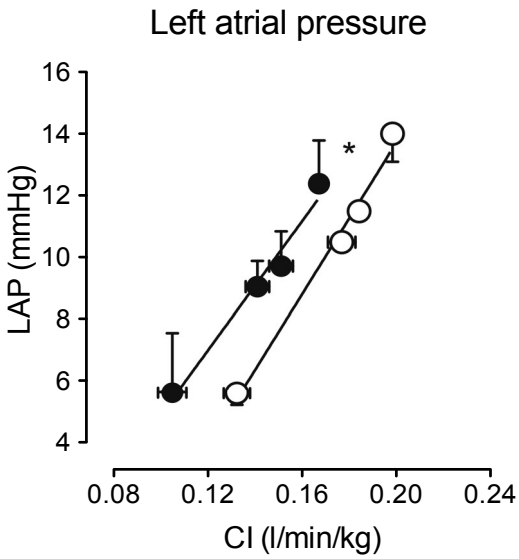
Increased maximal (F_{max}, *A*) and passive force (F_{pas}, *B*) were seen in DM + HC + HT as compared to Controls. Cardiomyocyte data were averaged for all measured cells and group averages are shown. Titin N2BA/N2B isoforms ratio was significantly decreased in DM + HC + HT animals (*C*). Typical examples of E and A waves in Control (*D*) and DM + HC + HT (*E*) animals and pressure–volume relationships (*G*) are presented. The early to late filling (E/A) ratio tended to be lower in the DM + HC + HT animals (*F*). End-diastolic elastance (slope of EDPVR, sEDPVR) was significantly increased (*H*) while a trend towards significance in the end-systolic elastance (slope of ESPVR, sESPVR) was recorded (*I*), $^{*}P < 0.05$, $^{\#}P = 0.10$ DM + HC + HT vs. Control.

3.6 Cardiac MRI and global LV function

MRI and PV loop variables as well as typical examples of E and A waves (*Figure 6D* and *E*) and pressure–volume loops (preload reduction, *Figure 6G*) are shown in *Table 2* and *Figure 6*. LV EDV and SV, indexed to body weight, and ejection fraction were not different between the two groups,

measured by either MRI or PV loop catheter (*Table 2*). Late gadolinium enhancement did not show any difference between the groups (data not shown), despite an increased collagen content in the LV of DM + HC + HT animals as measured histologically (*Figure 5A*). However, E/A ratio tended to be lower in the DM + HC + HT group, suggestive of early diastolic dysfunction (*Figure 6F*). These findings were complemented by the pressure–volume data, showing that DM + HC + HT animals had increased LV end-diastolic elastance, indicative of increased passive stiffness of the LV *in vivo* (*Figure 6H*, $P < 0.05$). The increase in LV end-diastolic elastance correlated with the increased collagen deposition in the subendocardium (see Supplementary material online, Figure S1I, $P = 0.05$). LV end-systolic elastance also tended to be higher in these animals, although this failed to reach statistical significance (*Figure 6I*, $P = 0.10$), which correlated well with the maximal force development of single cardiomyocytes ($P = 0.006$). Although no significant group differences were observed in LV end-diastolic pressure, dP/dt_{max}, LV dP/dt_{min}, or tau (*Table 2*), we did observe a shift in the relation between left atrial pressure and cardiac index (*Figure 7*) in chronically instrumented DM + HC + HT compared to control swine during exercise ($P < 0.05$). These data indicate that in swine in the awake state, at similar levels of cardiac index higher atrial filling pressures were observed in DM + HC + HT compared to control swine.

Figure 7



Relation between left atrial pressure (LAP) and cardiac index (CI) at rest and during treadmill exercise in chronically instrumented Control ($N = 4$; 97 ± 6 kg, white circles) and DM + HC + HT ($N = 4$; 94 ± 7 kg, black circles) swine. *$P < 0.05$ DM + HC + HT vs. Control.

Table 2. LV function in Control and DM+HC+HT swine under anaesthesia.

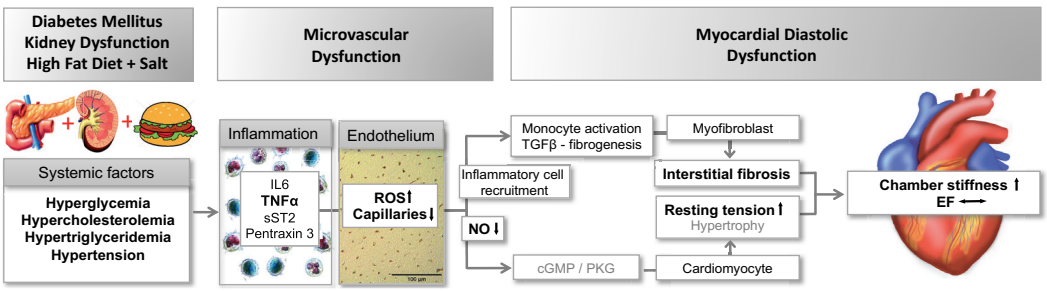
Parameter	Control	DM+HC+HT
Body weight (kg)	102 ± 4	79 ± 3*
Pressure/Volume Catheter	***N=8***	***N=10***
Heart rate (bpm)	85 ± 3	91 ± 5
LV EDV (ml)	174 ± 22	113 ± 11*
LV EDVi (ml/kg)	1.5 ± 0.2	1.5 ± 0.2
SV (ml)	75 ± 3	55 ± 4*
SVi (ml/kg)	0.75 ± 0.03	0.74 ± 0.08
Ejection fraction (%)	47 ± 5	50 ± 3
Millar Catheter	***N=8***	***N=10***
Heart rate (bpm)	88 ± 4	85 ± 3
dP/dt max (mmHg/s)	1470 ± 138	1604 ± 214
dP/dt min (mmHg/s)	-2510 ± 322	-2687 ± 266
Tau (ms)	49 ± 3	48 ± 3
LV EDP (mmHg)	9 ± 2	9 ± 2
MRI	***N=6***	***N=7***
Heart rate	89 ± 7	71 ± 3*
LV EDV (ml)	189 ± 12	162 ± 11
LV EDVi (ml/kg)	1.8 ± 0.1	2.1 ± 0.1
SV (ml)	98 ± 9	73 ± 7*
SVi (ml/kg)	0.93 ± 0.07	0.94 ± 0.06
Ejection fraction (%)	51 ± 3	45 ± 4

LV= left ventricle, SV=stroke volume, EDV=End-diastolic volume, EDVi=End-diastolic volume indexed for body weight, ESV= End-systolic volume, ESVi= End-systolic volume indexed for body weight, dP/dT min and dP/dT max = minimum and maximum rate of pressure change in the left ventricle, LV EDP=left ventricle end diastolic pressure, Tau=time constant of isovolumic relaxation; *P<0.05 compared to Controls.

4. Discussion

In the present study, we report that in the absence of major geometrical alterations in the heart, the co-existence of three common comorbidities leads to LV diastolic dysfunction as evidenced by increased passive LV stiffness and a trend towards reduced LV diastolic early-to-late filling velocities, while EF was still preserved. The cascade of events leading to diastolic dysfunction is summarized in *Figure 8*,[19] in which the events that were observed in the present study are highlighted in bold. These include the presence of chronic systemic inflammation and decreased endothelium-dependent vasodilation in the coronary arteries as assessed *in vitro.* We also observed reduced capillary-to-fiber ratio, elevated superoxide production due to NOX activation, eNOS uncoupling, and reduced myocardial NO production, all likely to be important mechanisms for the observed increases in LV collagen content and cardiomyocyte passive force contributing to LV stiffening and thereby reduced diastolic function.[12]

Figure 8



In a large animal, chronic exposure to multiple common comorbidities results in systemic inflammation, endothelium-dependent coronary artery dysfunction, capillary rarefaction, oxidative stress, and perturbed nitric oxide production, which are associated with increased myocardial fibrosis and passive cardiomyocyte stiffness, resulting in LV diastolic dysfunction. Adapted with permission from Ter Maaten et al.[19] The findings of the present study that are in agreement with the hypothesis are presented in bold.

Paulus and Tschöpe[12] proposed that a systemic inflammatory state produced by cardiovascular comorbidities is common in many HF patients, particularly those with HFpEF.[20,21] The inflammatory state promotes coronary microvascular endothelial dysfunction, which is characterized by generation of reactive oxygen species and reduced NO bioavailability, and leads to a cascade of signaling events that ultimately promotes cardiac fibrosis and myocyte stiffness. Indeed, it is now well

established that multiple common comorbidities, including obesity, DM, and HT, are strongly associated with HFpEF.[5–7,22–24] This cascade of events could be faithfully recapitulated in the present study, although the causality between the various steps remains to be tested. Firstly, a pro-inflammatory state was documented by markedly elevated levels of circulating TNF-α in diseased animals. TNF-α values correlated with the levels of glucose and cystatin C, confirming that DM and kidney dysfunction (as evidenced by high tubulo-interstitial damage score, increased cystatin C levels, and impaired GFR, despite normal urea and creatinine levels), are important contributors to increased systemic inflammation. Secondly, coronary small artery endothelial dysfunction was also evident as relaxation responses to bradykinin were diminished, while smooth muscle cell function was preserved as indicated by the preservation of vascular responses to the exogenous NO donor SNAP and the maintained cGMP-PDE5-PKG signaling, although the latter was measured in the myocardium and might also, partly, reflect cardiomyocyte signaling. Thirdly, and perhaps most importantly, ROS production was markedly increased and correlated with the decreased cardiac NO production. Specifically, the basal superoxide production was increased 3-fold in DM + HC + HT as compared to control swine. This increase was significantly suppressed by L-NAME as well as by VAS2870, indicating that both NOS and NADPH oxidase were sources of superoxide production. Upon stimulation of NOX, myocardial superoxide production was markedly increased in DM + HC + HT as compared to controls, confirming NOX as the major source of superoxide. This was probably due to the increase in NOX-activity, as myocardial gene expression was not significantly different between groups. Furthermore, although increased eNOS expression and phosphorylation levels were observed in DM + HC + HT animals, the monomer/dimer ratio was increased almost 3-fold, indicating that eNOS-uncoupling accounted for the aggravated NOS-dependent superoxide production. The increased myocardial superoxide levels in conjunction with low NO levels suggest direct quenching of NO by superoxide as a major factor contributing to the reduced NO bioavailability in the present animal model, both basal and agonist induced. This is in agreement with data from Paolocci et al. in isolated rat hearts.[25] In our model, the increase in superoxide production correlated with the levels of TNF-α, confirming that inflammation is one of the important mechanisms leading to the increase in oxidative stress in the diseased animals. Interestingly, catalase activity, as well as the expression of catalase and SOD-1 were also significantly increased in the diseased animals, representing a possible compensatory mechanism for increased oxidative stress as both expression levels correlated with superoxide production.

The observed functional alterations were associated with structural vascular impairments in the myocardium of DM + HC + HT animals. We observed capillary rarefaction as evidenced by lower capillary-to-fiber ratio in the diseased hearts. Capillary rarefaction has been asociated with metabolic syndrome in animal models[26] and has only been sparsely investigated in previous studies of diastolic

dysfunction, but our findings are in accordance with a study by Mohammed et al. who reported a reduced capillary density in the myocardium of HFpEF patients.[27] Capillary rarefaction may have important clinical implications, particularly when occurring simultaneously with the observed coronary arterial endothelial dysfunction, as it will impair myocardial oxygenation especially during physical exercise.[28] These microvascular alterations, at the level of both small arteries and capillaries, may contribute to reduced exercise tolerance in HFpEF patients.[29] The observed vascular changes were accompanied by an increase in total collagen, which together with the increased passive stiffness of single cardiac myocytes (likely due to the shift towards the stiffer titin N2B isoform), translated into higher LV stiffness[30–32] as demonstrated by increased LV end-diastolic elastance and the trend towards a decreased E/A ratio, both clear indicators of diastolic dysfunction. Indeed, the increase in collagen correlated with the increased LV end-diastolic elastance, indicating collagen deposition in the myocardium as an important determinant of increased stiffness. The activation of pro-fibrotic pathways in the myocardium and the resulting increase in collagen deposition is likely due to the increased oxidative stress and inflammation modulating the TGF-beta/SMAD3 signalling pathway.[33] Furthermore, Westermann et al.[34] have shown in patients with HFpEF that cardiac inflammation results in accumulation of extracellular matrix contributing to the development of diastolic dysfunction. Disturbed turnover of extracellular matrix, mediated through dysregulation of MMPs and their tissue inhibitors (TIMPS) is likely to play a role in this process. Our data suggest a reduced, rather than increased, matrix turnover in the myocardium of the animals with comorbidities at this stage of the disease. In addition, data obtained in a dog model of subacute heart failure indicate that persistent neurohumoral activation, which is also likely to be present in our porcine model, may result in high-energy phosphate depletion and enhancement of AMP deaminase activity contributing to myocardial stiffening irrespective of changes in extracellular matrix.[35] The structural abnormalities observed in the present study translated into a shift in the relation between left atrial pressure and cardiac index in exercising swine, indicating that also during exercise higher filling pressures were required to achieve a similar level of cardiac index, consistent with a stiffer heart. Interestingly, the increased maximal force of single cardiomyocytes correlated with the observed trend towards increased LV end-systolic elastance. It could therefore be speculated that the increase in contractility acted as a compensatory mechanism to maintain LV function particularly in the face of the elevated blood pressure in the absence of LV hypertrophy.[36]

4.1 Aspects of the experimental model

The animal model presented here is complementary to a recently reported porcine model of diastolic dysfunction.[37] Schwarzl et al. induced HT and HC using DOCA-salt and western diet containing high amounts of salt, fat, and cholesterol. An important difference between the two studies, besides the longer follow-up in our study (6 months vs. 12 weeks), was the induction of diabetes and kidney dysfunction in our model. The type of diabetes induced in our study is not consistent with either DM type I or with early DM type II, but rather with a late stage DM type II with impaired insulin production. In addition, the animals with comorbidities had a lower body weight, despite increased lipid plasma levels which may be due to growth retardation, which has also been shown in children with chronic kidney disease.[38] Although Schwarzl et al.[37] reported several similar functional findings, including increased LV end-diastolic stiffness, increased superoxide production, and eNOS uncoupling, some findings are distinctly different. Diabetes, which is a common comorbidity in HFpEF patients, likely contributed to the endothelial dysfunction and activation of NOX, which are also observed in HFpEF patients.[18] Moreover, whereas concentric LV hypertrophy occurred in response to the pressure-overload in the study of Schwarzl et al. cardiomyocyte area was reduced in our model, indicating that in the present model, myocyte hypertrophy is not a factor contributing to myocardial stiffening. The lack of myocyte hypertrophy, is in agreement with the maintained PKG activity levels in our animals and in accordance with the concept that severe hyperglycemia induces muscle cell atrophy both in skeletal[39,40] and cardiac muscle.[41,42] Furthermore, myocyte size inversely correlated in our animals with the levels of cystatin C suggesting a role of the chronic kidney dysfunction in this process. Schwarzl et al. only evaluated renal function by creatinine and urea, but not inulin clearance and cystatin C. Our study shows that marked reductions in glomerular filtration rate are not detected by urea and creatinine in hypertensive swine. Increased LV fibrosis was documented in the present study and correlated with the increased diastolic elastance, but was not observed in the study by Schwarzl et al., most likely because hyperglycemia in addition to inflammation is an important trigger for extracellular matrix remodeling in HFpEF.[43,44] Diabetes is known to lead to vascular deficiency, mediated by miR-320 according to a recent study[45] as documented here as capillary rarefaction. In the study by Schwarzl et al.[37] reduced phosphorylation of titin, the giant molecular 'spring' that is considered as one of the most important factors responsible for cardiomyocyte passive stiffness, was observed and a shift towards its stiffer isoform N2B was reported, supporting the concept that both collagen and titin contribute to myocardial stiffness in HFpEF patients.[30] In the present model, while the shift in titin isoform towards the stiff N2B isoform was also documented, a change in total phosphorylation was not observed. Altogether, data from both models suggest that the presence of different comorbidities may initiate partially different pathologic pathways, that may provide

potential targets for further study and mechanistic interventions. Based on the data from the present animal model, chronic treatment with antioxidants aiming at increasing NO bioavailability and alleviating oxidative stress, lowering inflammation, as well as sGC stimulators might result in reduction of collagen deposition, cardiomyocyte stiffening, and delay of onset of diastolic dysfunction.

4.2 Conclusions

As summarized in *Figure 8*, the present study demonstrates that in a large animal, chronic exposure to multiple common comorbidities results in systemic inflammation, endothelium-dependent coronary microvascular dysfunction, capillary rarefaction, oxidative stress, and perturbed nitric oxide production, which are associated with increased myocardial fibrosis and passive cardiomyocyte stiffness in the absence of myocyte hypertrophy, resulting in LV diastolic dysfunction. Future studies are needed to address the exact mechanisms connecting the different steps in the process; however, this large animal model provides an excellent translational tool for improving our understanding of the early pathophysiology of heart failure with preserved ejection fraction and for testing novel therapeutic interventions, including drugs, exercise, and diet interventions, for the treatment of the patients with this type of heart failure.

Supplementary material Supplementary material is available at *Cardiovascular Research* online. (https://academic.oup.com/cardiovascres/article/114/7/954/4844872)

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Supplementary Results

To exclude an effect of the surgical procedures on the plasma levels of TNF-α, we measured TNF-α levels in a subgroup of control animals (N=3) both prior to surgery and 2-3 weeks after surgery. We observed similar levels of TNF-α at both time points: 27.3±5.1 at 3 weeks after surgery vs 24.7 ±5.4 pg/ml before surgery, indicating that the sustained inflammatory response was due to the risk factors and not the result of surgical interventions.

Supplementary Table 1 Composition of the high fat diet.

Ingredients	%
Soybean hulls	19.65
Potato protein	2.5
Wheat gluten flour	8.6
Sugar	10
Fructose	15
Pea starch	15
Lard	25
Premix vitamins	1
Chalk	0.96
Monocalciumphosphate	1.08
Salt	0.47
Cholesterol	1
Lysine HCl	0.24
Sodium cholate	0.7

Supplementary Table 2 Primer sequences.

	Forward sequence	Reverse sequence
Beta-actin	GCATCCTGACCCTCAAGTAC	CACGCAGCTCGTTGTAGAAG
PDK4	TGCAATGAGGGCTACAGTCG	CGGTCAATGATCCTCAGGGG
SOD-1	TGGGCAATGTGACTGCTGGC	TCATGGACCACCATTGTGCGG
Catalase	TGCCACCGGCAACTATCCCT	TCGCTGTGAGGCCAAACCTTG
NOX 2	CCGCATTGTTGGCGACTGGA	CCCGTCCACAGCGATCTTAGG
NOX 4	ACCAGATGCTGGGGGATTGTG	CCTCGAAGGTAAGCCAGGAGTGT
ACTA 1	GAGAGCAGCAGAAACCCGACG	ACGATGGACGGGAACACAGC
ACTA 2	ATGGTGGGAATGGGACAA	GTGATGATGCCGTGTTCT
UBE2H	GAGGCGGATGGACACAGACG	CCTTCATACGGTGTTCCCTGTGG
NPPA	ACCGTGAGCTTCCTCCTCGT	TCCAAGTGGTCCAGCAAATTCTTG
ATF4	TTAGGCCATGGCGCTTCTCAC	TCGGCCATGTTGCGGAGTTT
MMP 2	GCAGTGATGGCAAGTTGTGG	TTGACATCGTCGTGGGACAG
MMP 9	TCGACGTGAAGACGCAGAAG	ACCTGATTCACCTCGTTCCG
TIMP 1	GATCTATGCTGCTGGCTGTGA	GTCTGTCCACAAGCAGTGAGT
TIMP 2	TTGCAATGCAGACGTAGTGA	GCCTTTCCTGCGATGAGGT

PDK4= Pyruvate Dehydrogenase Kinase isosyme 4; SOD 1= Superoxide Dismutase 1; NOX 2= NADPH Oxidase 2; NOX 4= NADPH oxidase 2; ACTA 1= Alpha Actin 1; ACTA 2= Alpha Actin 2; UBE2H= Ubiquitin-Conjugated Enzyme E2H; ANF/NPPA= Atrial Natriuretic Factor; ATF4= Activating Transcription Factor 4, MMP 2, 9= matrix metalloproteinase 2, 9, TIMP 1,2= tissue inhibitor of matrix metalloproteinase 1, 2.

Supplementary Table 3 Kidneys and heart weights at sacrifice.

		Controls (N=8)	DM+HC+HT (N=10)
Body weight (kg)		102 ± 4	79 ± 3*
Kidneys			
Left kidney	- absolute (g)	176 ± 12	127 ± 7*
	- relative (g/kg)	1.69 ± 0.09	1.64 ± 0.10
Right kidney	- absolute (g)	173 ± 14	132 ± 10*
	- relative (g/kg)	1.66 ± 0.12	1.71 ± 0.14
Heart			
LV	- absolute (g)	231 ± 17	184 ± 9*
	- relative (g/kg)	2.2 ± 0.1	2.3 ± 0.1
RV	- absolute (g)	73 ± 8	58 ± 3
	- relative (g/kg)	0.68 ± 0.08	0.74 ± 0.02
LA	- absolute (g)	21 ± 1	16 ± 1*
	- relative (g/kg)	0.20 ± 0.01	0.21 ± 0.01
RA	- absolute (g)	14 ± 1	13 ± 1
	- relative (g/kg)	0.13 ± 0.01	0.17 ± 0.01*

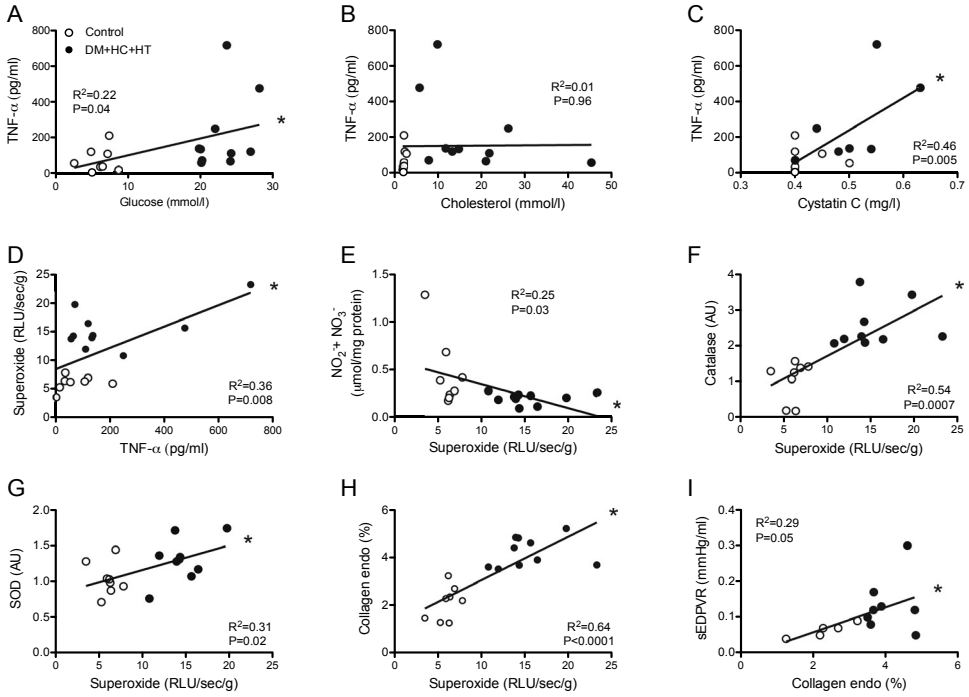
LV= left ventricle, RV= right ventricle, LA= left atrium, RA= right atrium. Data are presented as absolute value and as relative values normalised to body weight. *P<0.05 DM+HC+HT vs. Controls.

Supplementary Table 4 Gene expression in left ventricular myocardium

	Controls (N=8)	DM+HC+HT (N=10)
Beta-actin	1.00 ± 0.44	1.03 ± 0.26
PDK4	1.00 ± 0.22	1.91 ± 0.24*
SOD-1	1.00 ± 0.08	1.30 ± 0.09*
Catalase	1.00 ± 0.22	2.44 ± 0.21*
NOX 2	1.00 ± 0.12	1.52 ± 0.38
NOX 4	1.00 ± 0.13	0.91 ± 0.15
ACTA 1	1.00 ± 0.22	0.88 ± 0.03
ACTA 2	1.00 ± 0.16	1.37 ± 0.16
UBE2H	1.00 ± 0.10	1.26 ± 0.09
ANF/NPPA	1.00 ± 0.47	0.72 ± 0.23
ATF4	1.00 ± 0.08	1.51 ± 0.12*
MMP 2	1.00 ± 0.06	0.59 ± 0.05*
MMP 9	1.00 ± 0.26	0.73 ± 0.08
TIMP 1	1.00 ± 0.04	1.03 ± 0.06
TIMP 2	1.00 ± 0.09	0.73 ± 0.07*

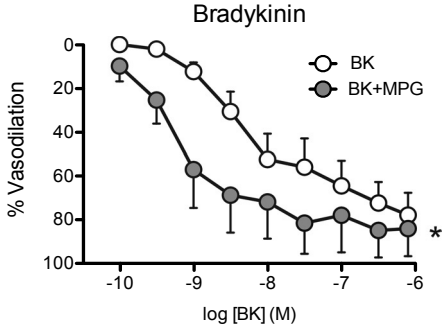
PDK4= Pyruvate Dehydrogenase Kinase isosyme 4; SOD 1= Superoxide Dismutase 1; NOX 2= NADPH Oxidase 2; NOX 4= NADPH oxidase 2; ACTA 1= Alpha Actin 1; ACTA 2= Alpha Actin 2; UBE2H= Ubiquitin-Conjugated Enzyme E2H; ANF/NPPA= Atrial Natriuretic Factor; ATF4= Activating Transcription Factor 4, MMP 2, 9= matrix metalloproteinase 2, 9, TIMP 1,2= tissue inhibitor of matrix metalloproteinase 1,2. Results were normalized to the housekeeping gene beta-actin and relative changes in expression levels were calculated relative to the Control group, and presented as Mean ±SEM. *P<0.05. DM+HC+HT vs. Controls.

Supplementary Fig. 1

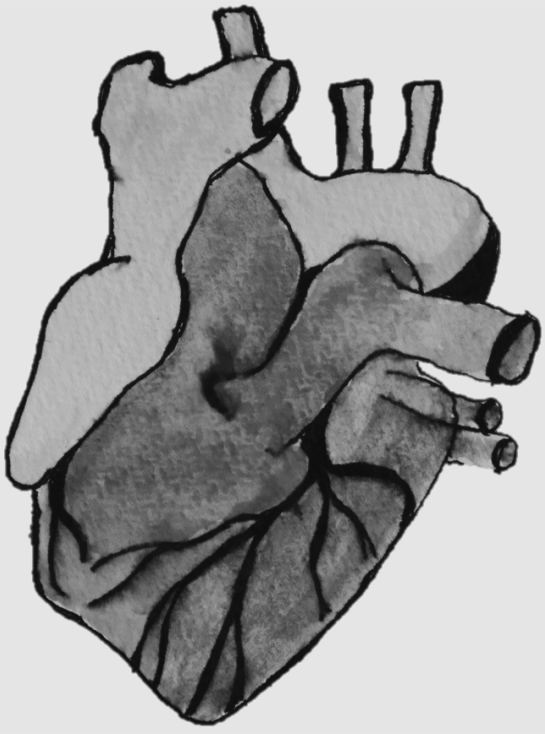


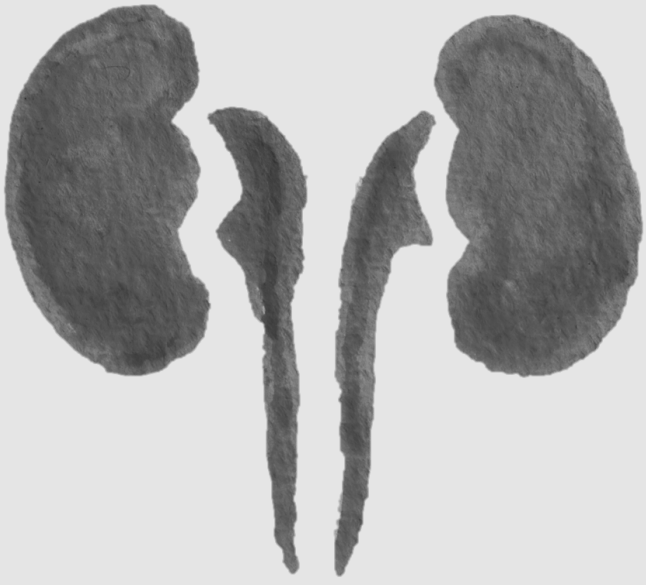
Correlations between the risk factors, inflammatory state, oxidative stress in the myocardium, fibrosis and left ventricular end diastolic elastance.

Supplementary Fig. 2



Concentration-response curves to bradykinin (BK) in baseline conditions and upon pretreatment with the free radical scavenger N-2-mercaptopropionyl glycine (MPG) in DM+HC+HT animals.





Chapter 8

Perturbations in myocardial perfusion and oxygen balance in swine with multiple risk factors: a novel model of ischemia and no obstructive coronary artery disease

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8

Abstract

Introduction: Comorbidities of ischemic heart disease, including diabetes mellitus (DM), hypercholesterolemia (HC) and chronic kidney disease (CKD), are associated with coronary microvascular dysfunction (CMD). Increasing evidence suggests that CMD may contribute to myocardial 'Ischemia and No Obstructive Coronary Artery disease' (INOCA). In the present study, we tested the hypothesis that CMD results in perturbations in myocardial perfusion and oxygen delivery using a novel swine model with multiple comorbidities.

Methods and Results: DM (streptozotocin), HC (high fat diet) and CKD (renal embolization), were induced in 10 female swine (DM+HC+CKD-group), while 12 healthy female swine on a normal diet served as controls (Normal). After 6 months, at a time when coronary atherosclerosis was still negligible, myocardial blood flow, metabolism and function were studied at rest and during treadmill exercise. DM+HC+CKD animals showed hyperglycemia, hypercholesterolemia and impaired kidney function. During exercise, DM+HC+CKD demonstrated perturbations in myocardial blood flow and oxygen delivery, necessitating a higher myocardial oxygen extraction – achieved despite reduced capillary density – resulting in lower coronary venous oxygen levels. Moreover, myocardial efficiency was lower, requiring higher oxygen consumption for a given level of myocardial work. These perturbations in myocardial oxygen balance were associated with lower myocardial lactate consumption, stroke volume and $LVdP/dt_{max}$, suggestive of myocardial ischemia and dysfunction. Further analyses showed a reduction in adenosine-recruitable coronary flow reserve, but this was exclusively the result of an increase in basal coronary blood flow, while maximal coronary flow per gram of myocardium was maintained; the latter was consistent with the unchanged arteriolar wall/lumen ratio, arteriolar density and peri-arteriolar collagen content. However, isolated small arteries displayed selective blunting of endothelium-dependent vasodilation in response to bradykinin in DM+HC+CKD swine, suggesting that changes in coronary microvascular function rather than in structure contributed to the perturbations in myocardial oxygen delivery.

Conclusion: Common comorbidities in swine result in CMD, in the absence of appreciable atherosclerosis, which is severe enough to produce perturbations in myocardial oxygen balance, particularly during exercise, resembling key features of INOCA.

Introduction

Common comorbidities of cardiovascular disease, including diabetes mellitus (DM), hypercholesterolemia (HC), chronic kidney disease (CKD), are well-known risk factors for the development of coronary artery disease of both large epicardial arteries and smaller coronary arteries.[1-4] While it is well-established that obstructive coronary artery disease (CAD) is a major cause of myocardial ischemia[5], there is increasing evidence that coronary microvascular dysfunction (CMD) also contributes to myocardial ischemia, not only in the presence of obstructive CAD[6-9], but also in patients without obstructive CAD, a situation referred to as 'Ischemia and No Obstructive Coronary Artery disease' (INOCA).[2, 9-11] Clinical studies have shown that INOCA is present in approximately one-third of men and two-thirds of women undergoing angiography for suspected ischemic heart disease and that cardiovascular death or myocardial infarction occurred in 6.7% of the patients without any signs of CAD and in 12.8% of patients with non-obstructive CAD.[12, 13]

Although the mechanisms underlying INOCA remain incompletely understood, there is increasing evidence that CMD, in particular impaired endothelium-dependent vasodilation, plays an important role.[3, 10, 14, 15] In agreement with these clinical observations, experimental data obtained in swine, chronically exposed to multiple comorbidities, also demonstrate endothelial dysfunction of isolated small coronary arteries studied in vitro, in the absence of obstructive CAD.[16-18] However, whether these perturbations of coronary microvascular endothelial function translate into impaired myocardial perfusion and oxygen delivery *in vivo*, i.e. result in INOCA, was not assessed in these studies. Consequently, we tested the hypothesis that combined comorbidities as frequently present in patients, result in perturbations in myocardial perfusion and oxygen delivery, causing a shift towards anaerobic metabolism and cardiac dysfunction, particularly during increased myocardial oxygen demand. To test our hypothesis, we studied swine that were chronically (six months) exposed to a combination of three common comorbidities – DM+HC+CKD, and have been extensively phenotyped in our previous study.[16] Here, swine were chronically instrumented after 5 months exposure to DM+HC+CKD to allow assessment of systemic and coronary hemodynamics as well as myocardial metabolism and function in the awake state, at rest and during treadmill exercise.

Materials and Methods

Animals

All animal experiments were approved by the Animal Care Committee at the Erasmus University Medical Center (Rotterdam, The Netherlands) and performed in accordance with the "Guiding Principles in the Care and Use of Laboratory Animals" as approved by the Council of the American Physiological Society. Ten female Yorkshire x landrace swine (25±1 kg) were included in the DM+HC+CKD group while 12 healthy female Yorkshire x landrace swine of similar age and weight were used as controls (Normal). Two swine assigned to the DM+HC+CKD group died prematurely. One animal died two weeks after CKD induction and one animal died during chronic instrumentation due to surgical complications. In the Normal group, also two swine were lost. One animal died during chronic instrumentation, while another animal experienced severe lameness prior to chronic instrumentation and was excluded from further study. Four additional swine (2 Normal and 2 DM+HC+CKD) were included for measurement of coronary flow reserve (CFR) *in vivo* and small artery function *ex vivo*. An overview of the experimental design and technical procedures is presented in **Figure 1**.

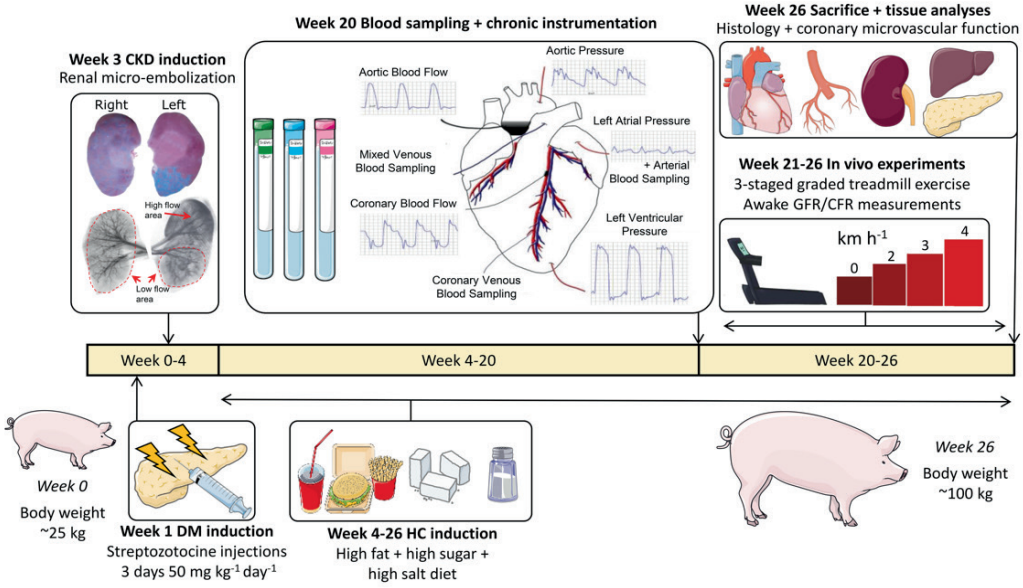
Induction of comorbidities

The induction of comorbidities in the DM+HC+CKD group has been described in detail elsewhere.[16] Briefly, DM was produced by injection of streptozotocin (Bio-connect B.V., Huissen, The Netherlands) in a dose of 50 mg kg^{-1} day^{-1} i.v. on 3 consecutive days. The severity and stability of DM was monitored bi-weekly by measurement of blood glucose and ketone levels.

Two weeks after DM induction, animals were sedated with intramuscular injection of a cocktail of Zoletil (tiletamine/zolazepam; 5 mg kg^{-1}), Sedazine (xylazine; 2.25 mg kg^{-1}) and atropine (2 mg) and artificially ventilated (O_2 and N_2 [1:2 vol/vol], to which 1–2% (vol/vol) isoflurane was added for anesthesia). CKD was produced by micro-embolization of the global right kidney as well as the lower pole of the left kidney. For this purpose, the renal arteries were catheterized under fluoroscopy guidance (right renal artery and selective catheterization of the artery perfusing the left lower renal pole) with a Swan-Ganz catheter, inserted through a 9F sheath in the right common carotid artery. Following inflation of the balloon to prevent back-flow into the aorta, 75 mg of polyethylene microspheres with a diameter of 38-42 µm (Cospheric, Santa Barbara, CA, USA) were infused in each kidney. The wound was closed and the animals were allowed to recover.

One week after CKD induction, a high fat and high sugar diet containing 10% sucrose, 15% fructose, 25% saturated fats and 1% cholesterol (Research Diets Services BV, Wijk bij Duurstede, The Netherlands) supplemented with sodium chloride (20 g day^{-1}) was gradually introduced. The Normal group continued to receive regular swine-chow. Animals were housed in pairs but were fed separately and had *ad libitum* access to drinking water.

Figure 1



Experimental timeline of the DM + HC + CKD swine from induction of the comorbidities to the termination. High-fat + high-sugar +high-salt diet was composed of 10% sucrose, 15% fructose, 25% saturated fats, and 1% cholesterol supplemented with sodium chloride (20 g day^{-1}). Normal swine were weight- and age-matched to the DM + HC + CKD swine, were fed normal chow without receiving the induction of any comorbidities, and underwent chronic instrumentation, in vivo experiments and termination according to a similar protocol as DM + HC + CKD swine. DM diabetes mellitus, CKD chronic kidney disease, HC hypercholesterolemia, GFR glomerular filtration rate, CFR coronary flow reserve

Chronic Instrumentation

After an overnight fast, Normal swine and DM+HC+CKD swine (5 months after CKD induction) were sedated with an intramuscular injection of a cocktail of Zoletil (tiletamine/zolazepam; 5 mg kg^{-1}), Sedazine (xylazine; 2.25 mg kg^{-1}) and atropine (2 mg), and artificially ventilated (O_2 and N_2 [1:2; vol/vol]), to which 2–2.5% (vol/vol) isoflurane was added. As described in detail elsewhere[19], a thoracotomy was performed in the fourth left intercostal space under sterile conditions and fluid-filled polyvinylchloride catheters were placed in the left ventricle, aorta, pulmonary artery and left atrium for pressure measurements and blood sampling. Additionally, two flow probes (Transonic Systems, Ithaca, NY) were placed, one around the aorta for cardiac output measurement, and one around the proximal left anterior descending coronary artery for measurement of coronary blood flow. Finally, two small angio-catheters (one as back-up) were inserted into the anterior inter-ventricular vein for coronary venous blood sampling. In one Normal and in one DM+HC+CKD animal the coronary venous angio-catheters lost patency prior to the exercise study. Electrical wires and catheters were tunneled subcutaneously to exit at the back and protected with a vest. Then, the chest was closed and animals were allowed to recover, receiving analgesia (0.3 mg buprenorphine i.m.) and a slow-release fentanyl patch (50 µg h^{-1}) for 6 days, and antibiotic prophylaxis (25 mg kg^{-1} amoxicillin i.v.) for 7 days. All catheters were flushed daily with heparinized saline (1,000–5,000 IU ml^{-1} saline) to prevent the formation of blood clots and to maintain catheter patency. One DM+HC+CKD swine had a malfunctioning coronary flow probe.

In Vivo Experiments in Awake Swine

Experiments started one week after surgery. First, the glomerular filtration rate (GFR) was measured at rest, using continuous inulin infusion [16]. On a subsequent day, myocardial perfusion and function at rest and during exercise were assessed using a motor-driven treadmill exercise protocol. Briefly, resting hemodynamic measurements, blood samples, and rectal temperature were obtained with swine standing quietly on the treadmill. Then, swine were subjected to a three-stage incremental treadmill exercise protocol (2, 3 and 4 km h^{-1} at 0% inclination, 3 min per stage). Hemodynamic variables were continuously recorded digitally on a Codas workstation (ATCODAS, Dataq Instruments, Akron, OH) with blood samples collected during the final 30 s of each exercise stage when steady-state hemodynamics had been achieved. Blood samples were analyzed for pO_2, pCO_2, pH, bicarbonate, O_2 saturation, and hemoglobin concentration (ABL-800, Radiometer, Copenhagen).

Coronary blood flow was measured in awake resting swine (4 Normal and 4 DM+HC+CKD) under basal conditions and during maximal coronary vasodilation using intravenous infusion of adenosine (0.5 mg kg^{-1} min^{-1}) in combination with phenylephrine (5-7.5 µg kg^{-1} min^{-1}) to maintain mean arterial pressure at baseline levels.[6] CFR was calculated as maximal coronary blood flow divided by basal coronary blood flow.

Sacrifice

At sacrifice, swine were sedated by intramuscular injection with a cocktail of Zoletil (tiletamine/zolazepam; 5 mg kg^{-1}), Sedazine (xylazine; 2.25 mg kg^{-1}) and atropine (2 mg) and anesthetized with pentobarbital (9 mg kg^{-1} h^{-1} i.v.). Subsequently, a sternotomy was performed and ventricular fibrillation was induced using a 9 V battery, and immediately the heart, kidneys, liver and pancreas were excised, weighed and prepared and stored for later biochemical, molecular and histological analyses.

In vitro coronary small artery function

In a subgroup of animals (3 Normal and 3 DM+HC+CKD), coronary small arteries (~300 µm diameter) were isolated from the epicardial surface of the left ventricular apex and studied *in vitro* using a Mulvany wire myograph (DMT, Aarhus, Denmark). Vasodilation to $3x10^{-9}$, 10^{-8} and $3x10^{-8}$ mol L^{-1} of the endothelium-dependent vasodilator bradykinin (BK, Sigma–Aldrich, Zwijndrecht, The Netherlands) and to 10^{-7}, $3x10^{-7}$ and 10^{-6} mol L^{-1} of the NO-donor, S-nitroso-N-acetylpenicillamine (SNAP, Sigma–Aldrich) were measured following preconstriction with 10^{-6} mol L^{-1} of the thromboxane-A2 analogue U46619 (Sigma–Aldrich).

Plasma and Tissue Analyses

Fasting arterial blood samples were obtained at the time of instrumentation and stored at -80 °C, for later determination of plasma glucose, triglycerides, total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), albumin, sodium and creatinine, as previously described.[16] Arterial plasma concentrations of tumor necrosis factor alpha (TNF-α, R&D Systems Europe Ltd., Abingdon, UK), neutrophil gelatinase-

associated lipocalin (Pig NGAL, BioPorto Diagnostics A/S, Hellerup, Denmark) and fasting insulin (Porcine Insulin, Mercodia AB, Uppsala, Sweden) were determined using ELISA kits.

Samples of the pancreas, liver, kidney, left anterior descending artery, right coronary artery, left circumflex artery and left ventricular anterior free wall were excised, cryo-embedded in Tissue-Tek or fixated in 4% buffered formaldehyde and embedded in paraffin for histological analyses. 4.5 µm thick slides of the pancreas were stained for insulin (FLEX polyclonal anti-insulin, Agilent Technologies, Santa Clara, CA). Six to eight fields at 20x magnification were analyzed and data were averaged per animal. Cryosections of the liver (4 µm thick) were stained with oil red O (Oil Red O, Sigma-Aldrich) for quantification of liver fat deposition. Five to seven liver lobes were analyzed and data were averaged per animal.

From formaldehyde-fixed, paraffin-embedded kidneys, 3 µm sections were sliced and stained. For the analysis, the upper pole of the left kidney, that was not embolized, was used. Tubulo-interstitial damage and glomerulosclerosis were scored on Periodic-Acid Schiff stained sections in a blinded manner. The tubulo-interstitial damage was scored in at least 20 different non-overlapping fields per animal at a magnification of 200x. The amount of inflammatory infiltrate between tubuli, interstitial fibrosis, tubular atrophy and dilatation were scored on a scale of 0-5, with 0 indicating not present, and 5 indicating that >75% of all tubuli were affected. A total TI damage score was calculated by summing the scores for the four variables. Glomerular score was scored at a magnification of 400x on 50 separate glomeruli by quadrants, with 0 indicating that no quadrant was affected and 4 indicating that the whole glomerulus was affected. Scored variables were matrix expansion, sclerosis, adhesion of Bowman's capsule and dilation, from which a total glomerulosclerosis score was then calculated. Glomerular volume was assessed using a computerized image analysis system consisting of a high-resolution digital camera (Leica ICC50 W) attached to a microscope (Leica DM750). All renal histological analyses were performed by observers blinded to the treatment of the animal.

Left ventricular anterior wall sections (4.5 µm thick) were stained for quantification of myocardial collagen deposition, myocyte size and capillary density. Six to eight fields were examined in the endocardial part of each slide, at 20× magnification. Collagen deposition was assessed using picrosirius red staining, with interstitial and perivascular collagen deposition being analyzed separately. Using light microscopy, interstitial collagen was measured as area occupied by all collagen fibers and expressed as a percentage of the myocardial area, perivascular collagen being excluded from this analysis. Using a linear polarization filter, the percentage area of the myocardium occupied specifically by collagen type I and III fibers was measured.[20] Perivascular collagen deposition was measured for all coronary arterioles (diameter 10-100µm) encountered in the left ventricular section

at 20x magnification (± 150mm^2) and expressed as a percentage of the perivascular area. The latter was defined as the area delineated by two times the distance from the lumen center to the external elastic lamina in all directions. Cross-sectional areas of cardiomyocytes with clearly visible nuclei were measured for each slide, using a Gomori silver stain. Capillary density was quantified using an endothelial cell staining with biotin-labeled lectin (lectin 1/100 in 1% bovine serum albumin in PBS, Sigma-Aldrich). All vessels smaller than 10 µm in diameter and without vascular smooth muscle cells were counted. Capillary to fiber ratio was calculated by dividing capillary density by the total number of cardiomyocytes per mm^2.

Right coronary artery, left circumflex artery, left anterior descending artery and left ventricular sections were stained with resorcin-fuchsin solution to assess media-to-lumen ratios of large coronary arteries and coronary arterioles (10-100µm inner diameter). Smooth muscle actin was stained (EnVision G|2 doublestain rabbit/mouse, Agilent Technologies, Santa Clara, CA, USA) in left ventricular sections to determine coronary arteriolar densities, according to an earlier described protocol[21], in short we counted arterioles of 20-100µm inner diameter with >2 smooth muscle layers. All measurements, except for renal histological measurements, were performed using a microscopy image analysis system (Impak C, Clemex Vision Image analysis system, Clemex Technologies, Quebec, Canada).

Data Analysis and Statistics

Data are presented as mean ± SEM. Digital recording and offline analysis of hemodynamic data obtained at rest and during exercise. Body O_2 consumption (BVO_2) was calculated as the product of cardiac output and the difference in O_2 content between arterial and mixed venous blood. Systemic vascular conductance (SVC) was computed by dividing the cardiac output by the mean arterial pressure. Myocardial O_2 and lactate delivery were computed as the product of coronary blood flow and arterial blood O_2 content or arterial lactate concentration. Myocardial O_2 (MVO_2) and lactate consumption were calculated as the product of coronary blood flow and the difference in O_2 content or lactate concentration between arterial and coronary venous blood. Myocardial oxygen extraction was computed as 100% • MVO_2/MDO_2. Myocardial work was computed as the product of cardiac output • systolic arterial pressure. Systemic parameters, including cardiac output, BVO_2, systemic vascular conductance, cardiac work and stroke volume, were normalized for body weight in kilograms (kg). Myocardial parameters, including MVO_2, coronary blood flow, myocardial oxygen delivery and

myocardial lactate consumption were normalized per gram (g) of myocardium perfused by the left anterior descending coronary artery, which was estimated to be 40% of the left ventricle.[22, 23]

Statistical analysis was performed in SPSS Statistics 21.0 (IBM Corp, Armonk, NY). Two-way ANOVA for repeated measures was used to analyze tabular *in vivo* hemodynamic and myocardial oxygen balance responses to graded treadmill exercise as well as the *ex vivo* microvascular function with Bonferroni post-hoc testing when appropriate. A two-way ANCOVA was used to analyze hemodynamic and myocardial oxygen balance data expressed as a function of whole body - or myocardial - oxygen consumption. Comparison of other variables between the two groups was performed by unpaired Students t-test. Correlations were calculated by Pearson's correlation. Statistical significance was accepted when $p<0.05$ (two-tailed).

3. Results

Model characteristics

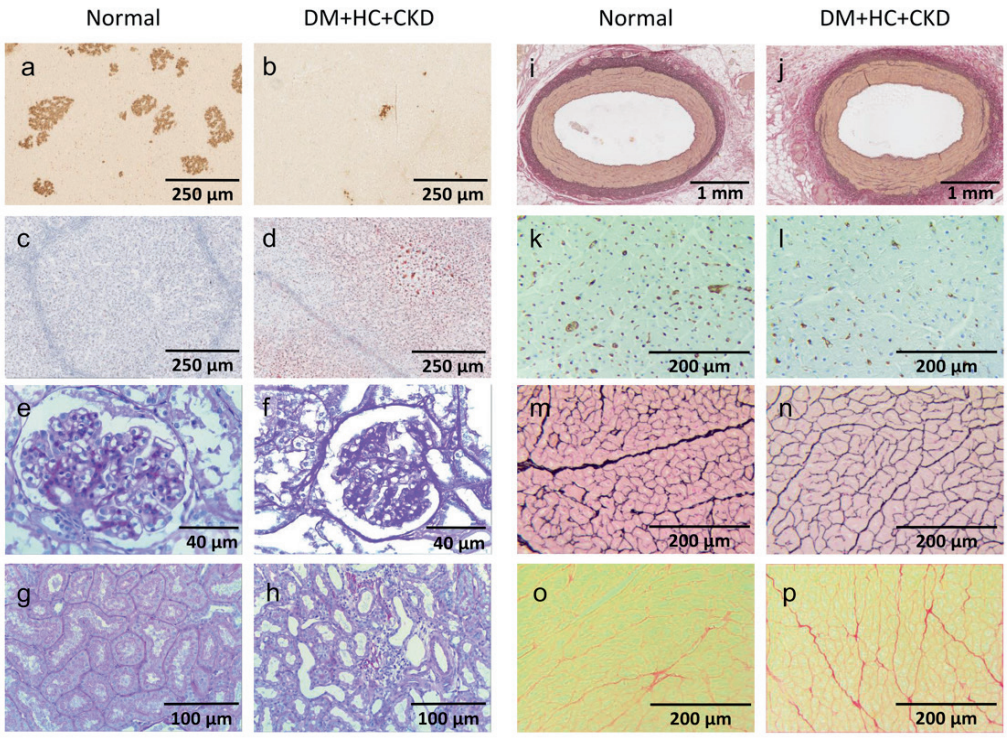
Metabolic, renal, inflammatory, and cardiac characteristics of the DM+HC+CKD swine model at 5 months follow-up are presented in **Table 1**, while representative histological sections of pancreas, liver, kidney, left anterior descending coronary artery and left ventricular anterior wall are shown in **Figure 2**. Metabolic dysfunction was present in DM+HC+CKD swine, with markedly elevated levels of plasma glucose, total cholesterol and LDL/HDL ratio, and similar triglyceride levels ($p=0.10$) as compared to healthy Normal swine. Pancreas staining demonstrated an ~80% reduction of the insulin-producing ß-cells in the islets of Langerhans of the pancreas in DM+HC+CKD swine, but insulin plasma levels were maintained. There was no difference in ASAT plasma levels between groups, while ALAT levels even decreased in DM+HC+CKD compared to Normal swine. In addition, DM+HC+CKD showed

Table 1: Metabolic, renal, inflammatory and myocardial characteristics of Normal and DM+HC+CKD swine

	n[a]	n[b]	Normal	DM+HC+CKD
Body weight (kg)	10	9	93 ± 6	104 ± 6
Metabolic function				
Plasma fasting glucose (mmol L^{-1})	10	9	8.4 ± 0.8	19.2 ± 1.5*
Plasma insulin (µg L^{-1})	9	9	0.08 ± 0.03	0.26 ± 0.16
HOMA-IR	8	9	1.13 ± 0.60	7.36 ± 4.61
β-cells islets of Langerhans (%)	6	7	100 ± 12	17 ± 4*
Plasma total cholesterol (mmol L^{-1})	10	9	1.72 ± 0.08	8.28 ± 0.86*
LDL/HDL cholesterol ratio	10	9	1.19 ± 0.08	3.51 ± 0.57*
Plasma triglycerides (mmol L^{-1})	10	9	0.20 ± 0.03	0.29 ± 0.05
Plasma ALAT (U L^{-1})	10	9	52 ± 4	24 ± 4*
Plasma ASAT (U L^{-1})	10	9	32 ± 7	23 ± 3
Liver steatosis (%)	6	7	0.04 ± 0.02	1.41 ± 0.80
Renal function and structure				
Plasma creatinine (µmol L^{-1})	10	9	122 ± 4	170 ± 11*
Glomerular filtration rate (ml min^{-1})[c]	7	7	197 ± 10	132 ± 14*
Plasma sodium (mmol L^{-1})	10	9	141 ± 1	134 ± 1*
Plasma albumin (g L^{-1})	10	9	40 ± 1	31 ± 2*
Plasma NGAL (ng ml^{-1})	6	9	128 ± 16	164 ± 16
Right kidney weight (g)	10	9	200 ± 14	184 ± 20
Left kidney weight (g)	10	9	207 ± 11	177 ± 22
Right kidney weight/BW (g kg^{-1})	10	9	1.96 ± 0.15	1.80 ± 0.13
Left kidney weight/BW (g kg^{-1})	10	9	2.06 ± 0.13	1.75 ± 0.14
Tubulo-interstitial injury score	6	5	2.1 ± 0.4	4.3 ± 0.6*
Glomerular sclerosis score	6	5	18.2 ± 3.0	32.4 ± 3.7*
Inflammation				
TNF-α (pg ml^{-1})	8	9	25 ± 5	52 ± 5*
Cardiac structure				
Left ventricular weight (g)	10	9	277 ± 12	251 ± 16
Left ventricular weight/BW (g kg^{-1})	10	9	2.9 ± 0.2	2.4 ± 0.2
Cardiomyocyte CSA (μm^2)	10	7	582 ± 44	663 ± 110
Capillary density (# mm^{-2})	10	7	1921 ± 157	1381 ± 172*
Capillary to fiber ratio	10	7	1.33 ± 0.17	0.84 ± 0.09*
Total Collagen (% of myocardium)	10	7	5.7 ± 0.7	9.6 ± 1.9*
Large coronary structure				
LAD media-to-lumen ratio	10	7	0.55 ± 0.05	0.63 ± 0.11
LCX media-to-lumen ratio	7	6	0.70 ± 0.09	0.57 ± 0.10
RCA media-to-lumen ratio	9	7	0.72 ± 0.10	0.78 ± 0.10

[a]n number of animals analyzed in Normal group, [b]n number of animals analyzed in the DM+HC+CKD group, [c]GFR obtained in awake animals, 1 week after surgery. Plasma samples were obtained under anesthetized basal conditions after onset of surgery. HOMA-IR homeostatic model assessment-insulin resistance, ALAT alanine aminotransferase, ASAT aspartate aminotransferase, NGAL neutrophil gelatinase-associated lipocalin, BW body weight, TNF- α tumor necrosis factor alpha, CSA cross sectional area, LAD left anterior descending artery, LCX left circumflex artery, RCA right coronary artery. Data are mean±SEM. *p<0.05 Normal vs DM+HC+CKD.

Figure 2



Representative histological sections of pancreas, liver, kidney, coronary artery and left ventricle of Normal and DM+HC+CKD swine. Representative insulin stained pancreas sections (**a** and **b**), Oil Red O stained liver sections (**c** and **d**) at 100x magnification, scale bar=250µm. Periodic acid Schiff stained sections of a glomerulus (**e** and **f**) at 400x magnification, scale bar=40µm and tubuli (**g** and **h**) from the top part of the left kidney at 200x magnification, scale bar=100µm. Representative resorcin-fuchsin stained sections of the left anterior descending artery (**i** and **j**) at 50x magnification, scale bar=1mm. Representative sections of the endocardium of the left ventricle, lectine stained (**k** and **l**), Gomori stained (**m** and **n**), picrosirius red stained (**o** and **p**) at 200x magnification, scale bar=200µm.

a trend towards an increase in liver fat deposition (p=0.09), which correlated positively with plasma levels of total cholesterol (r^2=0.519, p<0.05) and correlated inversely with ASAT (r^2=0.466, p<0.05). Although kidney weights were not different from Normal swine, renal dysfunction was present in DM+HC+CKD swine reflected in increased creatinine plasma levels and a significantly impaired glomerular filtration rate (GFR) as measured by inulin clearance, increased histological scores of tubulo-interstitial injury and glomerular sclerosis. Metabolic and renal dysfunction resulted in elevated TNF-α plasma levels. Absolute and relative left ventricular weights were not different between groups. Similarly, cardiomyocyte cross-sectional area showed no significant differences

between groups. In contrast, total collagen deposition (collagen type I and III) was elevated in DM+HC+CKD compared to Normal swine. Interestingly, this elevation was due to deposition of collagen type I fibers, known to form thick and stiff bundles, in DM+HC+CKD compared to Normal while deposition of the compliant thin collagen fibers (collagen type III) was unaltered (Supplemental Figure). Furthermore, significant decreases in left ventricular subendocardial capillary density as well as capillary-to-fiber ratios were observed in DM+HC+CKD swine. Macroscopic and microscopic examination of the large coronary arteries, showed no signs of atherosclerosis and no changes in media-to-lumen ratio in DM+HC+CKD swine.

Systemic Hemodynamics and Metabolism

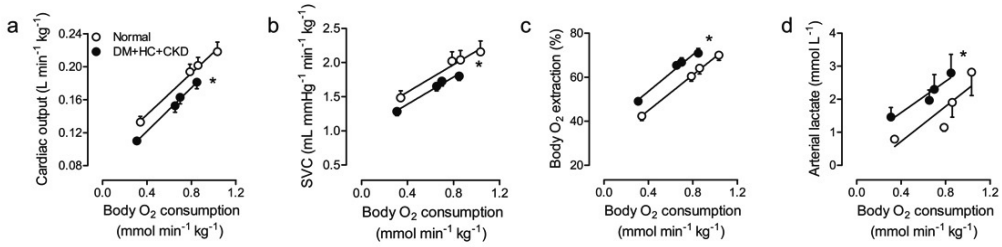
The impact of comorbidities on systemic hemodynamics and metabolism was assessed at rest and during graded treadmill exercise (**Table 2**). Although heart rates were similar at rest, the exercise-induced increase in heart rate was blunted in DM+HC+CKD compared to Normal swine, suggestive of chronotropic incompetence. Stroke volume was lower in DM+HC+CKD swine at rest (**Table 2**). These alterations in cardiac function resulted in a lower cardiac output in DM+HC+CKD compared to Normal both at rest and during exercise (**Table 2, Figure 3a**). Although renal embolization resulted in an acute increase in blood pressure, which we previously showed to be sustained for at least 2 months[16], hypertension was no longer present at 6 months after renal embolization. The normalization of mean aortic blood pressure was likely the result of the reduction in cardiac output, as systemic vascular conductance was still lower in DM+HC+CKD swine (**Figure 3b**). The lower cardiac output during exercise was only partly compensated for by an increase in body oxygen extraction (**Figure 3c**). Consequently, the exercise-induced increase in BVO_2 was blunted in DM+HC+CKD animals, which was associated with increased circulating levels of lactate (**Figure 3d**), indicative of systemic anaerobic metabolism and hence exercise intolerance. Both arterial pCO_2 and bicarbonate levels (arterial HCO_3^- at rest DM+HC+CKD 23.6±0.7 vs. Normal 26.7±0.3 mmol L^{-1}, p=0.01) were lower in DM+HC+CKD swine at rest and during low intensity exercise, while arterial pH was not different between groups (**Table 3**), suggesting full respiratory compensation of metabolic acidosis.

Table 2 Systemic hemodynamics and metabolism of Normal and DM+HC+CKD swine at rest and during treadmill exercise

			Rest	Exercise (km h^{-1})		
		n		2	3	
Heart rate (beats min^{-1})	Normal	10	122 ± 3	184 ± 11*	184 ± 10*	232
	DM+HC+CKD	8	117 ± 4	164 ± 9*	177 ± 11*†	197
Cardiac index (ml min^{-1} kg^{-1})	Normal	10	127 ± 6	188 ± 7*	195 ± 8*	212
	DM+HC+CKD	8	107 ± 4†	150 ± 7*†	160 ± 7*†	150
Stroke volume (ml kg^{-1})	Normal	10	1.07 ± 0.05	1.04 ± 0.05	0.96 ± 0.06*	0.93
	DM+HC+CKD	8	0.92 ± 0.03†	0.92 ± 0.03	0.92 ± 0.03	0.89
$LVdP/dt_{max}$ (mmHg s^{-1})	Normal	4	3390 ± 230	4660 ± 660	4970 ± 760	5310
	DM+HC+CKD	7	2990 ± 370	3800 ± 490*	4060 ± 610*	4070
$LVdP/dt_{min}$ (mmHg s^{-1})	Normal	4	-2330 ± 180	-2740 ± 210	-3050 ± 250	-3450
	DM+HC+CKD	7	-2360 ± 200	-2770 ± 200*	-2910 ± 220	-2950
mLAP (mmHg)	Normal	9	6 ± 1	13 ± 2*	15 ± 1*	18
	DM+HC+CKD	7	5 ± 1	9 ± 1*	11 ± 2*†	13
MAP (mmHg)	Normal	10	89 ± 2	95 ± 3*	98 ± 3*	100
	DM+HC+CKD	8	87 ± 2	94 ± 3*	95 ± 4*	96
SVC (ml $mmHg^{-1}$ min^{-1} kg^{-1})	Normal	10	1.49 ± 0.10	2.02 ± 0.14*	2.04 ± 0.14*	2.16
	DM+HC+CKD	8	1.27 ± 0.06	1.64 ± 0.07*†	1.72 ± 0.07*	1.84
Hemoglobin (mmol L^{-1})	Normal	10	9.7 ± 0.4	10.9 ± 0.5*	10.9 ± 0.5*	11.2
	DM+HC+CKD	8	9.5 ± 0.4	10.8 ± 0.5*	11.0 ± 0.3*	10.8
Arterial SaO_2 (%)	Normal	10	98 ± 1	98 ± 1	97 ± 1	97
	DM+HC+CKD	8	98 ± 1	97 ± 1	98 ± 1	98
Mixed venous SaO_2 (%)	Normal	10	57 ± 2	39 ± 2*	35 ± 3*	29
	DM+HC+CKD	8	50 ± 1†	34 ± 1*	33 ± 2*	28
BVO_2 (mmol min^{-1} kg^{-1})	Normal	10	0.33 ± 0.03	0.77 ± 0.05*	0.84 ± 0.07*	1.01
	DM+HC+CKD	8	0.31 ± 0.02	0.66 ± 0.05*	0.73 ± 0.07*	0.80

$LVdP/dt_{max}$ maximum rate of rise of left ventricular pressure, $LVdP/dt_{min}$ maximum rate of fall of left ventricular pressure, mLAP mean left atr
MAP mean arterial pressure, SVC systemic vascular conductance, SaO_2 oxygen saturation, BVO_2 body oxygen consumption. Values are mean±SI
versus rest within group; †p<0.05 versus corresponding Normal.

Figure 3



Systemic exercise response in DM + HC + CKD and Normal swine. DM + HC + CKD swine show a lower cardiac output (**a**), a lower systemic vascular conductance (SVC **b**), a higher body oxygen (O_2) extraction (**c**), and higher arterial lactate levels (**d**) for the same level of body oxygen consumption as compared to Normal. Data are mean ± SEM. DM + HC + CKD $n = 8$ and Normal $n = 10$. *$p < 0.05$ DM + HC + CKD versus Normal by repeated measures two-way ANCOVA.

Table 3 Myocardial metabolism of DM+HC+CKD and Normal swine at rest and during treadmill exercise

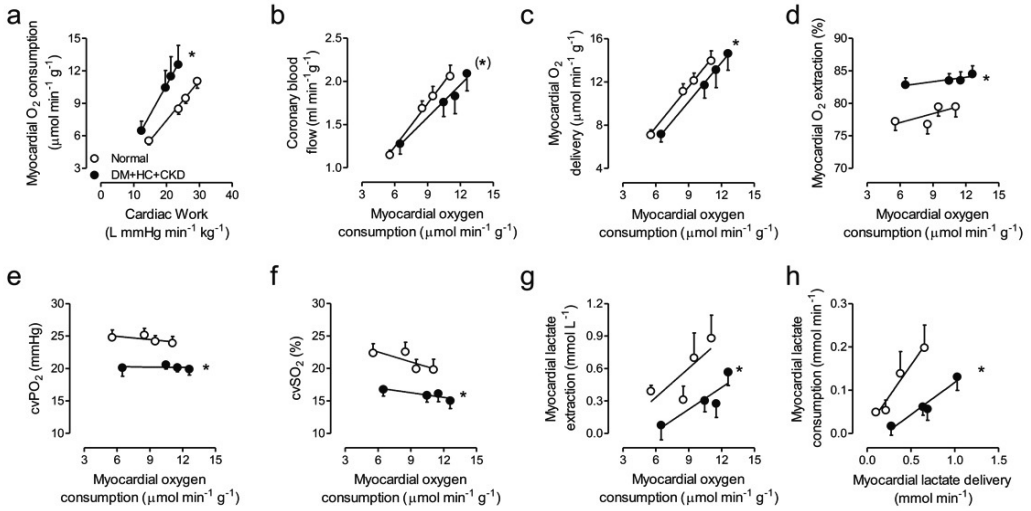
			Rest	Exercise (km h^{-1})		
		n		2	3	4
Art SaO_2 (%)	Normal	9	98 ± 1	98 ± 1	97 ± 1	97 ± 1
	DM+HC+CKD	8	98 ± 1	97 ± 1	99 ± 1	98 ± 1
CV SaO_2 (%)	Normal	9	22 ± 1	23 ± 1	20 ± 1	20 ± 2
	DM+HC+CKD	8	17 ± 1†	16 ± 1†	16 ± 1	15 ± 1†
Art pO_2 (mmHg)	Normal	9	109 ± 4	102 ± 5*	101 ± 5*	99 ± 4*
	DM+HC+CKD	8	110 ± 3	101 ± 3*	108 ± 5	107 ± 5
CV pO_2 (mmHg)	Normal	9	24 ± 1	25 ± 1	24 ± 1	24 ± 1
	DM+HC+CKD	8	20 ± 1†	21 ± 1†	21 ± 1†	20 ± 1†
Art lactate (mmol L^{-1})	Normal	9	0.76 ± 0.08	1.12 ± 0.12*	1.89 ± 0.45*	2.83 ± 0.70*
	DM+HC+CKD	8	1.84 ± 0.50†	2.56 ± 0.74	2.93 ± 0.88	3.85 ± 1.39
CV lactate (mmol L^{-1})	Normal	9	0.36 ± 0.04	0.81 ± 0.23	1.19 ± 0.32	1.95 ± 0.54*
	DM+HC+CKD	8	1.76 ± 0.49†	1.98 ± 0.63	2.65 ± 0.96	3.28 ± 1.44
Art pH	Normal	9	7.44 ± 0.01	7.46 ± 0.01*	7.46 ± 0.01*	7.46 ± 0.01*
	DM+HC+CKD	8	7.43 ± 0.01	7.46 ± 0.01*	7.47 ± 0.01*	7.47 ± 0.01*
CV pH	Normal	9	7.36 ± 0.01	7.35 ± 0.01	7.35 ± 0.01	7.33 ± 0.01
	DM+HC+CKD	8	7.36 ± 0.01	7.38 ± 0.01	7.38 ± 0.02†	7.36 ± 0.02
Art pCO_2 (mmHg)	Normal	9	39 ± 1	37 ± 1*	36 ± 1*	35 ± 1*
	DM+HC+CKD	8	36 ± 1†	34 ± 1†	31 ± 1*†	30 ± 1*†
CV pCO_2 (mmHg)	Normal	9	51 ± 1	50 ± 2	50 ± 2	48 ± 2
	DM+HC+CKD	8	46 ± 1†	44 ± 1†	42 ± 1*†	44 ± 2*
MVO_2 (µmol min^{-1} g^{-1})	Normal	9	5.6 ± 0.4	8.5 ± 0.5*	9.5 ± 0.5*	11.1 ± 0.7*
	DM+HC+CKD	7	6.5 ± 0.9	10.5 ± 1.6*	11.5 ± 1.6*	12.6 ± 1.8*

Art arterial, SaO_2 oxygen saturation, CV coronary venous, pO_2 partial pressure of oxygen, pCO_2 partial pressure of carbon dioxide, MVO_2 myocardial oxygen consumption per gram of myocardium. Values are mean±SEM. *p<0.05 versus rest within group; †p<0.05 versus corresponding Normal.

Myocardial Oxygen Balance, Perfusion and Metabolism

Compared to normal swine, DM+HC+CKD swine required higher levels of MVO_2 for each level of cardiac work, particularly during exercise, reflecting decreased myocardial efficiency (**Figure 4a**). The higher levels of MVO_2 in DM+HC+CKD swine were not fully met by commensurate increases in coronary blood flow (**Figure 4b**) and MDO_2 (**Figure 4c**), necessitating an increase in myocardial oxygen extraction (**Figure 4d**), that resulted in reductions in coronary venous pO_2 (**Figure 4e**) and coronary venous SaO_2 (**Figure 4f**) in DM+HC+CKD compared to Normal swine, both at rest and during exercise. Consistent with the impaired MDO_2 during exercise, a decrease in myocardial lactate extraction for a given level of myocardial oxygen consumption (**Figure 4g**) or myocardial lactate consumption for a given level of myocardial lactate delivery (**Figure 4h**) was observed in DM+HC+CKD swine compared to Normal, suggestive of anaerobic metabolism.

Figure 4

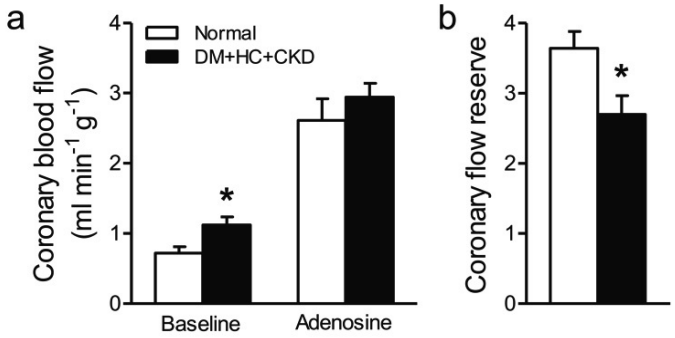


Myocardial blood flow and oxygen balance in DM + HC + CKD and Normal swine at rest and during graded treadmill exercise. Myocardium of DM + HC + CKD swine shows increased oxygen consumption for the same level of cardiac work (**a**), a trend towards impaired coronary blood flow especially during exercise (**b**), have a lower myocardial oxygen delivery (**c**), and a higher oxygen extraction (**d**), which results in lower coronary venous oxygen pressure ($cvPO_2$ **e**) and coronary venous oxygen saturation ($cvSaO_2$ **f**). Lower myocardial lactate extraction (**g**) and lower myocardial lactate consumption for a given level of myocardial lactate delivery (**h**) were measured in DM + HC + CKD as compared to Normal animals. Data are mean ± SEM. DM + HC + CKD: n = 7–8, Normal: n = 9. *$p < 0.05$ DM + HC + CKD versus Normal, (*)$p < 0.1$ DM + HC + CKD versus Normal by repeated measures two-way ANCOVA.

Coronary Flow Reserve, Structure and Endothelial Function

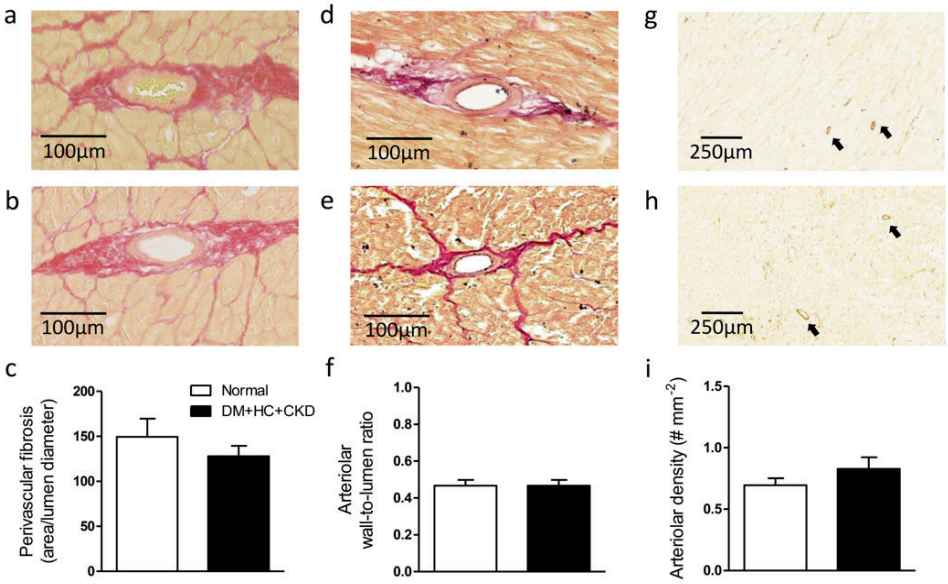
Consistent with an impaired recruitment of vasodilator reserve during exercise in DM+HC+CKD, CFR was reduced by 25% from 3.64±0.24 in Normal to 2.69±0.27 in DM+HC+CKD swine (p=0.038), which appeared principally due to a small increase in basal flow while maximal flow was unaltered (**Figure 5**). The latter was consistent with the lack of alterations in morphology or density of left ventricular small arterioles, evidenced by similar perivascular collagen content (**Figure 6a-c**), media-to-lumen ratios (**Figure 6d-f**) and arteriolar densities (**Figure 6g-i**) between groups. Interestingly, coronary microvascular function measurements *in vitro* confirmed coronary microvascular endothelial dysfunction as vasodilation to bradykinin was blunted in DM+HC+CKD compared to Normal (**Figure 7a),** while vascular smooth muscle cell function was maintained (**Figure 7b**).

Figure 5



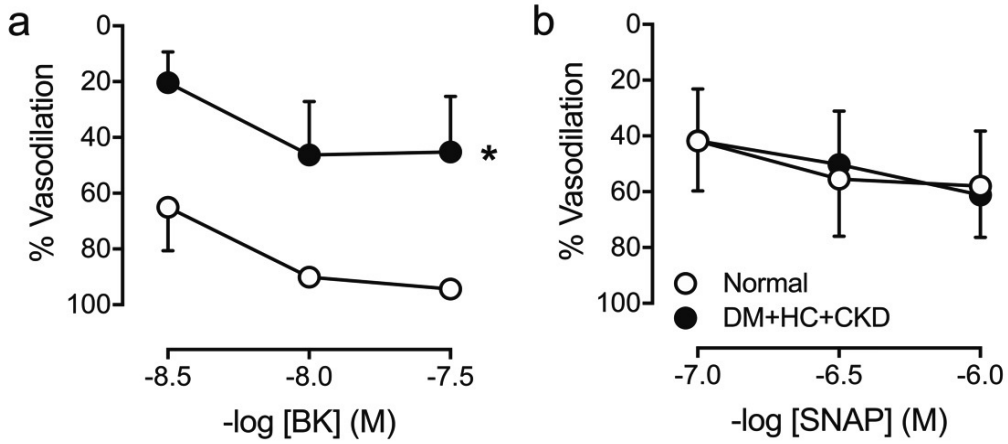
Coronary blood flow during baseline conditions and during maximal vasodilation to adenosine (**a**) resulting in a decrease in coronary flow reserve in DM + HC + CKD (**b**) compared to Normal swine at rest and awake state. Data are mean ± SEM. DM + HC + CKD: $n = 4$, Normal: $n = 4$. *$p < 0.05$ DM + HC + CKD versus Normal by unpaired t test.

Figure 6



Typical examples of small arterioles (< 100 µm) stained with picrosirius red staining of Normal (**a**) and DM + HC + CKD (**b**) and perivascular fibrosis quantification as area collagen corrected for lumen diameter (**c**). Typical examples of small arterioles (< 100 µm) stained with resorcin–fuchsin of Normal (**d**) and DM + HC + CKD (**e**) and media thickness corrected for lumen diameter (**f**). Typical examples of small arterioles stained for smooth muscle actin of Normal (**g**) and DM + HC + CKD (**h**) and quantification of coronary arteriolar density (< 100 µm, **i**). Normal $n = 10$, DM + HC + CKD $n = 7$. Data are mean ± SEM.

Figure 7

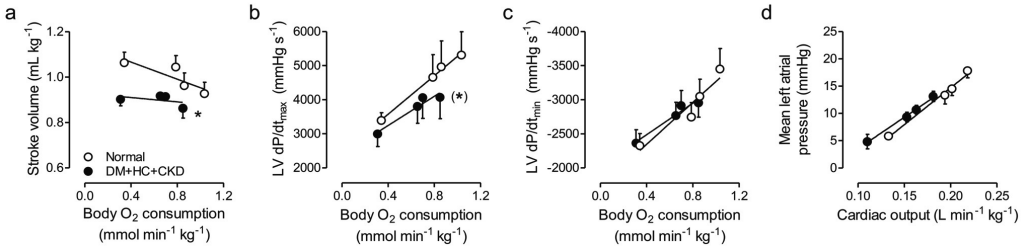


Ex vivo endothelium-dependent and endothelium-independent vasoreactivity of coronary microvessels. Small coronary arteries (~ 300 μm) of DM + HC + CKD have impaired bradykinin (BK)-induced vasodilation, suggesting endothelial dysfunction **(a**), while endothelium-independent vasodilation to nitric oxide donor sodium nitroprusside (SNAP) was unaltered, indicating maintained vascular smooth muscle cell function (**b**). Normal $n = 3$ and DM + HC + CKD $n = 3$. Data are mean ± SEM. Error bars are presented but might be too small to be visible. $*p < 0.05$ DM + HC + CKD versus Normal by repeated measures two-way ANOVA.

Left Ventricular Function

The perturbations in myocardial oxygen balance in DM+HC+CKD swine were associated with a lower stroke volume, both at rest and during exercise (Figure 8a), as well as a trend towards a lower $LVdP/dt_{max}$ during exercise (Figure 8b). No differences were observed in $LVdP/dt_{min}$ (Figure 8c) or left atrial pressure (Figure 8d), either at rest or during exercise, between the two groups.

Figure 8



Left ventricular function in DM + HC + CKD and Normal swine at rest and during graded treadmill exercise. DM + HC + CKD swine have a lower stroke volume (**a**), a trend towards an impaired left ventricular systolic function ($LVdP/dt_{max}$, **b**), similar left ventricular diastolic function ($LVdP/dt_{min}$, **c**) for the identical body oxygen consumption levels, and similar mean left atrial pressures for the same level of cardiac output (**d**) compared to Normal. Stroke volume, mean left atrial pressure and cardiac output measurements: DM + HC + CKD $n = 8$ and Normal $n = 10$; $LVdP/dt_{max}$ and $LVdP/dt_{min}$: DM + HC + CKD $n = 7$ Normal $n = 4$. Data are mean ± SEM. $^*p < 0.05$ DM + HC + CKD versus Normal, $(^*)p = 0.053$ DM + HC + CKD versus Normal by repeated measures two-way ANCOVA

4. Discussion

The present study tested the hypothesis that prolonged exposure to comorbidities results in perturbations in myocardial blood flow and oxygen delivery, leading to a shift towards anaerobic metabolism and cardiac dysfunction in exercising swine. The main findings were that (i) the combination of diabetes mellitus, hypercholesterolemia and chronic kidney disease resulted in lower cardiac output at rest and during exercise, which was accompanied by impaired systemic vasodilatation and increased circulating levels of lactate. (ii) Exposure to these comorbidities resulted in increased levels of oxygen consumption at similar levels of cardiac work, indicating reduced myocardial efficiency. (iii) The comorbidities also resulted in perturbations in myocardial perfusion and oxygen delivery, at a time when coronary atherosclerosis was negligible. (iv) The perturbations in myocardial oxygen balance were associated with lower lactate consumption and reductions in stroke volume and $LVdP/dt_{max}$, suggestive of myocardial ischemia and dysfunction. (v) Adenosine-recruitable coronary flow reserve was reduced, which was due to an increase in basal resting coronary blood flow per gram of myocardium. (vi) In contrast, maximal coronary blood flow per gram of myocardium was not altered, consistent with maintained arteriolar densities and wall/lumen ratios and maintained perivascular collagen content. (vi) Coronary small arteries demonstrated selective blunting of endothelium-dependent vasodilation. The implications of these findings will be discussed.

Coronary Microvascular dysfunction

The presence of risk factors, including DM, HC and CKD, has been associated with CMD and INOCA in both experimental[17, 18, 24] and clinical[10, 11, 25, 26] studies. For example, we previously showed that CMD was already present in swine 2.5 months after induction of diabetes and high fat diet in the absence of coronary atherosclerosis.[18] Furthermore, CMD remained present in swine with 15 months diabetes and hypercholesterolemia with modest non-obstructive atherosclerosis[17], and was also found in a swine model of familial hypercholesterolemia (FH) with moderate (20-60%) coronary plaque burden.[24] Here, we observed coronary microvascular endothelial dysfunction in isolated small arteries studied *ex vivo*, in absence of atherosclerosis which is in line with our previous study.[16] Taken together, these studies indicate that CMD is present well before coronary atherosclerosis occurs and remains present once the process of atherosclerosis advances.

The present study in chronically-instrumented swine demonstrates that comorbidities can cause significant perturbations in myocardial oxygen balance both at rest and particularly during exercise. Thus, DM+HC+CKD animals demonstrated increased myocardial oxygen consumption at a given level of cardiac work, particularly during exercise causing a counterclockwise rotation in the relation between cardiac work and oxygen consumption (**Figure 4A**). This reduced myocardial efficiency in DM+HC+CKD is commonly seen in metabolic disorders, including diabetes and dyslipidemia.[24, 27, 28] The mechanisms underlying the observed myocardial inefficiency in DM+HC+CKD swine were not investigated in the present study, but could be several-fold. First, a myocardial substrate shift towards free fatty acid utilization, leading to a reduced phosphate/oxygen ratio could have contributed to the increased oxygen consumption.[27, 29, 30] Second, and more likely, mitochondrial uncoupling[27, 28, 30, 31], possibly due to oxidative stress[27], could also lead to a decrease in phosphate/oxygen ratio, thereby increasing oxygen consumption at a given level of cardiac work.

Although mitochondrial function was not measured in the present study, a direct link between cardiac mitochondrial dysfunction and microvascular dysfunction in animal models of metabolic disease was recently proposed.[24, 28] In accordance with this concept, we observed a reduction in CFR of approximately 25% in DM+HC+CKD swine, as compared to Normal swine, which was principally due to an increase in basal coronary blood flow as a result of an increase in myocardial oxygen consumption. Strikingly, maximal myocardial blood flow was maintained, which was in accordance with the normal arteriolar morphology and density, and unaltered peri-arteriolar collagen content. Our finding of a reduction in CFR due to an increase in basal coronary flow, rather than a decrease in maximal flow, is in also good agreement with observations in a variety of patient groups. Thus, in INOCA patients with functional CMD[32], in patients with residual CMD after undergoing percutaneous

coronary intervention for obstructive CAD[33], and in patients with diabetes mellitus[34], an increase in basal coronary blood flow per gram of myocardium[32] or increases in basal coronary flow velocity[33, 34], as compared to healthy individuals, appears primarily responsible for the reduction in CFR. Moreover, patients with a reduced CFR and an increased basal blood flow demonstrate an increased cardiovascular mortality risk compared to patients with normal basal coronary blood flow and CFR.[35] Also, among patients with diabetes, women had a lower CFR than men due to higher basal myocardial blood flow.[36] Interestingly, the increase in basal myocardial blood flow correlated with diastolic dysfunction in women, not in men, while CFR did not correlate with diastolic dysfunction in either sex.[36] In light of these observations, it was recently proposed that basal myocardial blood flow could represent a potentially superior marker of CMD in certain settings.[37]

The present study further shows that the basal higher oxygen consumption in DM+HC+CKD was not fully met by a commensurate increase in myocardial oxygen delivery, which necessitated an increase in myocardial oxygen extraction, resulting in lower levels of coronary venous oxygen content. Cardiovascular comorbidities can cause perturbations in myocardial oxygen delivery by affecting the coronary circulation at different levels, including proximal obstructive CAD, distal small artery and arteriolar dysfunction, and alterations in capillary structure and function.[38] In the present study, the increase in oxygen extraction occurred in the absence of coronary atherosclerosis and despite a reduction in capillary density, which acts to reduce oxygen extraction capacity.[38] Moreover, coronary microvascular structure and maximal coronary blood flow per gram of myocardium were maintained. Hence the increased oxygen extraction most likely reflects perturbations in the regulation of resistance tone – likely involving endothelial dysfunction – resulting in impaired myocardial blood flow and oxygen delivery in the face of increased oxygen consumption.[38, 39]

The perturbations in myocardial oxygen delivery were accompanied by a reduction in lactate consumption – particularly during exercise – indicating a shift towards anaerobic metabolism, suggestive of myocardial ischemia.[40] Although the reduction in lactate consumption may in part be caused by a DM-induced reduction in pyruvate dehydrogenase activity[41], in three out of eight DM+HC+CKD swine we observed net lactate production under resting conditions, which can only be explained by anaerobic metabolism.[40] Our observations are consistent with the concept that CMD, in the absence of coronary atherosclerosis, can impair myocardial oxygenation severely enough to produce myocardial ischemia.[3, 11-13, 42, 43] Furthermore, these findings are in agreement with accumulating clinical evidence that myocardial ischemia can also occur in the absence of obstructive CAD, termed INOCA[10, 11, 42-44], indicating that swine with DM+HC+CKD represent a *bona fide* large animal model of INOCA.

Coronary Microvascular Dysfunction and Diastolic Dysfunction

There is increasing evidence that comorbidities such as DM, dyslipidemia and CKD are linked not only to INOCA[2, 3, 10] but also to the development of diastolic dysfunction and heart failure with preserved ejection fraction (HFpEF), involving endothelial dysfunction.[45] It has thus been proposed that INOCA and HFpEF both originate from CMD but that the paracrine effect of endothelial dysfunction is exerted on distinctive cell types, i.e. arteriolar vascular smooth muscle cells in INOCA versus cardiomyocytes in HFpEF, respectively.[46] Although we did not observe perturbations in active left ventricular relaxation, as evidenced by the maintained relation between $LVdP/dt_{min}$ and body O_2 consumption, we observed in our previous study (using the same animal model), an increase in left ventricular passive stiffness evidenced by an increase in the slope of the end-diastolic pressure volume relation, in the presence of a maintained ejection fraction. The present study in the same animal model, shows that not only capillary rarefaction, but also impaired regulation of myocardial perfusion by the coronary resistance vessels occurs, which is associated with endothelial dysfunction.[16] These experimental findings are in agreement with recent clinical studies, demonstrating the coexistence of diastolic dysfunction or HFpEF and microvascular angina.[26, 46, 47] Reduced coronary or myocardial flow reserve, independent of coronary artery stenosis, is considered to be a marker of microvascular endothelial dysfunction and is present in patients with HFpEF or diastolic dysfunction.[47-49] Thus, the common denominator linking HFpEF and INOCA appears to be CMD induced by the comorbidities.[45, 50, 51]

Our DM+HC+CKD swine model represents a model of early diastolic dysfunction (or pre-HFpEF), as left atrial pressures were not elevated either at rest or during exercise. Nevertheless, cardiac output was decreased which was due to a decrease in stroke volume as well as to chronotropic incompetence, i.e. limited capacity of the DM+HC+CKD swine to increase their heart rate during exercise. The latter is a common feature seen in HFpEF patients with CKD[52], as well as diabetic patients[53]. Chronotropic incompetence is thought to be the result of downregulation and/or desensitization of myocardial β-adrenergic receptors due to increased levels of catecholamines.[54] Although the HFpEF phenotype was still mild, advanced CMD was already present, indicating that CMD may precede advanced diastolic dysfunction and HFpEF. This was also suggested by a recent clinical study showing that in patients with a reduced CFR, diastolic function worsens progressively over time and is associated with an increased risk of HFpEF hospitalization.[47] Taken together, these findings are consistent with the concept of CMD being the primary defect that subsequently leads to diastolic dysfunction eventually progressing to overt HFpEF.

Methodological Considerations

Although several large and small animal models (for CMD) have been described, no single animal model perfectly emulates the human disease.[55] In the present study, CMD was induced by prolonged exposure to diabetes, high fat diet and CKD, risk factors commonly observed in patients with INOCA.[10] A type 2-like DM phenotype, with significant hyperglycemia was induced using multiple low dose streptozotocin injections combined with a high fat and high fructose diet, as previously described.[16-18, 55-57] Although disease development differs from the slow-onset DM type 2 in humans, this approach produces sustained hyperglycemia without insulin-dependency and results in progressive insulin resistance.[16-18] Moreover, in conjunction with the high fat and high fructose diet, this experimental approach results in dyslipidemia[16-18, 55], thereby mimicking several features of metabolic dysregulation as observed in the clinical setting.

It is increasingly recognized that CKD is an important risk factor for development of CMD.[58, 59] The exact mechanisms of the detrimental effects of CKD on coronary microvascular function are incompletely understood, but low grade inflammation and increased circulation of uremic toxins are proposed to play a role.[60] In humans, CKD is often the result of local renal inflammation, hypoxia, and loss of glomeruli and tubuli, with subsequent hyperfiltration of healthy regions, resulting in a vicious cycle of progressive kidney damage.[61] Animal models of renovascular hypertension, 5/6 nephrectomy and unilateral ureteric obstruction have been used by other investigators to mimic various aspects of CKD.[62, 63] Here, partial renal microembolization with microspheres was used to induce CKD. This method results in glomerulosclerosis and tubulointerstitial damage not only in the embolized areas, but also in the remodeled, non-obstructed upper pole of the left kidney.[16] These key features of human CKD resulted in a reduced GFR, measured by gold-standard inulin clearance, and increased creatinine levels. The combination of DM, HC and CKD resulted in a phenotype resembling INOCA in humans. However, the specific contribution of the individual factors and their potential synergistic action remains to be established.

The present study further demonstrates that functional changes in the coronary microvasculature are already present before overt plaque formation occurs. These early changes result in an impaired myocardial oxygen balance and reduced cardiac efficiency. The observation in a small group of animals that endothelial function in isolated coronary small arteries was perturbed, suggests a role for the loss of nitric oxide in mediating the impairments in myocardial oxygenation in DM+HC+CKD animals. Future studies should focus in more detail on the mechanisms underlying the increased myocardial oxygen consumption as well as the perturbations in oxygen delivery.

Although our data provide valuable information regarding the mechanisms of CMD at this early stage, without the influence of proximal obstructive CAD which can affect distal microvascular function and structure[6], it is increasingly recognized that there may be an interaction between non-obstructive and obstructive CAD. Thus, CMD may decrease proximal shear stress and aggravate proximal CAD, whereas proximal CAD may further induce CMD, potentially mediated by multiple processes involving microembolization and the release of vasoconstrictors.[6, 64, 65] Investigation of such interaction of obstructive CAD with CMD, by combining a chronic proximal coronary artery stenosis[6, 65] with the current model of comorbidities-induced CMD, should be the topic of future studies. Such studies should then also include the assessment of flow distribution across the left ventricular wall, as the presence of a coronary artery stenosis causes a regional flow redistribution away from the subendocardium towards the subepicardium[39], whereas comorbidities in the absence of obstructive CAD appear to result in more diffuse and transmurally homogeneous reductions in myocardial blood flow.[24, 32, 66]

Conclusion

The present study is the first to investigate the effects of three common comorbidities on myocardial oxygen balance in swine at rest and during graded treadmill exercise. Our findings demonstrate that, in the absence of coronary atherosclerosis, comorbidities can result in CMD that is severe enough to critically impair myocardial oxygenation, thereby resulting in anaerobic metabolism. Thus, our DM+HC+CKD swine model represents a *bona fide* large animal model of INOCA. A link between CMD and left ventricular diastolic dysfunction has recently been shown in clinical studies. In our model, overt CMD is present at a time when diastolic dysfunction is still modest.[16] These findings are in agreement with clinical observations[26, 46, 47] and support the concept that CMD is one of the drivers of diastolic dysfunction in patients with comorbidities, suggesting that CMD represents a prime target for therapeutic interventions in INOCA as well as diastolic dysfunction / HFpEF.

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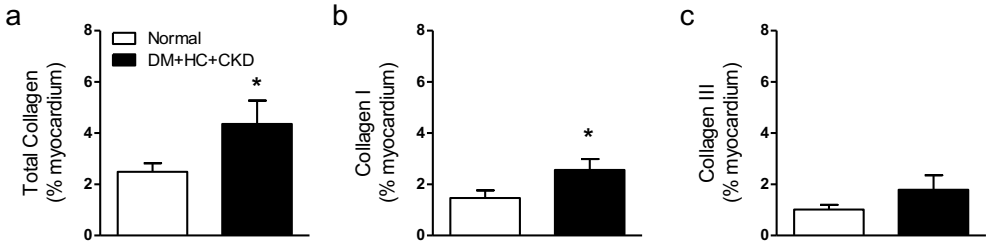
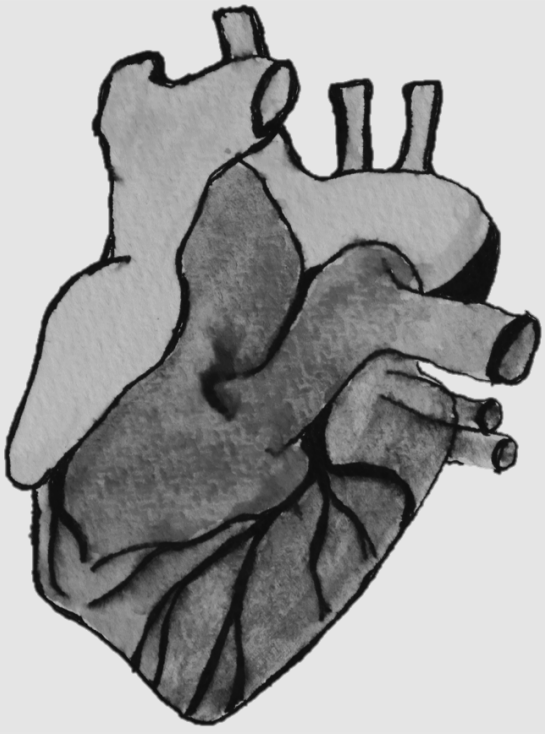
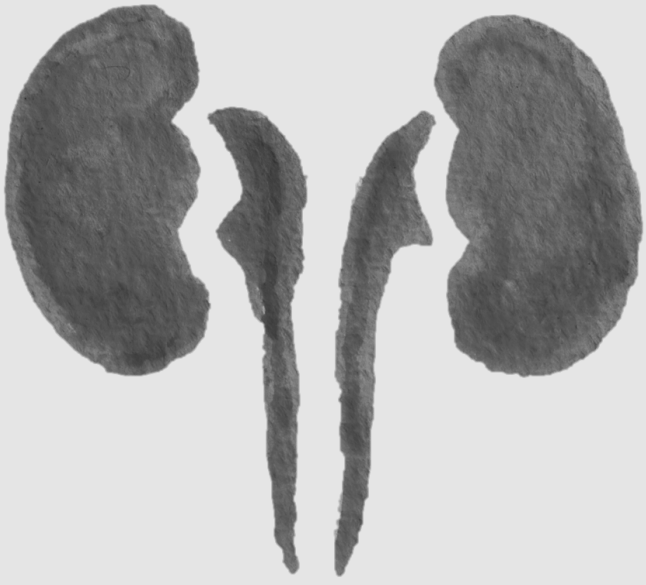
Supplementary results

Supplemental Fig



Supplemental Fig Total and specific collagen fiber content of interstitial fibrosis in Normal and DM+HC+CKD measured with a linear polarization filter. Significant increase in total collagen content of the left ventricle (**a**). This was mainly due to an increase in interstitial collagen I fiber content (**b**) while the collagen type III content was unchanged (**c**) in DM+HC+CKD swine compared to Normal. Normal n=10, DM+HC+CKD n=7. Data are mean±SEM.*p<0.05 DM+HC+CKD versus Normal by unpaired t-test.





Chapter 9

Reduced Nitric Oxide Bioavailability Impairs Myocardial Perfusion in Exercising Swine with Multiple Common Risk Factors

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Basic Research in Cardiology (invited revision)

9

Chapter 11

Summary, general Discussion and future perspectives

11

Summary, general discussion and future perspectives

In this thesis, we used large animal models to investigate functional and structural cardiovascular alterations in response to metabolic derangements in the absence (**Part I**) and presence (**Part II**) of chronic kidney disease (CKD). Our findings are highly relevant given the projected increase in the global prevalence of common cardiovascular risk factors in coming years[1, 2], not only in the high-income countries of North America and Western Europe but especially in low- and middle-income countries.[3] For example, the economic development of China and India and subsequent increase in socioeconomic status has resulted in a concomitant increase in cardiovascular disease risk.[3, 4] How the risk factors lead to cardiovascular disease development remains incompletely understood, and good treatment options are not always available. Therefore, there is a continuing demand for translational models to investigate the pathogenesis of cardiovascular disease, especially models that also recapitulate the multimorbidity state that is often observed in patients.[5] This is increasingly recognized by the scientific community, as also underlined by the scientific statements by the American Heart Association, concerning animal models for heart failure[6] and hypertension[7]. Moreover, improving the translational value of animal disease models will help to bridge the translational gap and implement findings from animal models to clinical practice more precisely, especially with regard to new treatment options.[5]

Alterations in cardiac function and structure due to multimorbidity

Metabolic derangements, such as diabetes mellitus (DM), dyslipidaemia and obesity, are the most well-known and common risk factors for cardiovascular disease.[8] For decades the focus of cardiovascular research has been on macrovascular disease and the development of atherosclerosis after chronic exposure to metabolic derangements. In **chapter 2,** we summarized the mechanisms which are at play in obesity-induced microvascular dysfunction in multiple organs. Although the specific underlying mechanisms differ between regional vascular beds, possibly due to inherent differences in specific organ physiology, microvascular dysfunction plays an important role in the development of multiple diseases. Indeed, in heart failure with preserved ejection fraction (HFpEF), coronary microvascular dysfunction (CMD) has been suggested to be the main contributor to cardiac dysfunction.[9] In addition to classical cardiovascular risk factors as discussed above, i.e. DM, dyslipidaemia, and obesity, less

conventional (non-cardiac) risk factors for the development of HFpEF have been described. Among these factors, which also include anaemia and chronic obstructive pulmonary disease, CKD is one of the most important factors.[9-11] In **chapter 6** we presented an overview of the pathophysiological processes by which CKD, mainly through CMD, can induce cardiovascular dysfunction and particularly HFpEF. CKD is not a completely new risk factor for cardiovascular disease, as dysfunction of heart and kidneys have been shown to be interrelated.[12] This so-called cardiorenal syndrome encompasses five different types of interactions between cardiovascular and renal diseases, of which type 4—chronic CKD resulting in heart failure[12]—is most relevant for our studies. Interestingly, the association between CKD and myocardial dysfunction has been suggested to be more pronounced in HFpEF patients when compared to heart failure with reduced ejection fraction.[10] In HFpEF, CKD is associated with worse myocardial function, and outcome.[11, 13] Uremic toxins in CKD patients can contribute to a state of systemic inflammation, multiorgan microvascular dysfunction and result in (ir)reversible effects in the myocardium, such as fibrosis and cardiomyocyte stiffening characteristic of HFpEF (**chapter 6**). To date, HFpEF remains one of the major challenges for researchers and clinicians. Currently, the European Society of Cardiology guidelines for treatment of HFpEF recommend treating underlying comorbidities in combination with diuretics to treat heart failure-related symptoms but, to date, no HFpEF-specific treatment to slow down progression and to reduce morbidity or mortality is available.[14] Life-style modifications, and exercise-training in particular, have shown some promise in that quality of life and cardio-respiratory fitness are improved in HFpEF patients. Yet, no beneficial effects were seen on systolic and diastolic function, and implementation of exercise training in an older patient cohort with multimorbidity is challenging.[15] In **chapter 6** we presented an overview of the clinical trials conducted for HFpEF treatments. Although most of these trials do not show an improvement in primary endpoint, some treatments should not be discarded yet, as patient-selection and -phenotyping is still proving difficult in HFpEF and, it can be argued that, with a better patient selection, some treatments should still be considered for specific HFpEF phenotypes.[16] Better understanding of the pathophysiological cascade of HFpEF is therefore imperative to determine different HFpEF phenotypes and the microvascular and cardiac mechanisms involved. Cardiopulmonary exercise testing (CPET) may facilitate delineation of microvascular and cardiac dysfunction and thereby expedite early recognition of HFpEF.[17-19] Furthermore,

animal models, in which more invasive measurements are possible can help to identify factors that discriminate between HFpEF phenotypes.

In **chapter 4**, we used a miniswine model exposed to 2 comorbidities, DM and dyslipidaemia, for 5 months to study the effect of exposure to these metabolic derangements, with a particular focus on myocardial function. We studied early myocardial changes at the molecular, mitochondrial and cellular level in conjunction with signs of early-stage diastolic dysfunction—left ventricular peak untwisting velocity and E/e'—as measured by echocardiography. Diastolic dysfunction appeared to be mainly due to intrinsic cardiomyocyte stiffening, as no structural changes were observed in the left ventricular myocardium. Indeed, isolated cardiomyocytes showed increased passive force and maximal force in this model. Although we observed no shift in titin isoforms, associated to increased passive force, titin hypophosphorylation by increase in oxidative stress and subsequent impaired nitric oxide (NO) signalling can also cause a passive force increase, as shown in patient studies.[9, 20-22] Indeed, increased left ventricular oxidative stress was observed with reduced NO-levels and endothelial NO synthase (eNOS) uncoupling, increasing vascular oxidative stress.[21] Additionally, impaired mitochondrial complex I respiration—both cause and consequence of increased oxidative stress—was present in this model, which can result in bioenergetic dysfunction and thus increase myocardial stiffness.[23] Furthermore, RNA sequencing revealed 63 genes to be differentially expressed in the left ventricular myocardium after 5 months exposure to metabolic derangements. Subsequent pathway analysis indicated that mainly glucose and free fatty acid metabolism pathways were altered. Healthy myocardium is able to switch between different substrates to meet metabolic demand and maintain oxygen utilization efficiency, which is impaired in metabolic derangements and contributes to cardiomyocyte dysfunction, by limiting ATP-bioavailability and increasing oxidative stress.[24] Mitochondrial dysfunction is thus suggested to be an important early mediator of myocardial dysfunction in metabolic derangements.[25, 26]

To gain more mechanistic insight in the relation between metabolic derangement, CKD and HFpEF, we developed and characterized a swine model with CKD as a comorbidity in addition to DM and dyslipidaemia. These three morbidities result in a systemic pro-inflammatory state (increased circulating tissue necrosis factor (TNF-)α levels), which interestingly, correlated strongest with a marker of CKD and less with glucose, while a correlation with total cholesterol levels was absent. This triple morbidity swine model, as

presented in **chapters 7-10**, represents a model of early diastolic dysfunction (or pre-HFpEF), evidenced by an upward shift in the end diastolic pressure-volume relationship measured by pressure volume loop and a trend towards a decrease in MRI-derived E/A ratio, under anaesthesia, although left atrial pressures were not elevated either at rest or during exercise. In **chapter 7**, we dissected some of the pathological cardiac processes leading from the initial induction of risk factors to diastolic dysfunction. In agreement with our findings in **chapter 4**, we showed that these three morbidities resulted in a pro-inflammatory state, uncoupling of eNOS, an increased myocardial and vascular oxidative stress—correlating with TNF-α—associated with reduced NO-bioavailability. However, in contrast to the findings in the animal model used in **chapter 4,** in the triple morbidity animal model we did observe structural myocardial alterations—a loss of capillary density and increased collagen deposition—that are both also observed in patients with HFpEF.[27] Furthermore, the increase in cardiomyocyte passive force, as also observed in **chapter 4**, was accompanied by a titin isoform- shift towards N2B, the stiffer titin isoform.

Five months of exposure to DM, hypercholesterolemia and CKD also resulted in CMD, evidenced by impaired endothelium-dependent vasodilation, while endothelium-independent vasodilation was maintained in isolated small coronary arteries. Endothelium-dependent vasodilation was restored by anti-oxidant treatment, which is in line with findings in clinical studies, demonstrating that increased vascular oxidative stress is an important pathophysiological mechanism in morbidity-induced diastolic dysfunction.[9, 21, 22] Altogether, the findings presented in both **chapter 4** and **7** demonstrated that both animal models recapitulate features of HFpEF and elucidated some of the mechanisms by which metabolic derangements alone or in combination with CKD can induce left ventricular diastolic dysfunction associated with increased oxidative stress, CMD and loss of NO. Our findings are in line with findings in HFpEF patients[9, 28, 29] and the ZSF-1 HFpEF rat model[21, 30]. Importantly, the findings in chapter 4 and 8 suggest that adding CKD results in a faster development and/or a more severe phenotype of diastolic dysfunction.

In **chapter 8** we further studied CMD in relation to myocardial oxygen balance and cardiac function in the triple morbidity animal model in the awake state, using chronic instrumentation and exercise testing to reveal more subtle alterations. We observed reduced myocardial oxygen utilization efficiency at rest and during exercise, evidenced by the higher myocardial oxygen consumption for the same level of cardiac work, which could be attributed

to a myocardial substrate shift or a reduction in mitochondrial function consistent with the findings from **chapter 4**.[31] We showed several mechanisms which might underlie the observed mitochondrial dysfunction; i.e. oxidative stress (**chapter 4** and **7-9**), lipotoxicity (**chapter 4**), lower specific mitochondrial complex respiration (**chapter 4**) or upregulation of uncoupling protein 3 (**chapter 4**). In line with our findings, myocardial mitochondrial dysfunction is also observed in diabetic patients undergoing elective cardiac surgery[32-35], as well as in swine models of familial hypercholesterolemia[36, 37] and metabolic derangement and moderate atherosclerosis.[38] Metabolic derangement-induced mitochondrial dysfunction is also linked to a loss of coronary vasodilator function and a reduction in myocardial oxygen efficiency (relationship between myocardial blood flow and cardiac work), which could all be restored by restoring mitochondrial function.[39] In **chapter 8-9**, we observed a similar loss of endothelium-dependent vasodilation in isolated small coronary arteries *in vitro* as well as at rest and during exercise *in vivo*, using chronic instrumentation, as evidenced by perturbations in myocardial oxygen delivery and reduced coronary flow reserve (CFR). This shows that advanced CMD was already present, indicating that CMD may precede advanced diastolic dysfunction and HFpEF. Indeed, reduced coronary flow reserve (CFR), in the absence of obstructive coronary artery stenosis, is considered to be a marker of microvascular (endothelial) dysfunction and is present in patients with HFpEF or diastolic dysfunction.[40-42] Furthermore, HFpEF development and prognosis are also predicted by CFR, as shown by a recent clinical study demonstrating that, in patients with a reduced CFR, diastolic function worsens progressively over time and is associated with an increased risk of HFpEF hospitalization.[42] In **chapter 8-9** we observed CMD both at rest and during exercise with evidence of anaerobic metabolism, suggestive of myocardial ischemia, providing further support for a link between diastolic dysfunction and ischemia and no obstructive coronary artery disease (INOCA). These findings are in agreement with recent clinical studies, demonstrating the co-existence of diastolic dysfunction or HFpEF and INOCA.[42-44] In **chapter 9** we further explored the mechanisms underlying CMD in small coronary arteries, responsible for impaired myocardial oxygen delivery, showing that CMD was mediated by loss of endothelium-dependent vasodilation principally due to a loss of NO bioavailability (**figure 1**), possibly through NO-scavenging by reactive oxygen species. Such increased microvascular oxidative stress and loss of NO-bioavailability in HFpEF was demonstrated in **chapter 4** and **7.** Whereas in HFpEF, CMD in the capillary endothelial compartment results in a loss of the

paracrine effect of NO signalling in the cardiomyocytes (**chapter 4** and **7**), CMD in endothelium of coronary small arteries and arterioles, with a loss of NO bioavailability, results in impaired control of coronary resistance vessels and subsequent INOCA features (**chapter 8-9**). CMD precedes INOCA and is the most common cause of myocardial ischemia in INOCA.[45, 46] Thus, the common denominator linking HFpEF and INOCA appears to be CMD (most notably loss of NO signalling) induced by the comorbidities (**figure 2**).[21, 27, 47] Notwithstanding the importance of the available research investigating the link between HFpEF and INOCA, a significant knowledge gap persists that warrants further research. For example, it is still unclear which syndrome, HFpEF or INOCA, presents in which patients, so that there might either be a timing effect, or there may be modulating factors that determine the development of the different syndromes (**figure 2**).[48]

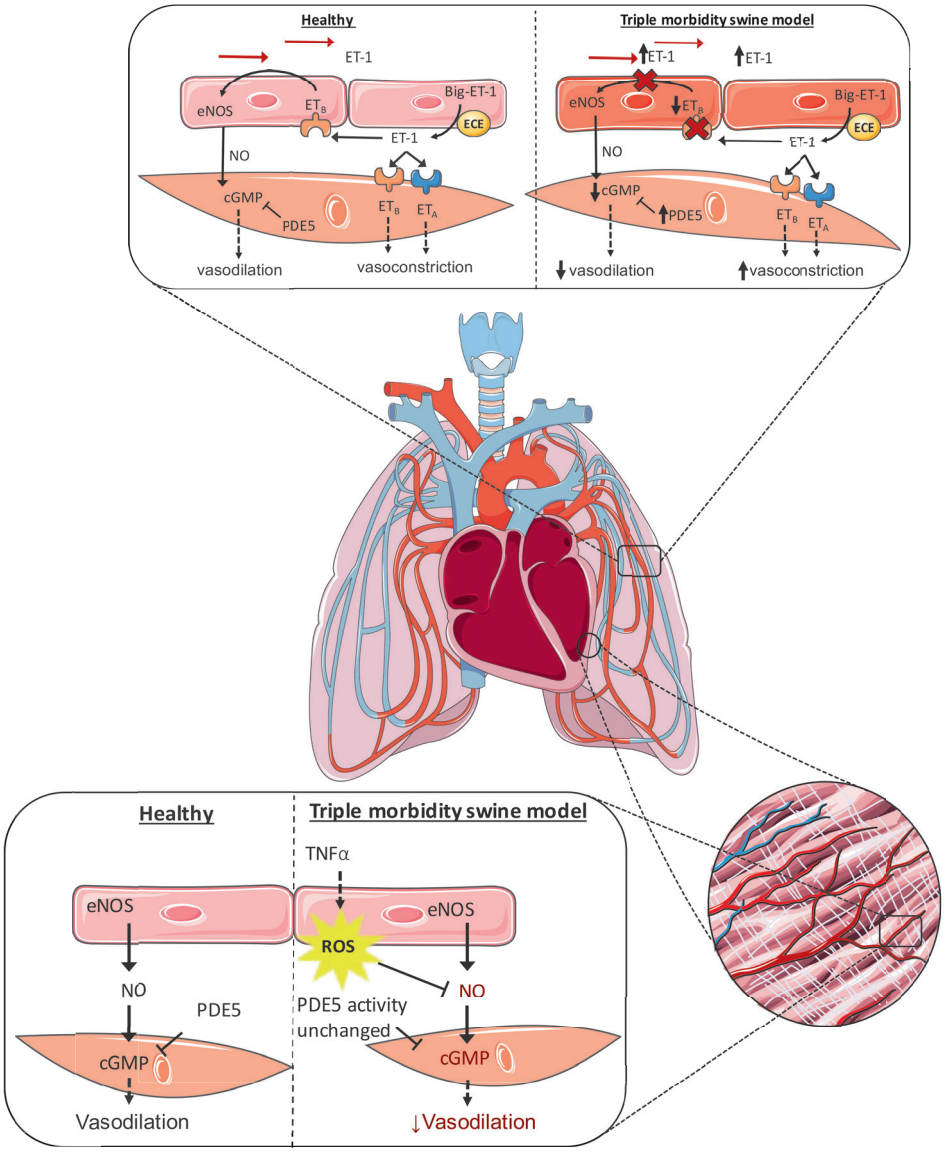
Multiple studies tried to unravel the different phenotypes within the HFpEF continuum and different classification methods can be employed.[49] At the moment, 3 major HFpEF phenotypes have been suggested, based on phenomapping, composed of a younger group with low brain natriuretic peptide, a group with high prevalence of obesity and DM and a group with CKD and pulmonary disease, phenotype 1, 2 and 3 respectively.[50] Our triple morbidity model may represent a combination of phenotype 2 and 3, as in **chapter 9** we demonstrate that early pulmonary vascular changes are already present in response to metabolic derangements and CKD. As presented in **chapter 7,** after exposure to DM, dyslipidaemia and CKD for 6 months, we demonstrated that both intrinsic cardiomyocyte stiffening and extracellular matrix expansion by increased collagen deposition occurred which can both contribute to increased myocardial stiffening. From our study in **chapter 7** we could not determine the contribution of each risk factor to the decreased left ventricular diastolic function. However, in **chapter 4**, we showed that, in swine with metabolic derangements without CKD, diastolic dysfunction was principally mediated by intrinsic cardiomyocyte stiffening as no extracellular matrix expansion was observed. These findings demonstrate that although diastolic dysfunction might be present in a variety of patients with multimorbidity, the underlying mechanisms could be different per patient. Additionally, CKD might aggravate the diastolic dysfunction phenotype with extracellular matrix expansion being associated advanced disease. Indeed, in HFpEF patients, intrinsic cardiomyocyte stiffness[51, 52] and extracellular matrix deposition[27, 51] are also the main determinants of myocardial stiffening, and good phenotyping is essential to evaluate what underlying mechanism(s) should be

treated in which patient.[53] Although all animal models have advantages and disadvantages[54] and modelling of HFpEF proves to be complex[55], animal models are needed to study underlying HFpEF pathophysiology, to help delineate which risk factor contributes to a specific phenotype and to test new treatments directed at these processes specifically. Herein lies the biggest challenge for future clinical trials for the treatment of HFpEF—good phenotyping of your population and targeting the right patients—ultimately leading to personalized medicine for the individual patient.[56]

Effect of multimorbidity on coronary microvascular function and INOCA

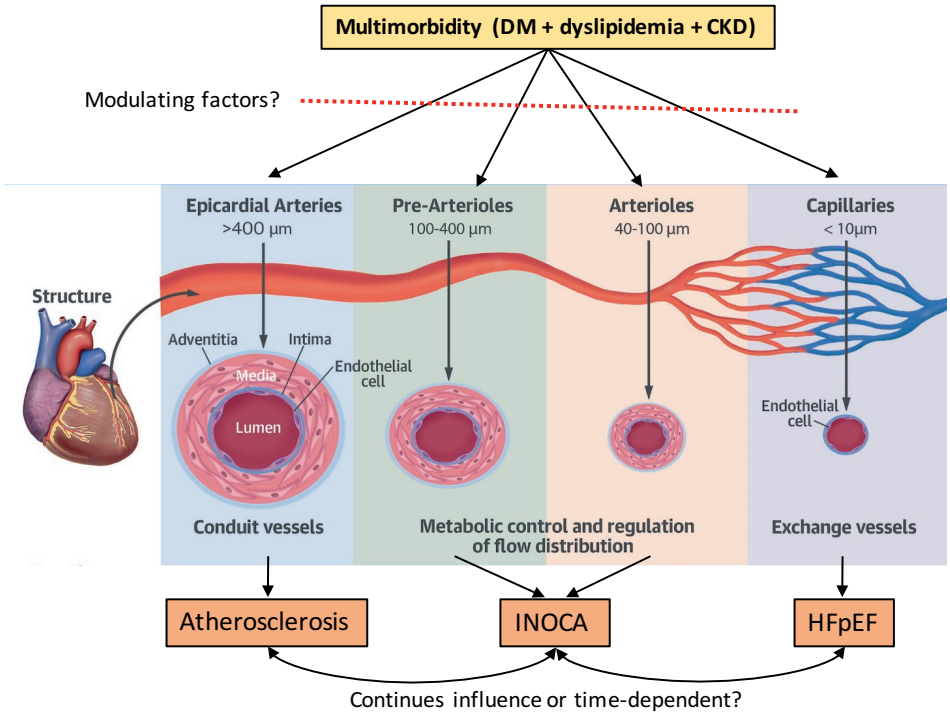
For decades, the focus of cardiovascular research has been on macrovascular disease and the development of atherosclerosis after chronic exposure to metabolic derangements. Consequently, the coronary microvasculature has been under-investigated with regard to cardiovascular disease. However, more than 50% of the patients with signs and symptoms of myocardial ischemia do not have flow-limiting coronary obstructions, suggesting that the microvasculature is also dysfunctional.[45, 46] Therefore, investigating pathophysiology and treating (coronary) microvascular dysfunction might prove to be the new frontier in cardiovascular health and disease.[57] Indeed, as we summarized in **chapter 2,** the possible underlying mechanisms for metabolic derangement-induced microvascular dysfunction are numerous, and in the coronary circulation can result in HFpEF as well as INOCA, as shown in **chapter 6-7**. The lack of a deeper understanding of the pathophysiological cascade of INOCA, impairs a designated diagnostic workup and no evidenced-based treatment options. In the clinical setting, diagnosing INOCA is still challenging due to the complexity of the syndrome and the current workup for chest pain is not yet optimized for diagnosing INOCA and the underlying aetiologies.[58] Additionally, the options of assessing coronary microcirculatory endothelial function in the clinical setting is still rather limited, thereby complicating the diagnosis of INOCA.[59]

Figure 1 Main alterations in vasomotor control in the pulmonary and coronary microcirculation of the triple morbidity swine model as presented in this thesis



ET-1 endothelin 1, eNOS endothelial nitric oxide synthase, ET_B endothelin receptor B, ET_A endothelin receptor A, ECE endothelin converting enzyme, NO nitric oxide, cGMP cyclic guanosine monophosphate, PDE5 phosphodiesterase, ROS reactive oxygen species.

Figure 2 Proposed hypothesis of coronary microvascular dysfunction in different vessels sizes in response to multimorbidity



Diabetes mellitus (DM), dyslipidaemia and chronic kidney disease (CKD) induces coronary microvascular dysfunction which can result in atherosclerosis, ischemia with no obstructive coronary artery (INOCA) and heart failure with preserved ejection fractions (HFpEF) by affecting vessels of different sizes. Which vessels are affected might be determined by modulating factors or all might be present (yet subclinical) and influence each other continuously or in a time-dependent manner, starting with the vessels most sensitive to metabolic changes (capillaries->arterioles->small arteries->large arteries). Adapted from Taqueti and Di Carli.[60]

Current consensus on the diagnostic flowchart incorporates invasive coronary angiography for the evaluation of coronary obstructions with invasive diagnostic fractional flow reserve if needed, coronary flow reserve (CFR) measurements for the evaluation of microvascular dysfunction, and a vasoreactivity test to acetylcholine and a nitrate for the assessment of endothelial dysfunction with/without vasospasm.[45, 46, 58] Although, great efforts have been undertaken to improve the diagnostic workup for INOCA, current testing methods only differentiate between an endothelium-dependent or –independent cause of CMD.[58] Testing

for specific underlying mechanisms is not yet applicable, which in part is due to a lack of pathophysiological knowledge, which also limits the therapeutic options available for the treatment of INOCA. Indeed, treatment of myocardial ischemia has traditionally been stenosis-centred, but recognition of the clinical syndrome INOCA requires are new and different treatment approach. This notion is further supported by the recent findings of the ISCHEMIA trial, which demonstrated that, in patients with moderate or severe ischemia and stable coronary artery disease, invasive treatment was not superior to conservative treatment.[61] Unfortunately, an evidenced-based (conservative) treatment for INOCA specifically, besides treating underlying comorbidities, is currently not available in clinical practice.[45] This is explained in part by the fact that INOCA is only recently being recognized as a syndrome of myocardial ischemia, but is also due to a lack of translational animal models which can be used for investigation of pathophysiological pathways and testing of novel compounds.[54]

As we showed in **chapter 8 and 9**, CFR, as measured as the relative increase in coronary blood flow in response to intravenous adenosine infusion in awake animals, was reduced in swine with triple morbidity reflecting the clinical characteristics of INOCA. Furthermore, we demonstrated in the same model, that not only pharmacologically-induced vasodilation was impaired but also resting and exercise coronary resistance vessel function. We observed these alterations both *in vivo*, evidenced by a reduced myocardial oxygen delivery, and *in vitro*, in isolated coronary vessel experiments. CMD reduced myocardial oxygen delivery and induced anaerobic metabolism as evidenced by a reduced myocardial lactate consumption and, in some animals, even net lactate production. This together with a decrease in myocardial oxygen utilization efficiency, as discussed above, resulted in an impaired myocardial oxygen balance, aggravating myocardial anaerobic metabolism suggestive of myocardial ischemia. As presented in **chapter 9**, we observed a loss of endothelium-dependent vasodilation, which was principally mediated by a loss of NO bioavailability, and appeared to be the consequence of increased NO scavenging by reactive oxygen species rather than dysfunctional eNOS, as levels of eNOS protein and activity were similar between groups (**Figure 1**). To compensate for the loss of NO bioavailability, vascular smooth muscle cell sensitivity to NO was increased *in vivo* in our swine model with multiple morbidities. Previous research showed similar results using *in vitro* techniques in swine with combined high fat diet-induced obesity and hypertension[62], in obese rats[63] and even in

hypertensive and obese patients[64]. Possible underlying mechanisms include increased soluble guanylyl cyclase (sGC) activity[65], or may act downstream of sGC by potentiating protein kinase G activity[66]. Phosphodiesterase 5 (PDE5)-activity was also similar between groups, suggesting that the impaired vasodilator response was not due to alterations in PDE5 activity nor was there a compensatory downregulation of PDE5 activity. Interestingly, the observed CMD appeared to be due to functional changes in vasomotor control, as we observed no structural vascular alterations, which is in line with recent findings in INOCA patients.[67, 68] These findings might help us understand which pathophysiological mechanisms play a role in INOCA, especially during exercise-induced angina.

CMD with loss of endothelium-dependent vasodilation has been previously described in coronary microvessels isolated from swine models of metabolic derangements with[62, 69] or without[70] CKD, or familial hypercholesterolemia[36], from obese rats[63] and also from DM patients[71]. Although there was quite some variation in the exposure time, type and combination of risk factors between studies, the common mechanism was a decrease in endothelium-dependent vasodilation. A combination (high fat diet and hypertension) of risk factors induced a more pronounced attenuation of endothelium-dependent vasodilation than one single risk factor, suggesting a synergistic effect.[62] Consistent with our findings, in the majority of these studies the loss of NO-bioavailability was the principal mechanism underlying CMD[62, 63, 70-72], although loss of endothelium-derived hyperpolarizing factor with maintained NO-mediated vasodilation has also been reported.[36] Some small randomized clinical trials have been conducted in the past which fit in the concept presented in the current thesis that endothelial dysfunction plays a central role in CMD, and also show that improvement of endothelial dysfunction/NO-bioavailability restore microvascular function. Treatment with statins alone[73-75] as well as in combination with a calcium channel blocker[76] or angiotensin-converting enzyme inhibitor[77] resulted in beneficial effects in INOCA patients. These treatments reduced exercise-induced ischemia, increased CFR or restored flow-mediated vasodilation, while endothelium-independent vasodilation was unchanged. These effects were independent of the lipid-lowering effects of the statins and was associated with an improved endothelial function, or NO-bioavailability more specifically[76-78] which was associated with reduced oxidative stress.[77] Additionally, long-term enalapril alone has also been shown to increase NO bioavailability and thus improve coronary flow reserve and myocardial ischemia symptoms in patients with INOCA[79] as well as improve exercise-induced

angina.[80] Moreover, 6 months of oral supplementation of L-arginine, the substrate for eNOS required to form NO, results in increased coronary blood flow response to acetylcholine and decreased symptoms scores in INOCA patients, further underlining the importance of altered NO signalling in INOCA.[81] Although all aforementioned drugs or supplements have different primary sites of action, their common mechanism of action is through increasing NO bioavailability.

Other therapies targeting the NO signalling pathway that are currently available include NO-donors, sGC-activators or -stimulators and PDE5 inhibition. NO-donors can be divided into direct NO donors, i.e. sodium nitroprusside (SNP) and S-Nitroso-N-acetyl-DL-penicillamine SNAP, which do not need an enzymatic conversion to release NO and (in)organic nitrates (i.e. nitroglycerine and isosorbide dinitrate) which act as pro-drugs and do need enzymatic conversion.[82, 83] Interestingly, previous research has shown that acute treatment with organic nitrates such as isosorbide dinitrate might even have a detrimental effect on coronary microcirculation and angina symptoms while it has a beneficial effect on patients with obstructive coronary artery disease.[84-86] It is hypothesized that high doses of organic nitrates, and to a lesser extent SNP[87], are necessary to achieve sufficient vasodilation of small coronary arterioles as opposed to large arteries and collaterals.[82] SNP, which we used *in vivo* in this thesis, would be the most suitable compound for inducing coronary arteriolar vasodilation and it has been used effectively in patients for decades. However it is only used for the treatment of severe hypertension, as it can only be administered parentally and has a short half-life, complicating chronic treatment.[83, 88] Additionally, SNP can induce coronary steal syndrome, reducing regional myocardial blood flow in patients with coronary artery disease[89] and increase myocardial arterio-venous shunting[90], which could be detrimental in myocardial ischemia due to regional perfusion deficits such as INOCA. Moreover, there may be organ-specific sensitivity to NO-donors both in health and disease[91], resulting in systemic side-effects before noteworthy coronary vasodilation is achieved. We observe a similar phenomenon in **chapter 9**, as in our three comorbidities swine model we had to abort the infusion of the NO donor SNP in some animals due to dangerously low systemic blood pressure, while coronary vascular conductance was just moderately increased. In contrast to direct NO-donors, prolonged treatment with organic nitrates can result in nitrate-tolerance and also has been shown to be detrimental to endothelial function as it also plays a role in the redox balance and induce increased oxidative stress, limiting the role of organic nitrates in the

treatment of INOCA.[82] Altogether, the use of organic nitrates or direct NO-donors seem to have some important limitations for the treatment of INOCA, therefore other therapeutic options which can increase NO-bioavailability indirectly should be considered. Inorganic nitrates, available via bioconversion from dietary sources, do not result in tolerance, induce less oxidative stress and have been investigated in angina patients with obstructive coronary artery disease.[92] In a recent study, inorganic nitrates improved nitrite and nitrate plasma levels as well as maximum exercise time, but failed to reach statistical significance on time to 1mm ST-depression on electrocardiogram treadmill testing (P=0.10), suggesting a possible small antianginal effect of inorganic nitrates in these patients.[93] However, conflicting results have been published concerning the effect of inorganic nitrates on endothelial function, necessitating further research.[92]

In **chapter 9**, we observed only a modest coronary microvascular vasodilator response to PDE5 inhibition in both groups. In patients with INOCA, limited clinical data about the effect of PDE5 inhibition are available and the two clinical studies that are available show conflicting results.[94, 95] These considerations suggest that the potential of PDE5 inhibition in the treatment of CMD in INOCA patients is uncertain, but should be further investigated. sGC activators and stimulators are two novel classes of drugs, which can induce NO-independent stimulation of the NO-sGC-cGMP pathway.[96, 97] In healthy animals and models of pulmonary hypertension these drugs were capable of inducing dose-dependent vasodilation in the pulmonary circulation as well as in the systemic circulation.[98] In a rat model of isoproterenol-induced myocardial ischemia, the sGC activator cinaciguat was able to restore myocardial damage, reduce oxidative stress and improve myocardial function.[99] In a canine model of global myocardial ischemia/reperfusion by cardiac bypass clamping, cinaciguat restored left ventricular function, coronary blood flow and acetylcholine-induced coronary vasodilation *in vivo* and *in vitro* in canine coronary arteries stimulated with peroxynitrite.[99, 100] In a proof of concept study, acute decompensated heart failure patients showed improved hemodynamics with cinaciguat infusion.[101] Besides the beneficial effect of these drugs on the myocardium in congestive heart failure, renal blood flow increased and GFR was maintained without activation of the renin-angiotensin-aldosterone system, making them particularly interesting in patients with cardiorenal syndrome.[102, 103] Notwithstanding the importance of these findings, the therapeutic properties of these compounds on the coronary circulation should

be determined in more depth, but might prove valuable new drugs in the treatment of both INOCA and HFpEF.

Multimorbidity-induced generalized endothelial dysfunction

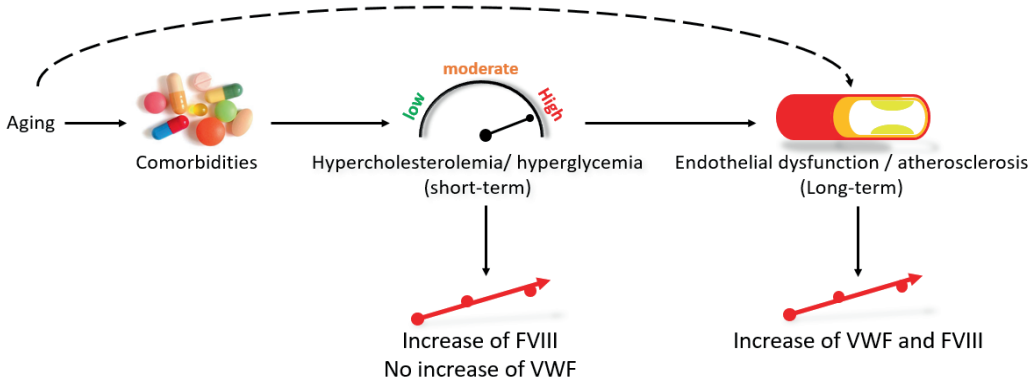
In **chapter 2** we summarized the mechanisms which are at play in obesity-induced microvascular dysfunction in multiple organs. Although the specific underlying mechanisms differ between regional vascular beds, possibly due to inherent differences in specific organ physiology, microvascular dysfunction plays an important role in the development of many diseases of multiple organs. As stated before, multimorbidity results in HFpEF due to a systemic pro-inflammatory state, therefore non-cardiac microvascular beds and organs are involved in HFpEF pathogenesis.[53] Pulmonary hypertension (PH) is present in about 36-83% of the HFpEF (HFpEF-PH) patients[104, 105], and it is of clinical importance, as multiple studies have demonstrated that HFpEF-PH patients have a more severe phenotype with a worse outcome than patients without PH.[104, 106] In part this is due to PH, with subsequent right ventricular dysfunction, being considered a late (end-stage) complication in HFpEF due to left ventricular backward failure.[107] However, it has also been suggested that a portion of the HFpEF-PH patients might be erroneously classified as type 2 PH (due to left heart disease) as it resembles pulmonary arterial hypertension (type 1 PH), a pre-capillary PH phenotype.[107-109] The pathophysiology of how comorbidities associated with HFpEF result in pulmonary vascular disease remains unclear. In conjunction with the lack of clear pathophysiological mechanisms, an evidenced-based treatment regimen for HFpEF-PH specifically is not yet available.[110] In **chapter 10** we showed that in our triple morbidity swine model with diastolic dysfunction, pulmonary vascular resistance was increased and pulmonary vasomotor control was impaired. Although no clear increase in pulmonary artery pressure was observed, which was due to a lower cardiac output, these early changes demonstrate that pulmonary microvascular dysfunction is already present before overt PH and right ventricular dysfunction occur. We observed no changes in pulmonary arterial structure in our model. However, we observed an increase in endothelin 1-mediated vasoconstriction, in combination with an increased PDE5 activity, contributing to an increased pulmonary vascular resistance (**figure 2**). Consistent with our findings, in HFpEF-PH patients increased circulating endothelin 1 has been observed, which was associated with a higher pulmonary vascular resistance, an abnormal

pulmonary vasodilation and a more severe HFpEF-PH phenotype.[111] Despite these observations that endothelin 1 may play a detrimental role in development of pulmonary vascular disease in HFpEF, no beneficial effects of 12 weeks of endothelin-receptor antagonism with either bosentan or macitentan were observed in patients with HFpEF-PH in the BADDHY[112] and the MELODY-1[113] trials respectively. Nevertheless, our study suggests that endothelin receptor blockade may be beneficial, particularly in early pulmonary vascular disease secondary to multimorbidity. In **chapter 10** we also demonstrated that in the presence of NO synthase inhibition the pulmonary vasodilator effect of endothelin receptor blockade was enhanced in control swine while, surprisingly, it was abolished in the triple morbidity swine, suggesting an altered interaction between NO and endothelin. It has been shown in the past that the NO and endothelin interact on multiple levels. For example, the endothelial ET_B receptor promotes NO production by eNOS.[114] Vice versa, NO inhibits endothelin-mediated contraction and endothelin 1 production/release.[115] In contrast, it was shown that, further downstream of NO, an increased PDE5 activity, resulting in reduced cGMP levels, can induce an increased endothelin vasoconstrictor influence in lung vasculature.[116] In **chapter 10** we also observed an increase PDE5 influence in pulmonary vasomotor control while NO bioavailability was unaltered. Although the role of PDE5 inhibition in the pathogenesis of HFpEF-PH has not been thoroughly investigated, due the success of PDE5 inhibition in type 1 PH resulted in multiple trials which tested PDE5 inhibition in HFpEF-PH. However, conflicting results have been published with respect to the efficacy of PDE5 inhibition in HFpEF-PH, and recent meta-analyses concluded that there is no beneficial effect of PDE5 inhibition on pulmonary hemodynamics or exercise capacity[117, 118], although some individual studies do show a beneficial effect. It should be noted, however, that there were substantial differences in study populations. Thus, the studies with negative results had a higher proportion of post-capillary PH patients[119, 120], while the patients included in the study by Guazzi *et al.*[121] had higher pulmonary vascular resistance resembling the pre-capillary arterial phenotype present in our swine model. Stratifying patients based on underlying disease may prove critical, to identify subgroups of HFpEF-PH patients that may benefit from endothelin receptor antagonism or PDE5 inhibition. Moreover, combined treatment might be beneficial as we demonstrated that there is an altered NO-endothelin interaction, and PDE5 inhibition can reduce endothelin vasoconstrictor influence.[116] This altered interaction should be further explored in our triple morbidity swine model by combined acute treatment at rest and during

exercise, and subsequently the effects of chronic treatment could be explored in the future. Personalized medicine by careful patient selection, together with earlier detection, possibly by incorporation of CPET into routine HFpEF diagnostics[18, 122], and prevention of progression towards end-stage HFpEF-PH might help to improve clinical status of HFpEF-PH patients.

Besides the role of the vascular endothelium in regulating resistance vessel tone, and hence tissue perfusion, microvascular endothelium in particular also plays an important role in maintaining coagulation homeostasis. Von Willebrand Factor (VWF) is mainly produced in endothelial cells and is a key pro-coagulant protein that mediates platelet adhesion and aggregation.[123] VWF also functions as carrier protein in the circulation for factor VIII, another important protein in the coagulation cascade, thereby preventing degradation of factor VIII. Recently, it was demonstrated that the age-related increase in circulating VWF is associated with an increased prevalence in comorbidities in an ageing population.[124] Moreover, higher circulating levels of VWF and also factor VIII have been associated with an increase in cardiovascular disease and worse cardiovascular outcome.[125] However it is presently unclear whether this association reflects a direct effect of the comorbidities on VWF levels or whether this occurs secondary to endothelial dysfunction and/or atherosclerosis. Therefore, in **chapter 5** we retrospectively studied vWF and factor VIII levels in the model described in **chapter 4**. VWF was not elevated after 5 months' exposure to DM and hypercholesterolemia at a time when coronary endothelial dysfunction and coronary atherosclerosis are not (yet) present. Conversely, factor VIII was increased in the group with metabolic derangements compared to the healthy controls (**Figure 3**). The dissociation between factor VIII and VWF might be due to the high levels of lipids as factor VIII can bind to lipids, prolonging its half-life.[126] Furthermore, hyperglycaemia can induce a loss of endothelial glycocalyx[127] and an increase in myocardial oxidative stress[128], both resulting in increased factor VIII. To further investigate the relation between VWF and comorbidities, these were measured in swine exposed to dyslipidaemia or DM+dyslipidaemia for 15 months, that showed overt coronary endothelial dysfunction and atherosclerosis. In this cohort, the increase in circulating VWF between 9 and 15 months exposure coincided with the atherosclerosis development and endothelial dysfunction, suggesting that VWF may represent a biomarker of advanced cardiovascular disease (**figure 3**). However, since VWF has a potent pro-coagulant effect, it might also be a mediator of cardiovascular diseases by promoting (micro)thrombi formation and thus could be regarded as a potential target in novel treatments.[129]

Figure 3 Summary of the association between comorbidities, endothelial dysfunction and atherosclerosis and circulating levels of von Willebrand factor and factor VIII.



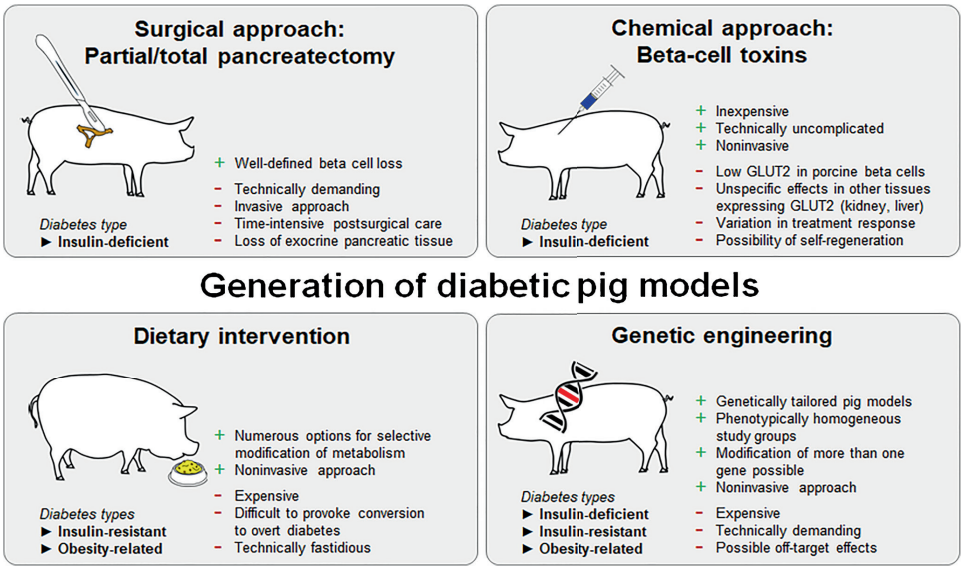
Proposed relationship between comorbidities (diabetes mellitus and dyslipidemia) and the coagulation proteins von Willebrand factor (VWF) and factor VIII (FVIII). Aging results in an increase in comorbidities which increase VWF mediated by coronary microvascular endothelial dysfunction and/or coronary atherosclerosis.

However, causality remains to be proven, as conflicting results have been published. For example, patients with von Willebrand disease, a bleeding disorder due to a complete or partial loss or a loss of function of VWF, have a lower prevalence of arterial thrombosis.[130] In contrast, in patients (>55 years of age) with high VWF due to a genetic variation, VWF-levels were not associated with coronary heart disease during a mean follow-up time of 10.8 years.[131] However, whether atherosclerosis is also prevented in patients with von Willebrand disease remains to be determined. Clinical observations in 47 von Willebrand disease patients suggest that atherosclerosis, measured by carotid and femoral artery intima-media thickness, does develop.[132] However, pigs with von Willebrand disease showed resistance to aortic atherosclerosis and there might also be resistance to coronary atherosclerosis, but evidence is still inconclusive.[133, 134] One explanation for these findings in pigs with von Willebrand disease, is that pigs also carry a polymorphism in apolipoprotein B100 which results in reduced diet-induced hypercholesterolemia, thus limiting atherosclerosis development.[135] Further research on the casual role of VWF in the development of endothelial dysfunction and/or atherosclerosis is required to determine its potential as a therapeutic target.

Current standings and future perspectives

CMD is clearly one of the new frontiers in cardiovascular research.[57] However, due to the inherent difficulties of CMD study in humans, animal models which emulate human CMD more precisely are needed. In **Chapter 3**, we present an overview of the currently available experimental animal models of CMD. Although small animal models have clear advantages with regard to costs, housing, handling and genetic manipulation, large animal models hold greater translational capacity to the human situation, given their close resemblance of cardiovascular anatomy, physiology and metabolism. Whereas the use of canine models has decreased in the last decade, mostly due to societal pressure, swine models have proven to be an excellent alternative. While some swine breeds, such as the Ossabaw and Rapacz swine, have a spontaneous genetic variation making them susceptible to cardiovascular disease, they are not widely available.[136] However, besides these two, most other swine breeds do not develop cardiovascular disease spontaneously in a timeframe suitable for research purposes.[136] Hence, induction of metabolic derangement in swine has been used in multiple studies and has been well validated. **Figure 4** gives an overview of the most commonly used methods, surgical or chemical reduction of β-cell mass, dietary intervention and genetic engineering, of inducing DM in swine used in cardiovascular research.[136] All of these methods have distinctive diabetic phenotypes as a result and all have their unique challenges and advantages. [136] In our studies, we used a combination of low-dose streptozotocin and a high-fructose, high-sucrose and high fat diet, which indeed induced pronounced hyperglycaemia and dyslipidaemia both in the miniswine model (**chapter 4-5**), the dyslipidemic farm swine model with DM (**chapter 5**), and the triple morbidity model (**chapter 7-10**). Although this regimen of streptozotocin injections induces a type 2 DM-like phenotype, no hyperinsulinemia was observed due to the use of streptozotocin.[137] In humans, DM with insulin resistance with hyperinsulinemia is observed in the majority of the patients, and it has been shown that hyperinsulinemia is also an important mediator of cardiovascular disease.[138, 139] Our data in swine exposed for 15 months to only high fat diet[140] showed only mild insulin resistance without hyperglycaemia in the absence of streptozotocin treatment, indicating that DM-induction without streptozotocin using only a diet is difficult in farm swine, time-consuming and expensive.[136] Furthermore, in view of the genetic selection for the meat industry, which has resulted in a rapid body growth, their increase in size and body weight limits follow-up time and age of inclusion. In accordance, farm swine are often studied at

Figure 4 Different methods for inducing diabetes mellitus in swine models for studying cardiovascular disease



relative young age (<9 months) due to their size and body weight increase and at this age they have just reached sexual maturity (~6 months).[141] Since most cardiovascular diseases occur principally in older individuals, this limits the translational capacity of these animal models. Additionally, younger animals likely have a higher regenerative/repair capacity, that alleviates organ damage over time, thereby complicating induction of morbidities, as has been observed in streptozotocin induced DM.[136] We observed a similar phenomenon after the induction of hypertension by CKD. Namely, in **chapter 7** we demonstrated that the mean arterial pressure increased (to ~110 mmHg) acutely after CKD-induction by subtotal renal embolization and remained elevated at least up to 12 weeks. However, at >20 weeks, mean arterial pressure (**chapter 8**) had returned to levels similar to control swine (~90mmHg). As summarized in **chapter 6**, there are multiple mechanisms by which CKD can induce CMD and cardiac diastolic dysfunction besides CKD-associated hypertension. Notwithstanding these methodological considerations, our triple morbidity model represents a relevant translational model for CMD with subsequent features of HFpEF and INOCA. Based on the findings in this thesis and findings discussed above, we recommend two different directions for future research.

Firstly, the current triple morbidity model has been phenotyped extensively and this animal model will prove a valuable tool to test new therapeutic strategies for CMD-associated syndromes, in particular HFpEF and INOCA. Depending on the disease of interest (INOCA or HFpEF) therapies that could be considered should target cardiac dysfunction, CMD or both. Previously we discussed sGC-activators and -stimulators as possible treatment options for CMD, given our findings in this thesis in conjunction with observations from other investigators. Such a compound, which targets both NO-cGMP signalling in resistance vessels (and thus INOCA) and in capillaries-cardiomyocytes (and thus HFpEF), should be investigated in future studies. Another class of drugs, sodium glucose cotransporter 2 inhibitors (SGLT2i), which were developed for the treatment of DM by promoting glycosuria and thus lowering of plasma glucose, may also target cardiac and vascular dysfunction directly. This class of drugs has been shown to lower the incidence of major adverse cardiac events in major clinical trials, independent of DM-status of the patients and their glucose-lowering effect.[142, 143] Indeed, a large clinical trial (EMPEROR-PRESERVED, NCT03057951) for the treatment of HFpEF with empagliflozin is currently being conducted.[144] However, exactly how SGLT2i influences cardiac function is presently unclear, although several mechanisms have been proposed and are worthy of further exploration.[144] Two of these proposed mechanisms are of interest for the findings in current thesis. Namely, improvement of myocardial oxygen efficiency by a metabolic substrate shift towards ketone bodies[145] and reduction of endothelial oxidative stress and thus improved endothelial function, NO-bioavailability and subsequent increased coronary blood flow.[146, 147] This combined effect on both cardiac and microvascular function makes SGLT2 inhibitors a promising therapy in cardiovascular diseases associated with CMD. In addition, compounds that improve mitochondrial function such as elamipretide which targets cardiolipin in cardiomyocytes are also of potential interest for the treatment of HFpEF.[148, 149] Since, mitochondrial dysfunction also likely contributes to the impaired myocardial oxygen balance in our triple morbidity model, this might be a good therapeutic option to test whether by improving mitochondrial function it can also improve myocardial oxygen balance and mitigate diastolic dysfunction. Finally, new therapeutic targets may arise from the current animal model. Thus, we are currently exploring an –omics approach, including transcriptomics, proteomics and metabolomics to find novel targets for HFpEF as well as for INOCA. These relatively novel techniques are developing to become widely applicable, sensitive and less expensive. These unbiased approaches may aid in discovering

new leads for pathophysiological mechanisms and therapeutic targets.[150] However, validation in human disease is needed to eliminate species-specific pathways or targets.

Secondly, improving the current animal model to further enhance translational power should help to narrow the translational gap, for instance by using older animals. Age can influence cardiovascular disease on multiple levels; the risk factors that a person develops with aging, which are mimicked in our studies by the induction of risk factors, age-related changes in sex hormones (i.e. puberty and menopause)[151] or alterations in the immune system, such as immune senescence.[152] This is just a selection of the age-related effects that influence cardiovascular (dys)function. While some of these factors might be possible to mimic also in young animals, such as menopause by ovariectomy, mimicking all of the age-related effects will be virtually impossible. Since using older animals in farm swine is practically challenging, switching to a miniswine, such as the Göttingen miniswine as employed in **chapter 4-5**, is a viable alternative. Another option is to genetically engineer swine to undergo accelerated aging, which has been successfully done in mouse models.[153, 154] In the last two decades, genetically engineering of swine has become easier, more accessible and successful, mostly due to CRISPR-Cas technology.[155] This has resulted in the development of new DM type 1 and type 2 swine models.[136] For instance, swine with a deficient glucose-dependent insulinotropic polypeptide receptor, showed a pre-diabetic phenotype with impaired glucose tolerance at younger age (11 weeks of age) that progressed to loss of pancreatic β-cell mass at 5 months and 11 months.[156] Interestingly, this model also developed renal dysfunction from a young age onward (8 weeks).[157] Such genetic-engineered models might be interesting to study in combination with a western diet to aggravate the cardiovascular phenotype and study cardiac and microvascular function, both at a young age, but especially at an older age. Altogether, genetic engineering of swine, particularly in a miniswine background, to make them susceptible to develop DM, obesity, dyslipidaemia and/or CKD, will tackle some of the remaining disadvantages of using swine for cardiovascular research and will further improve translation from pre-clinical research into the clinic.

Conclusion

The findings in this thesis indicate that (coronary) microvascular disease, due to metabolic derangement, is common in a variety of cardiovascular diseases. Chronic kidney disease, a common non-classical cardiovascular risk factor, can aggravate cardiovascular dysfunction in combination with metabolic derangement. The pathophysiological mechanisms involved in microvascular dysfunction are multifold and—importantly—appear to be organ-specific. CMD with a loss of NO-bioavailability is a hallmark of both HFpEF and INOCA and seems to be the linking factor between these syndromes, as we demonstrated in our newly developed multimorbidity swine model. Strikingly, in this model, pulmonary vascular disease was already present before overt left ventricular backward failure occurred, with increased vasoconstrictor influence of PDE5 and endothelin 1, distinctively different from the coronary circulation. Besides altering vascular tone control, metabolic derangement also affects coagulation homeostasis, another important endothelial (micro)vascular function. Factor VIII increased directly in response to metabolic derangement without an increase in von Willebrand Factor, the latter becoming increased when overt CMD and atherosclerosis were present. Altogether, these findings demonstrate the variety of functions of the microcirculation and show that metabolic derangements and CKD can disrupt these functions in an organ-specific manner, causing multiple clinical syndromes in need of further phenotyping and novel targeted treatment options.

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Appendix

Nederlandse samenvatting
Curriculum Vitae
List of publications
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Dankwoord

Nederlandse samenvatting

In 2018 waren naar schatting, in Nederland 1,6 miljoen mensen met cardiovasculaire ziekten waarvan 37.769 mensen zijn overleden (25% van alle sterfte in Nederland).[1] Volgens de World Health Organisation betreft de wereldwijde cardiovasculaire sterfte ongeveer 17.9 miljoen mensen per jaar, en is daarmee de meest voorkomende doodsoorzaak wereldwijd.[2] De belangrijkste risicofactoren voor het ontstaan van cardiovasculaire ziekten en uiteindelijk hartfalen zijn diabetes mellitus (DM), obesitas en hypertensie.[1,2] Deze risicofactoren zullende komende jaren naar verwachting alleen nog maar in prevalentie toe gaan nemen[3, 4], voornamelijk in ontwikkelingslanden met lage- tot middeninkomens.[5] Dit gaat gepaard met de gelijktijdige toename in sociaaleconomische status, zoals in landen als China en India.[5, 6] Hoe deze risicofactoren leiden tot cardiovasculaire ziekten, en met name tot hartfalen, is nog niet geheel opgehelderd, zeker als er meerdere risicofactoren in één patiënt voorkomen. Er wordt gedacht dat niet alleen de grote bloedvaten, maar vooral de microcirculatie gevoelig is voor metabole veranderingen en in een vroeg stadium wordt aangetast door deze risicofactoren. Een voorbeeld van een cardiovasculaire aandoening waar de microcirculatie is aangedaan, is hartfalen met een behouden ejectie fractie (HFpEF), wat in ongeveer de helft van de hartfalen patiënten voorkomt.[7] Het onderzoeken van de microcirculatie, zeker die van hart en longen, is in mensen niet eenvoudig vanwege het gebrek aan niet-invasieve, sensitieve methoden. Om de microvasculaire (dys)functie te onderzoeken, de pathofysiologie te begrijpen en om nieuwe therapieën te ontwikkelen zijn diermodellen nodig die deze cardiovasculaire ziekten met de onderliggende risicofactoren kunnen nabootsen. In dit proefschrift hebben we onderzoek gedaan naar de invloed van veelvoorkomende risicofactoren, zoals DM, dyslipidemie en chronische nierschade, op het ontstaan van cardiovasculaire ziekten en dit in twee verschillende delen besproken.

In **deel I** van dit proefschrift beschreven we wat de invloed is van de metabole veranderingen DM en dyslipidemie, op de myocardiale en coronaire functie. In **hoofdstuk 2** hebben we een overzicht gegeven van de verschillende mechanismen die microvasculaire dysfunctie kunnen induceren, zowel functioneel als structureel, in dyslipidemie geassocieerd met obesitas. In dit hoofdstuk gaven we weer dat, alhoewel er overeenkomstige mechanismen zijn in de verschillende organen die we beschreven hebben, er juist ook verschillende mechanismen tussen organen die een rol kunnen spelen. Dit benadrukt dat de microcirculaties van de verschillende organen uniek zijn, waarschijnlijk vanwege hun eigen kenmerkende fysiologie. In **hoofdstuk 3** gaven we een overzicht van verschillende kleine en grote diermodellen met metabole ontregeling met coronair microvasculaire dysfunctie (CMD) tot gevolg. Daarnaast beschreven we welke pathofysiologische mechanismen

aangetoond zijn in die diermodellen. We bespreken in dit hoofdstuk dat er tussen verschillende diersoorten een grote variatie bestaat in cardiovasculaire anatomie, fysiologie en metabolisme, en concluderen dat de grote diermodellen op deze vlakken het meest overeenkomstig zijn met de mens. Alhoewel kleine en grote diermodellen beide hun voor- en nadelen hebben, geen diermodel perfect de humane situatie nabootst en je keuze afhankelijk moet zijn van je onderzoeksvraag, zouden grote diermodellen over het algemeen de beste translatie naar de humane cardiovasculaire ziekten mogelijk maken. Daarom hebben we in het onderzoek beschreven in **hoofdstuk 4**, een Göttingen minivarken vijf maanden aan DM en dyslipidemie blootgesteld hebben om verandering in het linkerventrikel te kunnen bestuderen. Hier zagen we dat DM en dyslipidemie resulteerden in linkerventrikel diastolische dysfunctie, gemeten door middel van echocardiografie. Dit leek vooral het gevolg te zijn van intrinsieke veranderingen in de individuele cardiomyocyten, zoals toegenomen passieve kracht (stijfheid), aangezien er geen structurele veranderingen in het myocardium aangetoond konden worden. Tevens werd er toegenomen myocardiale oxidatieve stress aangetoond, welke geassocieerd zou kunnen zijn met de aanwezige mitochondriële dysfunctie. Daarnaast liet RNA-sequencing zien dat er 63 genen in het myocard anders gereguleerd waren en dat de glucosemetabolisme en vrije vetzuurmetabolisme netwerken vooral aangedaan waren. In **hoofdstuk 5** hebben we, in hetzelfde model, laten zien dat coronaire microvasculaire endotheelfunctie (nog niet) aangedaan was ten opzichte van gezonde controle varkens en dat er nog geen tekenen van coronaire atherosclerose waren. Dit was geassocieerd met een toename van factor VIII, terwijl von Willebrand Factor (VWF)-waarden onveranderd waren op dit tijdstip. Een langere (vijftien maanden) blootstelling aan dyslipidemie alleen of in combinatie met DM in boerderijvarkens, resulteerde wel in coronaire microvasculaire dysfunctie en atherosclerose met een gelijktijdige toename in VWF in de tijd, gedurende de blootstelling aan de risicofactoren. Dit suggereert dat een stijging van VWF mogelijk geassocieerd is met coronaire microvasculaire dysfunctie en/of atherosclerose, en niet direct met DM of dyslipidemie.

In **deel II** hebben we onderzocht wat de bijdrage is van chronische nierschade (CNS) naast DM, en dyslipidemie op de ontwikkeling van myocardiale, coronaire microvasculaire en pulmonale microvasculaire functie. In **hoofdstuk 6** hebben we een overzicht gegeven van hoe CNS kan lijden tot diastolische dysfunctie en HFpEF. Naast CNS-geïnduceerde hypertensie kan CNS via verschillende mechanismen HFpEF tot gevolg hebben. We hebben laten zien dat een deel van deze mechanismen hun effect direct op cardiomyocyten uitoefenen, terwijl het merendeel indirect hun effect uitoefenen via pro-inflammatie en CMD. Daarnaast hebben we in **hoofdstuk 6** een overzicht gegeven van reeds uitgevoerde klinische trials betreffende HFpEF en hebben we, op basis van de besproken mechanismen van CNS-geïnduceerde HFpEF, nieuwe behandelopties gesuggereerd die getest zouden

kunnen worden. **Hoofdstuk 7** beschrijven we een nieuw boerderijvarkenmodel waarin HFpEF werd geïnduceerd door middel van blootstelling aan DM, dyslipidemie en CNS, met een pro-inflammatoire staat tot gevolg. In dit model zagen we linkerventrikel diastolische dysfunctie, gemeten onder anesthesie met behulp van een druk-volume loop analyse, (grotere helling eind diastolische druk volume relatie). Daarnaast, vonden we structurele veranderingen in het myocardweefsel van deze dieren, namelijk een afname van capillaire dichtheid en toename in extracellulaire matrix. Tevens was hier sprake van een toegenomen passieve kracht van de individuele cardiomyocyten. Zoals eerder gezien in **hoofdstuk 4** was hier ook sprake van myocardiale oxidatieve stress, een verlies van stikstofmonoxide en dysregulatie van het enzym (eNOS), welke stikstofmonoxide produceert in de coronaire microcirculatie. Deze myocardiale afwijkingen waren geassocieerd met CMD, gemeten in geïsoleerde kleine coronaire arteriën. CMD leek dus te resulteren in het verlies van stikstofmonoxide geproduceerd door capillairen, welke normaal fungeert als signaalmolecuul voor cardiomyocyten, wat uiteindelijk leidde tot HFpEF. In **hoofdstuk 8** hebben we vervolgens in meer detail de cardiovasculaire functie onderzocht, in ditzelfde diermodel met drie risicofactoren, in de afwezigheid van atherosclerose. Dit hebben we gedaan met dieren in zowel wakkere toestand in rust als tijdens toenemende inspanning. De dieren met de risicofactoren toonden een afname van het hartminuutvolume, waardoor er een verstoring was in de systemisch zuurstofbalans met een verhoogde systemische lactaatproductie. Het linkerventrikel liet een verlaagde efficiëntie in zuurstofverbruik zien, welke in combinatie met een verlaagd zuurstofaanbod, door CMD, resulteerde in een verhoogde zuurstofextractie. Ondanks deze verhoogde extractie bleef er een tekort aan zuurstof, wat resulteerde in een verminderde myocardiale lactaatconsumptie, een teken van anaeroob metabolisme. Tevens werd een lichte mate van systolische linkerventrikelfunctie gezien tijdens rust en inspanning. De CMD, werd aangetoond door een afname in de farmacologisch-bepaalde coronaire flow reserve, en leek het gevolg te zijn van functionele veranderingen sinds er geen structurele veranderingen waren waargenomen. Dit model lijkt dus representatief te zijn voor CMD, waarbij de coronaire arteriolen en kleine coronaire arteriën zijn aangedaan, met daaruit volgend ischemie zonder obstructief coronair lijden (‘ischemia and no obstructive coronary artery disease’, INOCA). In **hoofdstuk 9** hebben we verder de CMD onderzocht in dit model met drie risicofactoren. Hierin lieten we zien dat CMD inderdaad functioneel van aard is en dat er een afname is van farmacologisch-geïnduceerde endotheel-afhankelijke relaxatie, gemeten in zowel in vivo in wakkere varkens als in vitro in geïsoleerde kleine coronair arteriën. De endotheel dysfunctie was het gevolg van een verminderde beschikbaarheid van stikstofmonoxide gemeten in vivo in rust en tijdens inspanning en in geïsoleerde kleine coronair arteriën, door middel van inhibitie van eNOS. Primair was dit niet het gevolg van een verandering in eNOS hoeveelheid of functie, maar door een verhoogde

oxidatieve stress welke stikstofmonoxide wegving. In de vasculaire gladde spiercel, verderop in de stikstofmonoxide signaleringscascade, bleek de phosphodiesterase-5 activiteit onveranderd. Vervolgens onderzochten wij in **hoofdstuk 10** de functie van de pulmonaire vaatbed in dit model. We lieten zien dat, ondanks dat er nog geen sprake was van linkerventrikelfalen met longoedeem, er al wel een verhoogde pulmonale vasculaire weerstand gemeten kon worden zowel in rust als tijdens inspanning. Histologisch onderzoek van de longen liet zien dat er geen veranderingen waren opgetreden in de pulmonale vaten die de verhoogde pulmonale vasculaire weerstand konden veroorzaken. Functioneel waren er geen veranderingen in stikstofmonoxide beschikbaarheid, terwijl er wel een toename was van phosphodiesterase-5 activiteit, wat zorgde voor verminderde cyclisch guanosinemonofosfaat beschikbaarheid en dus afgenomen vasodilatatie. Daarnaast was er ook een toename van circulerende endotheline-1 alsmede een toegenomen endotheline-1 gemedieerde pulmonale vasoconstrictie. Ondanks de toename in pulmonale vasculaire weerstand, was er op dit moment nog geen verandering is de functie van het rechterventrikel, zowel gemeten in rust als tijdens inspanning en onder anesthesie gemeten met MRI, wat suggereert dat het vroege pulmonale vasculaire dysfunctie betreft.

Concluderend laten we in dit proefschrift zien dat blootstelling aan veelvoorkomende risicofactoren zoals DM, obesitas en CNS een belangrijke rol spelen in de ontwikkeling van cardiovasculaire ziekten. Daarbij is er een belangrijke rol voor de microvasculatuur weggelegd die normaal gesproken de bloedtoevoer van organen en de uitwisseling van voedingsstoffen reguleert. Afhankelijk van het orgaan en van de grootte van de aangedane vaten kan dit resulteren in karakteristieke pathofysiologische veranderingen in orgaanfunctie en in karakteristieke cardiovasculaire syndromen zoals HFpEF of INOCA.

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Curriculum Vitae (Nederlands)

De auteur van dit proefschrift, Jens van de Wouw, werd geboren op 4 juli 1990 te 's-Hertogenbosch, Nederland. In 2008 behaalde hij zijn VWO diploma aan het Sint-Janslyceum te 's-Hertogenbosch, Nederland. Daarna heeft hij gedurende één semester een 'pre-medical program' gevolgd aan Green River College te Auburn, Washington, Verenigde Staten. In 2009 startte hij met de studie psychologie in Tilburg, Nederland. In 2010 maakte hij een overstap nadat werd hij toegelaten tot de geneeskundeopleiding aan het Erasmus Medisch Centrum te Rotterdam, Nederland. Tijdens zijn bachelor volgde hij het keuzeonderwijs '*Cardiovasculaire Aandoeningen*' en een minor '*Interne Geneeskunde*', waar hij een systematic review uitvoerde naar '*Non-Invasive Cardiac Output Measurements*' onder begeleiding van Dr. J. Alsma (internist acute geneeskunde). Door deze electieve onderwijsmomenten werd zijn interesse voor (cardiovasculaire) fysiologie gewekt. Tijdens zijn master geneeskunde (2013-2017) heeft hij, tevens onder begeleiding van Dr. J. Alsma, een multicenter onderzoek uitgevoerd naar somatisch onvoldoende verklaarde klachten op de spoedeisende hulp. Dr. J. Alsma heeft hem geïntroduceerd bij Dr. Anton van den Meiracker (vasculair internist, Erasmus MC), die betrokken was bij een onderzoeksproject naar de invloed van diabetes mellitus, hypercholesterolemie en chronische nierschade op de cardiovasculaire functie. Dit werd uitgevoerd op de afdeling Cardiologie, divisie Experimentele Cardiologie, Erasmus MC. Daaropvolgend startte hij in 2016 met zijn promotietraject op dit onderzoeksproject onder begeleiding van Prof. Dr. D.J. Duncker, Prof. Dr. D. Merkus en Dr. O. Sorop. De resultaten voortkomende uit dit promotietraject zijn in dit proefschrift beschreven. Na het afronden van zijn promotietraject zal hij gaan werken als ANIOS op de afdeling interne geneeskunde, om vervolgens te solliciteren voor de opleiding tot internist. Hij woont samen met zijn partner, Renée Hogenhout, te Rotterdam.

Curriculum Vitae (English)

The author of this thesis, Jens van de Wouw, was born on July 4th 1990 in ’s-Hertogenbosch, the Netherlands. In 2008, he completed his pre-university education at Sint-Janslyceum in ’s-Hertogenbosch, the Netherlands. Afterwards he followed one semester of a ‘pre-medical program’ at Green River College, Auburn, Washington, USA. In 2009, he started a bachelor in psychology, at Tilburg University, the Netherlands. In 2010, he got enrolled into the bachelor of medicine at Erasmus University Medical Centre Rotterdam, the Netherlands. During his bachelors, he followed the elective course ‘Cardiovascular Diseases’ and the minor ‘Internal Medicine’, during the latter he conducted a systematic review on ‘Non-Invasive Cardiac Output Measurements’ under supervision of Dr. J. Alsma (internist acute medicine). He got interested in (cardiovascular) physiology due to these elective courses. During his master in medicine (2013-2017) he conducted, also under supervision of Dr. J. Alsma, a multicentre study investigating medically unexplained physical symptoms at the emergency department. Dr. J. Alsma introduced him to Dr. Anton van den Meiracker (vascular internist, Erasmus MC), who was involved in a research project investigating the effect of diabetes mellitus, hypercholesterolemia and chronic kidney disease on cardiovascular function. This project was being conducted at the Department of Cardiology, Division of Experimental Cardiology, Erasmus MC. Subsequently, he started in 2016 with his PhD-project under supervision of Prof. Dr. D.J. Duncker, Prof. Dr. D. Merkus and Dr. O. Sorop. The results from his PhD-project are described in this PhD-thesis. After finishing his PhD-project, he will start working as a resident at the department of internal medicine and he will apply for the training to become an internist. He lives together with his partner, Renée Hogenhout, in Rotterdam.

List of Publications

Published articles:

Cellular, mitochondrial and molecular alterations associate with early left ventricular diastolic dysfunction in a porcine model of diabetic metabolic derangement.

Heinonen I, Sorop O, van Dalen BM, Wüst RCI, **van de Wouw J**, de Beer VJ, Octavia Y, van Duin RWB, Hoogstrate Y, Blonden L, Alkio M, Anttila K, Stubbs A, van der Velden J, Merkus D, Duncker DJ.
Sci Rep. 2020 Aug 6;10(1):13173. doi: 10.1038/s41598-020-68637-4. PMID: 32764569

Both male and female obese ZSF1 rats develop cardiac dysfunction in obesity-induced heart failure with preserved ejection fraction.

Nguyen ITN, *Brandt MM, ***van de Wouw J**, van Drie RWA, Wesseling M, Cramer MJ, de Jager SCA, Merkus D, Duncker DJ, Cheng C, Joles JA, Verhaar MC.
PLoS One. 2020 May 6;15(5):e0232399. doi: 10.1371/journal.pone.0232399. PMID: 32374790

Perturbations in myocardial perfusion and oxygen balance in swine with multiple risk factors: a novel model of ischemia and no obstructive coronary artery disease.

van de Wouw J, Sorop O, van Drie RWA, van Duin RWB, Nguyen ITN, Joles JA, Verhaar MC, Merkus D, Duncker DJ.
Basic Res Cardiol. 2020 Feb 25;115(2):21. doi: 10.1007/s00395-020-0778-2.PMID: 32100119

Experimental animal models of coronary microvascular dysfunction.

*Sorop O, ***van de Wouw J**, Chandler S, Ohanyan V, Tune JD, Chilian WM, Merkus D, Bender SB, Duncker DJ.Cardiovasc Res. 2020 Mar 1;116(4):756-770. doi: 10.1093/cvr/cvaa002. PMID: 31926020

Chronic Kidney Disease as a Risk Factor for Heart Failure With Preserved Ejection Fraction: A Focus on Microcirculatory Factors and Therapeutic Targets.

***van de Wouw J**, *Broekhuizen M, Sorop O, Joles JA, Verhaar MC, Duncker DJ, Danser AHJ, Merkus D.
Front Physiol. 2019 Sep 4;10:1108. doi: 10.3389/fphys.2019.01108. PMID: 31551803

Limited synergy of obesity and hypertension, prevalent risk factors in onset and progression of heart failure with preserved ejection fraction.

Brandt MM, Nguyen ITN, Krebber MM, **van de Wouw J**, Mokry M, Cramer MJ, Duncker DJ, Verhaar MC, Joles JA, Cheng C.

J Cell Mol Med. 2019 Oct;23(10):6666-6678. doi: 10.1111/jcmm.14542. PMID: 31368189

Multiple common comorbidities produce left ventricular diastolic dysfunction associated with coronary microvascular dysfunction, oxidative stress, and myocardial stiffening.

Sorop O, Heinonen I, van Kranenburg M, **van de Wouw J**, de Beer VJ, Nguyen ITN, Octavia Y, van Duin RWB, Stam K, van Geuns RJ, Wielopolski PA, Krestin GP, van den Meiracker AH, Verjans R, van Bilsen M, Danser AHJ, Paulus WJ, Cheng C, Linke WA, Joles JA, Verhaar MC, van der Velden J, Merkus D, Duncker DJ.

Cardiovasc Res. 2018 Jun 1;114(7):954-964. doi: 10.1093/cvr/cvy038. PMID: 29432575

Medically unexplained physical symptoms in patients visiting the emergency department: an international multicentre retrospective study.

Alsma J, **Wouw JV**, Jellema K, Coffeng SM, Tobback E, Delesie L, Brand CLVD, Vogelaers D, Mariman A, Paepe P, van Saase JL, Weiland A.

Eur J Emerg Med. 2019 Aug;26(4):249-254. doi: 10.1097/MEJ.0000000000000536. PMID: 29360692

The microcirculation: a key player in obesity-associated cardiovascular disease.

Sorop O, Olver TD, **van de Wouw J**, Heinonen I, van Duin RW, Duncker DJ, Merkus D.

Cardiovasc Res. 2017 Jul 1;113(9):1035-1045. doi: 10.1093/cvr/cvx093.PMID: 28482008

Published book sections:

Coronary Microvascular Dysfunction in Cardiovascular Disease: Lessons from Large Animal Models.

Sorop O, **Van de Wouw J**, Merkus D, Duncker DJ. In: Microcirculation: From Bench to Bedside. 1st ed. Editors: Dorobantu M. Badimon L. Cham, Switzerland: Springer Nature, 2020. doi: 10.1007/978-3-030-28199-1

Articles accepted for publications:

A Direct Comparison of Natural and Acoustic-Radiation-Force-Induced Cardiac Shear Waves.

Keijzer LBH, Caenen A, Voorneveld J, Strachinaru M, Bowen DJ, **van de Wouw J**, Sorop O, Merkus D, Duncker DJ, van der Steen AFW, de Jong N, Bosch JG, Vos HJ

Scientific Reports invited revision

Articles under review:

Reduced Nitric Oxide Bioavailability Impairs Myocardial Perfusion in Exercising Swine with Multiple Common Risk Factors.

van de Wouw J, Sorop O, van Drie RWA, Joles JA, Danser AHJ, Verhaar MC, Merkus D, Duncker DJ.

Basic Research in Cardiology invited revision

Impaired Pulmonary Vasomotor Control in Exercising Swine with Multiple Comorbidities.

***van de Wouw J**, *Steenhorst JJ, Sorop O, van Drie RWA, Wielopolski PA, Hirsch A, Duncker DJ, Merkus D.

Basic Research in Cardiology invited revision

Endothelial dysfunction and atherosclerosis increase von Willebrand factor and Factor VIII: a randomized controlled trial in swine.

*Atiq F, ***van de Wouw J**, Sorop O, Heinonen I, de Maat MPM, Merkus D, Duncker DJ, Leebeek FWG.

Thrombosis and Haemostasis invited revision

Invited Review:

Albumin: An Interface or a Sponge?.

van de Wouw J and Joles JA

Invited Review for Kidney International

PhD Portfolio

Name PhD-candidate:	Jens van de Wouw
Erasmus MC Department:	Department Cardiology, Division of Experimental Cardiology
Research School:	COEUR
PhD period:	2016-2020
Promotor 1:	Prof. Dr. D.J. Duncker
Promotor 2:	Prof. Dr. D. Merkus
Co-promotor	Dr. O. Sorop

Courses	**Year**	**ECTS**
Pathophysiology of ischemic heart disease Erasmus MC	2018	1.5
Article 9 course (small rodents) Erasmus MC	2016	4.5
Article 9 course (pig specific course) UMC Utrecht	2017	1.0
Basic-Training Ultrasound Erasmus MC	2017	0.6
NHS Course: Cardiac Function and Adaptation	2017	2.0
Hands-on training in systematic reviews of animal studies Radboud UMC	2018	0.2
Basic course on Radiation protection Erasmus MC	2018	0.2
English Biomedical Writing and Communication Erasmus MC	2018	4.0
Pathophysiology of ischemic heart disease Erasmus MC	2018	1.5
NHS Course: Vascular Biology	2018	2.0
	Total Courses:	**19.5**

Research Seminars and Lectures	**Year**	**ECTS**
COEUR PhD-day “valuable science”	2017	0.4
Sex and Gender in Cardiovascular Research	2018	0.5
COEUR PhD-day “responsible science”	2018	0.4
Prof. G. Kararigas “Sex-specific remodeling of the healthy and diseased heart”	2017	0.1
Prof. R. Kramann “Perivascular progenitor cells in cardiovascular and fibrotic disease”	2017	0.1
Prof. G. Agnetti “Understanding Heart Failure and the Function of Intermediate Filaments”	2020	0.1
	Total Research and Lectures:	**1.6**

Symposia and Congresses	**Year**	**ECTS**
Dutch Physiology days Maastricht	2016	0.6
Experimental Biology Chicago	2017	1.5
Netherlands Heart Institute 1st translational cardiovascular research meeting Utrecht	2017	0.6
Young@Heart Utrecht	2017	0.3
Frontier on CardioVascular Biology Vienna	2018	0.9
Netherlands Heart Institute 2nd translational cardiovascular research meeting Utrecht	2017	0.6
International Society for Heart Research European Section Meeting Utrecht	2018	1.2
European Society of Cardiology Congress Munich	2018	1.5
American Heart Association Scientific Meeting Chicago	2018	0.9
Dutch Endothelium Biology Society meeting Amsterdam	2018	0.9
	Total Symposia and Congresses:	**22.5**

Supervising Students and Teaching	**Year**	**ECTS**
Bachelor Thesis M. Sahin	2017	1.2
Bachelor Thesis D. Groeneveld	2017-2018	1.6
Bachelor Thesis M. Koster	2018-2019	1.2
Master Thesis F. Leandre	2019	0.6
Mentor Medicine Students Erasmus MC	2018-2020	1.2
Systematic Review Writing Medicine Students Erasmus MC	2018-2020	0.6
Teaching Exercise Physiology Medicine Students Erasmus MC	2019-2020	1
	Total Supervising Student and Teaching:	**7.4**

Total ECTS:	**51**

Awards	**Year**
First poster prize (€1000) 1st Translational Cardiovascular Research Meeting	2017
Best poster presentation (audience award) the PhD-course Dutch Heart Foundation	2017
Poster prize (€250) the International Society for Heart Research European Section Meeting	2018
Experimental Biology Young Investigator Travel Award	2019
Frontier in CardioVascular Biomedicine Travel Award by *Working Group: Coronary Pathophysiology and Microcirculation*	2018+ 2020
Scientific Sessions Travel Award by *Council on Atherosclerosis Thrombosis and Vascular Biology*	2018

Dankwoord

Het doen van goed wetenschappelijk onderzoek en daarmee ook het halen van een PhD is een teamprestatie. In het huidige wetenschappelijk klimaat is het noodzakelijk om samen te werken met experts uit verschillende gebieden. Zeker in translationeel onderzoek waarin we de brug proberen te slaan tussen fundamentele ontdekkingen en klinische vraagstukken. Tijdens mijn PhD heb ik mogen samenwerken met veel verschillende mensen, die ik graag daarvoor zou willen bedanken.

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Beste Dirk, mijn eerste ontmoeting met jou was tijdens de colleges in het eerste jaar. Daar stond je vol overtuiging en passie te vertellen over coronaire flowregulatie en linkerventrikelfunctie. Wellicht dat mijn interesse voor cardiovasculaire fysiologie daar al werd gewekt. Je bent een echte motivator en kan iedereen enthousiast krijgen voor nieuwe resultaten en nieuwe onderzoekideeën. Daarnaast heb je me geleerd altijd kritisch te denken en over alles te durven twijfelen, zowel over je eigen resultaten als die van anderen. Tevens ben ik onder de indruk van je punctualiteit en je oog voor detail. Hoe vaak ik wel niet bolletjes van 5 naar 4 heb gezet is niet op de handen van de gehele commissie te tellen. Ik hoop nog een hoop van je te kunnen leren in de toekomst. Bedankt voor de energie die je me hebt gegeven tijdens mijn promotietraject en de tijd en energie die je in de inhoud van dit proefschrift hebt gestoken.

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Daarnaast wil ik graag alle leden van mijn promotiecommissie, prof.dr. Verhaar, prof.dr. Danser, prof.dr. Zijlstra, dr. Eringa, dr. Joles bedanken voor het lezen en beoordelen van mijn proefschrift. Ik zie uit naar de gedachtewisseling.

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Mijn paranimfen Jarno en Tim wil ik graag bedanken dat ze naast mijn zijde zullen staan tijdens de gedachtewisseling.

Jarno, de man met vele namen. Niemand op de afdeling weet helemaal zeker hoe je nou echt heet, Jarno, Jors, Jurriën of toch gewoon Jens? Sterker nog, ik heb je een half jaar gemaild en kreeg nooit een mailtje terug. Nou ja uiteindelijk kreeg ik een mailtje dat ik moest stoppen met haar?? te mailen. We hebben veel leuke momenten met elkaar meegemaakt tijdens onze promotietrajecten, eigenlijk teveel om er maar eentje uit te lichten. We zijn over de loop de jaren ook goede vrienden geworden. Succes met je laatste jaar (<12 maanden…) als arts-onderzoeker op de experimentele cardiologie. Ik weet zeker dat je ook een prachtig proefschrift zult afleveren met kwalitatief goed en uniek onderzoek. Ik hoop dat we nog vaak na het werk samen op een terras/borrel/feestje terecht komen.

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Michelle, we hebben direct samen mogen werken aan ons review over HFpEF en CKD. Maar daarnaast zijn we regelmatig samen op pad geweest. Je bent altijd wel te porren voor een klein borreltje op vrijdagmiddag of een wat groter borreltje in het liftencomplex van de Ee-toren. Veel succes met het afronden van jouw PhD, alhoewel ik er niet aan twijfel dat het helemaal goed gaat komen.

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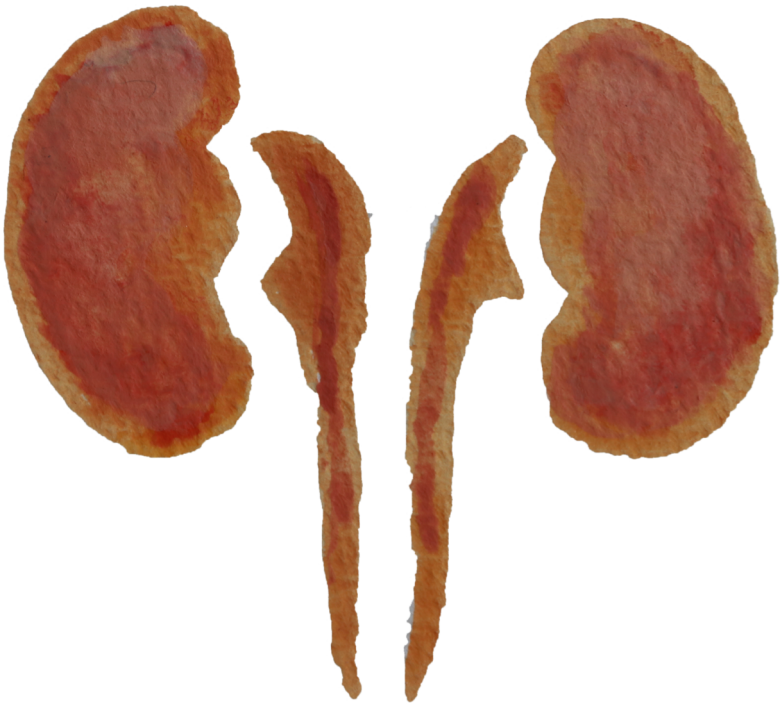

‘There is no science which does not spring from pre-existing knowledge’

- W. Harvey