

Insulin resistance and cardiovascular function

Observational, translational and
interventional studies

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Gothenburg, Sweden, 2016



UNIVERSITY OF
GOTHENBURG

Cover illustration by Cathrine Rohman-Fransson, Cat Kalligrafi

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– **Observational, translational and interventional studies**

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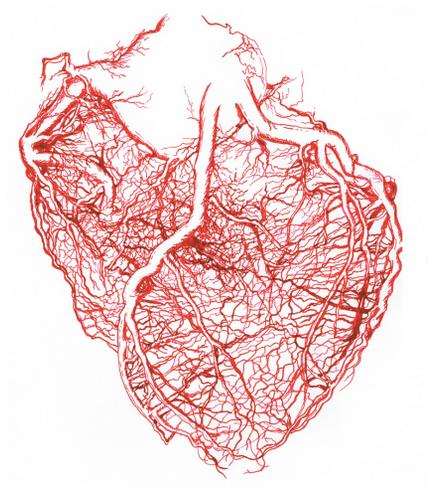
ISBN 978-91-628-9846-5 (Print)

ISBN 978-91-628-9847-2 (PDF/online)

E-publication: <http://hdl.handle.net/2077/42341>

Printed in Gothenburg, Sweden 2016

Ineko AB



*There is more to the
heart
than meets the eye*

Till min familj

– You miss a 100% of the shots you don't take
“Wayne Gretzky”

Abstract

Background: Microvascular disease is now recognized as an important driver for cardiovascular mortality and morbidity. Diabetic patients are known to suffer from this condition, leading to e.g. coronary ischemia as well as kidney dysfunction. Accumulating evidences indicate that the vascular pathological alterations may be a direct consequence of impaired glucose homeostasis and may occur long before diabetes is diagnosed. Early risk identification and a better understanding of associated mechanisms could be of great importance in disease management. Thus, the overall hypothesis of this thesis was that impaired glucose homeostasis already in the non-diabetic stage is associated with coronary and peripheral microvascular dysfunction and an unfavorable systemic risk profile, possibly facilitating progression of cardiovascular disease. For translational understanding, we hypothesized that obese insulin resistant leptin-deficient (ob/ob) mice could be a potential model for microvascular dysfunction and associated mechanisms. Finally, we hypothesized that short-term personalized lifestyle intervention may improve coronary microcirculation in healthy subjects.

Summary of results: My thesis shows that high insulin resistance assessed by the Homeostatic model assessment for insulin resistance (HOMA-IR) added independent prognostic value in patients with chest pain without myocardial perfusion defects. HOMA-IR was inversely associated with decreased peripheral vascular function, increased systemic pro-inflammatory state and decreased levels of pro-angiogenic vascular growth factors (Paper I). Also, impaired coronary flow reserve (CFR) predicted cardiovascular outcome in these patients and HOMA-IR was the strongest biochemical marker associated with decreased CFR. Interestingly, upon gender specific analysis, HOMA-IR seemed to be the strongest predictor of decreased CFR in men while systolic blood pressure was the strongest predictor in women (Paper II). Furthermore, impaired CFR and increased renal vascular resistance were observed in the ob/ob mice compared to lean controls. Possible mechanisms behind these observations were an impaired nitric oxide pathway as well as decreased renal vascular density (Paper III). Finally, CFR was improved with a personalized and supervised exercise and diet program in healthy volunteers (Paper IV).

Conclusions: This thesis suggests that insulin resistance measured by HOMA-IR confers independent prognostic information in non-diabetic pa-

tients with chest pain without myocardial perfusion defects. Furthermore, increased HOMA-IR is associated with poor cardiovascular status and there seems to be gender specific mechanisms associated with coronary microvascular dysfunction. In addition, the ob/ob mice may be a useful translational model for interventional studies to improve understanding of microvascular complications in impaired glucose homeostasis. Finally, three months of personalized life style intervention can enhance cardiovascular function in healthy subjects.

Keywords

Insulin resistance, microvascular function, peripheral vascular function, coronary flow reserve, prognosis, myocardial perfusion scintigram, animal model

Sammanfattning på svenska

Hjärtat består av kärl i flera storlekar, allt ifrån de största artärerna till de minsta kapillärerna. Det har länge varit känt att flödesbegränsande åderförkalkning i hjärtats kranskärl leder till syrebrist. Idag finns avancerade metoder för att lokalisera och åtgärda förträngningar i de stora kärlen vilket varje år räddar många människors liv. Intressant nog består hjärtats kärlbädd till ca 90% av småkärl (mikrovaskulära kärl) som traditionella metoder inte kan visualisera. Fokus riktas nu till de patienter med bröstsmärta som saknar flödesbegränsande förträngningar i de stora kranskärlen, men fortfarande har en misstanke om pågående syrebrist i hjärtat till följd av mikrovaskulär kranskärlsjukdom. Detta kan istället undersökas med andra bildgivningsmetoder så som kranskärlsultraljud.

Risikfaktorer för prediktion av hjärtkärlsjukdom och överlevnad är viktiga hjälpmedel för vårdpersonal och specifika riskfaktorer för mikrovaskulär kranskärlsjukdom är efterfrågade. Typ 2 diabetes är en sjukdom förenad med accelererad utveckling av kärldysfunktion och mikrovaskulära komplikationer samt en ökad risk för både hjärtkärlsjukdom och död. Det är välkänt att patienter med typ 2 diabetes har en minskad känslighet för insulin och försämrad glukoskontroll flera år innan de får sin diagnos. Denna okänslighet för insulin kallas insulinresistens och beskrivs som minskad förmåga till glukosupptag som svar på en normal insulin koncentration. Detta resulterar i högre cirkulerande blodnivåer av glukos och förhöjd produktion av insulin. Insulinresistens på en icke-diabetisk nivå är också associerat till ogynnsam påverkan på hjärta och kärl.

I denna avhandling har vi studerat icke-diabetiska patienter med bröstsmärta remitterade för misstänkt kranskärlsjukdom, men där man ej kunnat påvisa flödesbegränsande åderförkalkning. Vi fann att flera av dessa patienter har mikrovaskulär kranskärlsjukdom och att insulinresistens är viktig för prediktion av hjärtkärl-relaterade händelser så som död, hjärtinfarkt och kärlkramp. Intressant nog fann vi också att insulinresistens verkar vara en starkare riskfaktor hos män, medan förhöjt blodtryck var förenat med ökad association till mikrovaskulär sjukdom hos kvinnor. Det är känt att förhöjt blodtryck leder till dysfunktion i kärlen och dess koppling till mikrovaskulär sjukdom är högst relevant. Vid behov av ökat blodflöde har de mikrovaskulära kärlen en förmåga att vidga sig för att möta upp kravet. Vi fann att pati-

enter med ökad insulinresistens hade försämrad förmåga att vidga kärlen samt en låggradig systemisk inflammation och minskad möjlighet att bilda nya kärl. Alla dessa mekanismer kan tillsammans försämra funktionen hos de mikrovaskulära kärlen och slutligen leda till syrebrist i vävnaden.

För ökad förståelse kring bakomliggande mekanismer samt studera potentiella läkemedel är humanlika djurmodeller avgörande. Vi har med samma ultraljudsteknik studerat den mikrovaskulära kranskärlsfunktionen hos insulinresistenta möss. Vi fann att dessa möss har kranskärlsdysfunktion och att en potentiell bakomliggande mekanism är minskad förmåga att producera kväveoxid. Kväveoxid är viktigt för kärlens förmåga att vidga sig och vid brist dras småkärlen istället samman och blodflödet till muskel- och fettvävnad minskar, vilket tillslut kan leda till syrebrist.

Slutligen är det av stor vikt för individen, sjukvården och samhället att förebygga framtida utveckling av hjärtkärlsjukdom i den friska populationen. Preventiva åtgärder har visat sig ge effekt och idag tillhör fysisk aktivitet och rätt kost grundstenar för en god hälsa. Vi avsåg att med två olika program studera huruvida en livsstilsförändring innefattande kost och motion förbättrar den mikrovaskulära kranskärlsfunktionen hos friska individer. Vi rekryterade friska otränade försökspersoner, och lottade halva gruppen till att genomgå ett standard-träningsprogram med tillgång till lokalt gym samt övergripande medelhavsinspirerade kostråd. Den andra gruppen fick hemskickade lättillgängliga träningsprogram via sociala medier, hade personlig kontakt med professionell instruktör veckovis, samt fick mer detaljerade medelhavsdietinspirerade kostråd. Träningstiden för båda programmen var totalt tre timmar per vecka i tre månader. Resultaten visar att det personliga tränings- och kost programmet förbättrade den mikrovaskulära kranskärlsfunktionen jämfört med både en kontrollperiod och standard programmet.

Sammanfattningsvis visar mitt avhandlingsarbete att redan på ett icke-diabetiskt stadium har ökad insulinresistens negativ betydelse för mikrovaskulära kärlfunktionen och innebär en ökad risk för utveckling av hjärtkärlsjukdom. Den visar också att det kan finnas en könsrelaterad skillnad i riskfaktorer och att utöver insulinresistens har högt blodtryck viktig betydelse hos framförallt kvinnor. Slutligen visar jag att ett lättillgängligt träningsprogram innefattande kontinuerlig kontakt med personlig tränare och medelhavsinspirerade kostråd förbättrar hjärtkärlfunktionen hos friska på bara tre månader.

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

- I. Westergren HU, Svedlund S, Momo RA, Blomster JI, Wåhlander K, Rehnström E, Greasley PJ, Fritsche-Danielson R, Oscarsson J, Gan LM.

Insulin resistance, endothelial function, angiogenic factors and clinical outcome in non-diabetic patients with chest pain without myocardial perfusion defects

Cardiovascular Diabetology 2016;15: 36.

- II. Westergren HU, Michaëlsson E, Blomster JI, Miliotis T, Svedlund S, Gan LM.

Determinants of coronary flow reserve in non-diabetic patients with chest pain without myocardial perfusion defects

Resubmitted to PLoS One 2016.

- III. Westergren HU, Grönros J, Heinonen SE, Miliotis T, Jennbacken K, Sabirsh A, Ericsson A, Jönsson-Rylander AC, Svedlund S, Gan LM.

Impaired Coronary and Renal Vascular Function in Spontaneously Type 2 Diabetic Leptin-Deficient Mice

PLoS One 2015;10(6): e0130648.

- IV. Westergren HU, Gan LM, Månsson M and Svedlund S.

Effects of a Personalized Supervised Lifestyle Intervention Program on Cardiovascular Status in Sedentary Healthy Volunteers

Submitted 2016.

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Abbreviations

ADMA	Asymmetric dimethylarginine
CFR	Coronary flow reserve
CI	Confidence interval
DA	Discriminant analysis
DAPS	Diagnosis, Analysis, Personalization and Supervision
ECG	Electrocardiogram
EIF2	Eukaryotic initiation factor 2
HbA_{1c}	Haemoglobin A1c
HOMA-IR	Homeostatic model assessment for insulin resistance
HR	Hazard ratio
IMT	Intima media thickness
IQR	Interquartile range
MAPK	Mitogen activated protein kinase
OPLS	Orthogonal projection to latent structures by partial least square analysis
PI3K	Phosphatidylinositol 3 kinase
PLS	Partial least squares
VGEF	Vascular endothelial growth factors
WHO	World Health Organization

Introduction

General background underlying the current thesis

The definition of cardiovascular disease according to the World Health Organization (WHO) includes, the for many people frightening condition, coronary heart disease. The main focus of this condition has traditionally been on atherosclerosis burden and narrowing of the coronary arteries, leading to obstructive coronary artery disease. The progression of this pathology in patients is important and life threatening. However, the significance of coronary microvascular disease is now accepted and gaining increased attention. Indeed emphasizing its importance, the 2013 European Society of Cardiology guidelines include microcirculatory pathology as a possible feature underlying stable coronary artery disease (1).

Coronary microvascular disease, commonly called small vessel disease involves the network of arterioles, capillaries and venules, holding approx. 90% of the total myocardial blood volume. Dysfunction in the cardiac microcirculation can result from both structural as well as functional abnormalities and have been elusive investigation with traditional imaging techniques (2). Today, functional tests allow examination of coronary as well as peripheral microcirculation. In combination with traditional techniques, they have increased the possibility of detecting impaired microvascular function both in patients with and without flow-limiting, obstructive coronary artery disease. In 2007, four main types of classifications of coronary microvascular dysfunction were proposed based on the clinical setting in which it occurs: (i) coronary microvascular dysfunction in the absence of myocardial diseases and obstructive coronary artery disease, (ii) coronary microvascular dysfunction in myocardial diseases, (iii) coronary microvascular dysfunction in obstructive coronary artery disease, and (iv) iatrogenic coronary microvascular dysfunction (3). In this thesis the focus lies on (i) coronary microvascular dysfunction in the absence of myocardial diseases and obstructive coronary artery disease. In addition, the microcirculation is important not only in the heart but also in e.g. the kidney and peripheral extremities. Indeed, patients with coronary microvascular dysfunction have attenuated peripheral microvascular function (4). The systemic nature of microvascular

dysfunction is important and possible to study by combinations of imaging and functional techniques, a possibility taken advantage of in the current thesis.

Importantly, not only addressing microvascular dysfunction in the absence of obstructive coronary artery disease is of significance, but also evaluating possible risk factors associated with this disorder. Being able to do so, we must reflect on the multiple mechanisms known to contribute to microvascular dysfunction. These include oxidative stress, impaired vasomotor function, leukocyte-endothelial cell adhesion, endothelial dysfunction, altered microvessel density and thrombosis, all induced in e.g. hypertension, hypercholesterolemia, diabetes and obesity (5). Indeed, risk factors observed in patients with metabolic syndrome might have a greater impact on endothelial function than other risk factor combinations (6). Both traditional and non-traditional risk factors related to atherosclerosis and cardiovascular morbidity and mortality are associated with endothelial dysfunction (6). Traditional risk factors were recently shown to be less pronounced in coronary microvascular dysfunction (7) and the complement of additional biomarkers seems important (8, 9). Therefore, improved risk stratification is of value for guiding medical care in patients with suspected non-obstructive coronary artery disease. In the current thesis, we therefore aimed to further address the need of validated risk factors in non-diabetic patients without obstructive coronary artery disease, with focus on insulin resistance.

Finally, the importance of promoting a healthy lifestyle and preventing the progress of cardiovascular disease is well known and has been endorsed by recent guidelines (10). The development of cardiovascular disease is strongly connected to both an unhealthy dietary habit and physical inactivity, among others. The WHO states that healthy diets and physical activity are keys to good nutrition and necessary for a long and healthy life. However, the prevention of cardiovascular disease remains challenging (11). WHO recommends adults to perform exercise training at the minimum of 150 min per week, e.g. 30 min daily, 5 days a week (12). The percentage of citizens fulfilling these guidelines was recently reported high in Northern European countries. However, still an average of 28% of the adults in Europe do not comply with these recommendations (13). The challenge in fulfilling a program in the long term is sometimes eased with continuous feedback and personalization. Thus, the final paper implements a personalized, supervised lifestyle intervention program in sedentary healthy volunteers for studying its impact on coronary and peripheral vascular function.

Cardiovascular disease

The overall mortality rate due to cardiovascular disease is still higher than any other disease in Europe. In Europe, 43% among men and 51% among women die from cardiovascular disease, compared with 19% and 23%, respectively, for all cancers. In comparison to cerebrovascular- and other cardiovascular diseases, coronary heart disease causes the highest number of deaths in both men and women and accounts for almost 1.8 million deaths, or 20% of all deaths in Europe annually. Despite that the rate is decreasing in many European countries and our neighbours Norway and Denmark now belongs to the predominantly “high income” countries holding the lowest rate of mortality due to coronary heart disease (14), still a large proportion of the European populations will lose their lives prematurely due to heart disease. Subsequently, specific understanding of different subgroups of patients included in the broad field of this disease is of value. Historically, the main focus for coronary heart disease has been on atherosclerosis burden and obstruction of the coronary arteries. During the last decades, the importance of the coronary microvasculature has been highlighted (1) and at present time, many studies are performed within this area.

Microcirculation

The microcirculation represents about 7% of the human body volume with a ubiquitously distributed complex but highly organized branching pattern (15). The microcirculation consists of the vascular network build-up between the arteries and veins roughly defined to include small arterioles, capillaries and small venules (16). The microvessels have a high degree of heterogeneity meeting the regional variation in distances to the supplying tissue as well as to adapting to local metabolic demands and mechanic stimuli (17). The role of the microcirculation includes supplying oxygen, nutrients and hormones to the tissues, but also removing waste products, controlling inflammation, repair and fluid exchange within the surrounding tissue (18, 19). Furthermore, the quantitatively most substantial drop in hydrostatic pressure occurs at the level of the microcirculation and avoidance of large fluctuations in hydrostatic pressure at the capillary level and determining the overall peripheral resistance is important (20, 21).

All vessels have a vascular endothelium forming a monolayer of cells between the vessel lumen and the vascular smooth muscle cells or tissue, known to have an important role in modulation of both vascular function and structure (19). The healthy endothelium regulates vascular smooth muscle cell proliferation (22). In addition, it has anticoagulant and non-thrombogenic properties, including low-level expression of adhesion molecules maintaining a physiological

interaction between the endothelial cells and e.g. leukocytes and platelets. The endothelium also has an important role in controlling permeability across the vessel wall as well as in angiogenesis (the sprouting of microvessels from pre-existing vessels), a complex process tightly regulated by e.g. vascular endothelial growth factors (VEGF) (19).

Finally, the balance between vasodilation and vasoconstriction controls the vascular tone. This is further determined by the contractile state of vascular smooth muscle, regulated by e.g. (i) intrinsic properties of the vascular smooth muscle cells (myogenic tone), (ii) metabolic signals from adjacent tissue, (iii) endothelial cells influenced by the forces from the flowing blood and involved in signalling along the vessel wall (23) (Figure 1).

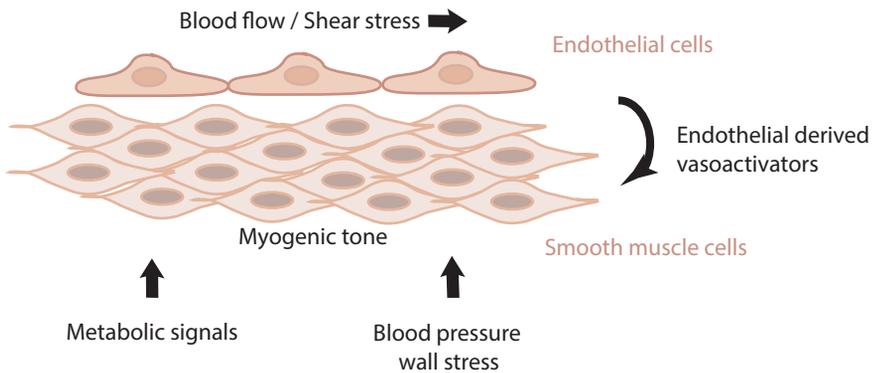


Figure 1 Mechanisms influencing microvascular tone

Both endothelial and smooth muscle cells responds to stimuli associated with regulation of microvascular tone, including hemodynamic forces as well as metabolic signals. Furthermore, endothelial cells produce vasoactive substances, influencing the vascular tone of the smooth muscle cells.

Both prostacyclin and endothelium-derived hyperpolarizing factor are able to modulate vascular tone by increased dilation. However, the predominant vasodilator released from endothelial cells is nitric oxide (24, 25). Nitric oxide is generated by endothelial cells from L-arginine by nitric oxide synthase, which converts L-arginine to nitric oxide and citrulline (26). Nitric oxide is released from endothelial cells mainly in response to shear stress elicited by the circulating blood. Nitric oxide diffuses to and stimulates relaxation of vascular smooth muscle cells. Additionally, nitric oxide also prevents leukocyte adhesion and migration, smooth muscle cell proliferation, platelet adhesion and aggregation, having an overall anti-atherogenic effect (27). In contrast to the vasodilating effect of nitric oxide, the endothelium-derived endothelin-1 has a vasocon-

striction effect. In the healthy condition, the balance between nitric oxide and endothelin-1 preserves the bioavailability of nitric oxide, favouring vasorelaxation (28).

Coronary microcirculation

The coronary circulation is unique in that sense that its perfusion is obstructed during the systolic phase of the cardiac cycle by the surrounding contracting muscle. During the systolic contraction of the left ventricle, the intramyocardial microvessels compress, closing the coronary arteries and preventing inflow. Conversely, during diastole, coronary arteries are opened and arterial inflow increases with a transmural gradient that favours perfusion to the sub-endocardial layers (29). The continuous beating heart consumes high amounts of oxygen already at rest. The oxygen extraction in the myocardium is therefore high during resting conditions, and increases in oxygen demand can only be met by increases in coronary blood flow (30). The coronary microcirculation includes vessels with diameters below approximately 300 μm with different mechanisms of vasoreactivity control, able to meet both instant and long-term changes in myocardial oxygen demand (23, 31). Under normal conditions, the pre-arterioles (200-500 μm) and arterioles (<200 μm) are the primary vessels controlling coronary vascular resistance, responsible for about 80% of the total coronary vascular resistance. The pre-arterioles are epicardial, extra-myocardial vessels reacting to changes in shear stress and intravascular pressure to retain a sufficient perfusion pressure in the distal arteriolar bed (32, 33). The arterioles regulate the intramyocardial coronary circulation and are usually subdivided into three categories, according to their size and predominant mechanism that regulates their tone (Figure 2). The larger arterioles are regulated by blood flow and shear stress causing flow-related vasomotor response by the release of vasoactive substances from endothelial cells (3, 33). The medium-sized microvessels are mainly modulated by myogenic control, including increased intraluminal pressure mediating contraction of vascular smooth muscle cells and, conversely dilation when the pressure decreases (34). Lastly, the tone of the smaller arterioles is modulated by the metabolic activity of the myocardium. Therefore, upon an increase of metabolic demand the smaller arterioles will dilate, leading to a reduction in pressure upstream in medium-sized microvessels causing myogenic dilation. This in turn increases blood flow further upstream, resulting in endothelium-dependent vasodilation (33). These mechanisms allow the microcirculation to regulate myocardial perfusion both at rest and at different levels of myocardial metabolic demand (2).

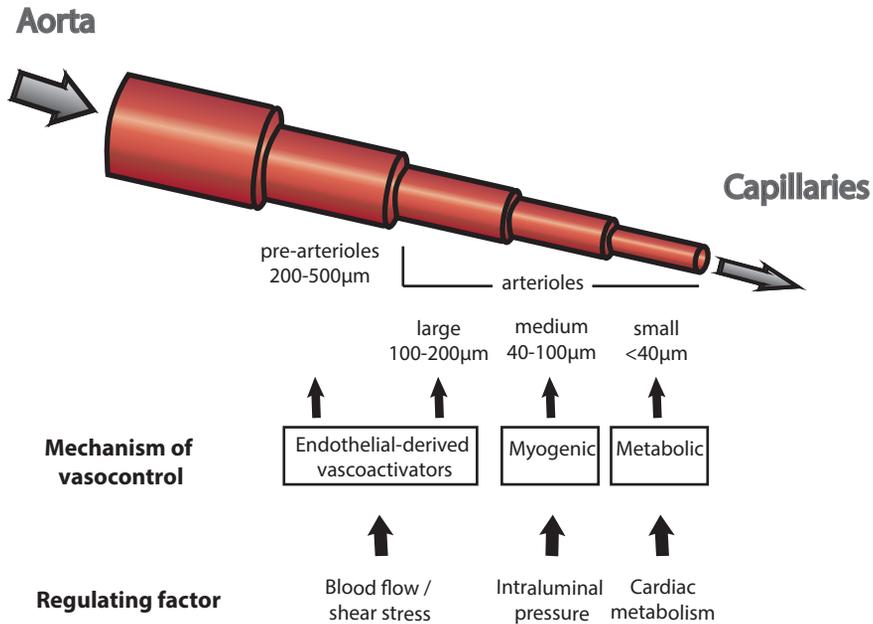


Figure 2 Classification of arterioles

Pre-arterioles and arterioles are subdivided into categories according to their size and the predominant mechanism regulating their tone.

Alterations in vascular tone causes instant changes, which alongside circumferential wall stress upon persistence can lead to long-termed changes. In addition, continuity of these conditions leads to generation of new vessels by angiogenesis or elimination of vessels by pruning (35, 36). Given the complex physiology and anatomy of the coronary microcirculation, there are many potential mechanisms contributing to coronary artery disease.

Coronary artery disease

Coronary heart disease is interchangeably called coronary artery disease as well as ischemic heart disease. This condition refers to failing circulation of the heart and includes acute coronary syndromes (angina pectoris, myocardial infarction, cardiac death) and chronic coronary heart disease. The most common cause of ischemic heart disease is atherosclerosis, underlying the obstructive plaque build-up occluding the coronary arteries and consequently decreasing oxygen

supply (37). Accordingly, during the past decades, obstructive coronary artery disease has been the main focus of international cardiology by successful implementation of e.g. percutaneous coronary intervention, which has saved many patients' lives, especially in those with hemodynamically significant obstructive coronary artery disease. However, non-obstructive coronary artery disease can also lead to myocardial infarction (38) among other severe cardiovascular events (39). Therefore, during the last decades, non-obstructive coronary artery disease has gained increased focus.

Non-obstructive coronary artery disease

Coronary angiography investigation is considered golden standard method for diagnosing obstructive coronary artery disease. Applying this technique, the definition of non-obstructive coronary artery disease is not always consistent in the literature. A large Danish study as well as the CONFIRM study defined normal coronary arteries as 0% stenosis in all coronary arteries. Non-obstructive coronary artery disease was defined as a lumen diameter reduction $\geq 1\%$ but $<50\%$ in any epicardial coronary artery (39, 40). Pepine et. al. recently shed light on the experience from the Women's Ischemic Syndrome Evaluation (WISE) study (41) concluding that almost any quantitatively measured luminal irregularity results in at least a 20% diameter reduction compared to completely normal-appearing adjacent segments. This means that patients with narrowing of any coronary epicardial vessel ranging from 0-19% may be defined as no coronary artery disease or no apparent coronary artery disease. Furthermore, at least one vessel with a lumen diameter narrowing $\geq 20\%$ but $<50\%$ defines non-obstructive coronary artery disease, while obstructive coronary artery disease includes at least one stenosis $\geq 50\%$ in one, two or three coronary epicardial vessels (42). This definition incorporates increased patient number within the category of non-obstructive coronary artery disease as compared to Veterans Administration CART National Registry using a similar definition as WISE, with the exception of non-obstructive as stenosis $\geq 20\%$, but $<70\%$ narrowing in any epicardial artery, or $\geq 20\%$ but $<50\%$ in the left main artery (43).

Prevalence and cardiovascular outcome in patients with non-obstructive coronary artery disease

Angiographically normal or near-normal coronary arteries are more common in women (44). Up to half of the women and one third of the men undergoing elec-

tive angiography due to non-acute chest pain have non-obstructive coronary artery disease (45, 46). The WISE study demonstrated that women with symptoms and/or signs of myocardial ischemia but without obstructive coronary artery disease are at elevated risk for cardiovascular events at both 5- and 10-years follow-up (41, 47). Between 2005 and 2009 the CONFIRM study included over 20 000 patients without known coronary artery disease undergoing coronary angiography. The study showed one third of the patients to have non-obstructive coronary artery disease (1-49% stenosis) and that both women and men above 65 years of age had 5-fold higher all-cause mortality risk than those below 65 years (40). A retrospective cohort study on over 11 000 patients referred to coronary angiography due to suspected stable angina pectoris found up to 65% of the women and 32% of the men to have non-obstructive coronary artery disease. During a 7-year follow-up, these patients had an increased risk for both major adverse cardiovascular events as well as for all-cause mortality with 85% and 52%, respectively, and with no difference between genders (39). A recent study highlighted the importance that non-obstructive coronary artery disease is associated with a higher 1-year rate of myocardial infarction as well as mortality compared to patients with no apparent coronary artery disease undergoing elective angiography (48). These studies among others have emphasized the need of moving risk stratification from obstructive coronary artery disease to also include identification and understanding of the pathology of non-obstructive coronary artery disease. Although coronary angiography is a powerful tool in identifying obstructive coronary artery disease, in the absence of flow-limiting stenosis more precise tools for risk-stratifying patients with non-obstructive coronary artery disease is needed (42).

Coronary microvascular dysfunction in non-obstructive coronary artery disease

The coronary microcirculation has been elusive traditional imaging techniques (Figure 3) contributing to less evaluation of the clinical importance of coronary microvascular dysfunction as compared to epicardial coronary artery disease (2).

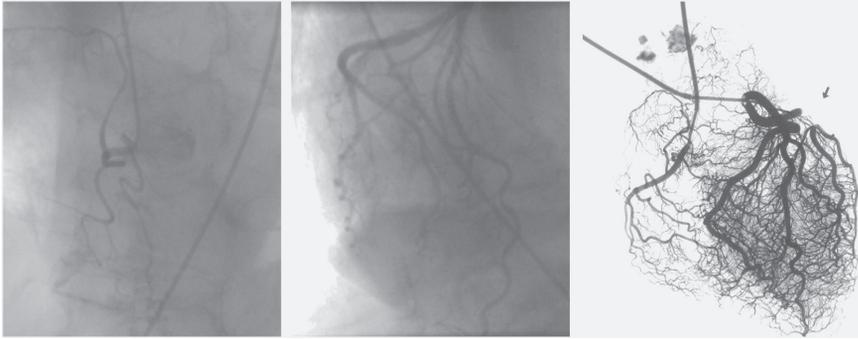


Figure 3 Visualisation of coronary circulation

Angiogram of the right coronary artery (left panel) and a dominant left coronary artery system (middle panel), as compared to the visualised microvascular network on an ex vivo arteriogram (right panel). Reprinted with permission from Oxford University Press. Herrman J et. al. Coronary microvascular dysfunction in the clinical setting: from mystery to reality. *European Heart Journal* (2012) 33, 2771–2781 (2).

In 1967, Likoff et.al. first suggested a possible coronary microvascular disorder in a cohort of patients with angina pectoris (49). Coronary microvascular dysfunction is likely to co-exist with obstructive epicardial coronary artery disease, as well as being proposed to contribute to the signs and symptoms of ischemia not associated with obstructive coronary artery disease (3, 9). Coronary microvascular dysfunction in the absence of obstructive coronary artery disease was recently shown to be prevalent in two thirds of a patient cohort with chest pain and non-obstructive coronary artery disease (7). In addition, the presence of microvascular dysfunction is associated with an adverse cardiovascular prognosis in both men and women (50).

Methods to assess coronary microvascular function

The presence of myocardial ischemia due to coronary microvascular dysfunction in the absence of obstructive coronary disease is not always evident or easy to determine. Many patients without obstructive coronary artery disease lack large regional myocardial perfusion defects. Consequently several conventional clinical methods to assess myocardial ischemia that rely on detecting relatively large regional differences in left ventricle perfusion and/or wall motion, are unable to determine ischemia in these patients. Instead, the functional state of coronary microcirculation is considered useful in diagnosing and risk stratifying these patients (42). During the last decades, non-invasive techniques of assessing coronary function has been developed (51). The most frequently used are those that measure coronary blood flow reserve with infusion of pharmacological vasodila-

tor stressors. These stressors include adenosine, which is endogenously released by myocardial cells during increased oxygen consumption. Adenosine fulfils its effect by stimulating increased vasodilation by activating arteriolar smooth muscle cell A2 receptors (52, 53), leading to decreased vascular resistance mainly in the coronary bed, but also to some extent reduced total peripheral resistance, increased heart rate and consequently slightly increased rate pressure product. In addition, the increased blood flow in the coronary microvascular bed stimulates further flow-mediated vasodilation in larger arterioles (54) through a nitric oxide dependent mechanism (55). Dipyridamole is another frequently used stressor, inhibiting adenosine deaminase and thereby the degradation of adenosine in the tissues (56). Acetylcholine is the most widely used substance that mediates its effect primarily by endothelial-dependent vasodilation. However, this substance requires intracoronary infusion and is thereby limited to invasive diagnostic use (57).

Positron emission tomography to assess coronary flow reserve

Positron emission tomography is non-invasive and considered the gold standard method, quantifying absolute myocardial blood flow in both global and regional sites. Positron emitting substances are used as tracers and absolute blood flow is averaged over a time-period of 4-5 minutes reflecting functional aspects of selected areas in the myocardium (51). Positron emission tomography is a highly reproducible method (58) known to predict cardiovascular outcome (59). Coronary flow reserve was recently shown to add incremental value in risk stratification of patients with non-obstructive coronary artery disease (50). However, this method is expensive and can only be performed at specialized centres. These limitations together with potential radiation exposure make it less suitable for repeated assessment of impaired coronary microvascular function.

Transthoracic Doppler echocardiography to assess coronary flow reserve

Transthoracic Doppler echocardiographic assessment of coronary blood flow reserve is a non-invasive, accessible and highly reproducible emerging tool to assess the extent of coronary microcirculatory dysfunction in the absence of epicardial stenosis (60, 61). Coronary flow velocity reserve is an indirect measure of coronary flow reserve (60), shown to have high agreement with positron emission tomography (60) as well as with invasive Doppler guide wire (61).

Coronary flow reserve is usually measured in the left anterior descending coronary artery (LAD), but can also be assessed in left circumflex artery as well as in right coronary artery (62, 63). Using this method to evaluate coronary flow reserve has prognostic value in patients with non-obstructive coronary artery disease (64). Transthoracic colour Doppler assessed coronary flow reserve is a relatively simple, inexpensive method suitable for serial measurements. However, this method requires an experienced operator to obtain reliable and reproducible measurements.

Peripheral vascular dysfunction in non-obstructive coronary artery disease

Endothelial dysfunction is a systemic process and not only coronary microvascular dysfunction is evident in patients with chest pain and normal angiography, they often exhibit a disturbance also in the peripheral circulation (4).

Reactive hyperaemic index to assess peripheral vascular function

The peripheral vasculature is anatomically complex, consisting of a dual circulation of arteriovenous anastomoses and nutritive vessels. The fingertip is rich in arteriovenous anastomoses and vascular tone is primarily modulated by the sympathetic nervous system (65), although nitric oxide and endothelial function have a partial role in the regulation of resting blood flow (66). Peripheral arterial tonometry evaluates arterial pulse wave amplitude changes in the fingertip at rest and following the induction of reactive hyperaemia (67). The hyperaemic response has been shown to be partly influenced by nitric oxide (66) in addition to other factors (68-70). Reactive hyperaemic index was demonstrated equivalent in patients with non-obstructive and obstructive coronary artery disease (71) and shown related to multiple traditional as well as metabolic risk factors (72). In patients with chest pain but without myocardial perfusion defects, reactive hyperaemic index improves risk stratification of patients in the intermediate and high Framingham risk groups, highlighting its potential value in non-obstructive coronary artery disease (73).

Pathogenesis in microvascular dysfunction

Microvascular dysfunction involves a complex pathology, including multiple mechanisms, all interacting with each other (3). Indeed, endothelial cells are

continuously exposed to fluid shear stresses modulating production of substances that regulate vasoconstriction, vessel growth, fibrinolysis and cell adhesion (74), all which may be affected in the pathological state.

Endothelial dysfunction

A healthy endothelium is needed for a balanced dilation and constriction of the arterial vessels, regulating and synchronizing the contractile state of the cardiomyocytes (31). Reactive oxygen species is produced by the endothelium and endothelial dysfunction is a consequence of increased oxidative stress including superoxide and reactive oxygen species (22). These mechanisms incorporate the reactive oxygen species increase of asymmetric dimethylarginine (ADMA) levels, inhibiting endothelial nitric oxide synthase (75-77) as well as decreased L-arginine and thereby decreased nitric oxide availability and blunted endothelium-dependent vasorelaxation (22). The decreased vasodilation capacity facilitates platelet aggregation, inflammation, vascular smooth muscle cell migration and proliferation, and leukocyte adhesion, which further promotes endothelial dysfunction (76). In addition, the production of endothelin-1 is increased, further favouring vasoconstriction which in turn may reduce the production of nitric oxide (28).

Blood viscosity and vascular inflammation

The vascular endothelium is both affected by and contributes to the inflammatory process that may lead to atherosclerosis progression. Pro-inflammatory factors cause endothelial cell activation, further promoting the inflammatory process and an atherogenic phenotype. Activation of endothelial cells results in synthesis of chemokines that contribute to transendothelial migration (74), expression and/or activation of integrins (78) and adhesion molecules (79), accelerating the inflammatory process. The initial interaction between leukocytes and endothelial cells is mediated by selectins expressed on the endothelial cell surface and ligands located on the leukocytes surface. This selectin-mediated interaction results in the rolling of leukocytes along the vessel wall. Consequently, upregulated adhesion molecules bind to their counter receptors on endothelial cells, primarily intercellular adhesion molecule-1, resulting in adhesion and transmigration of leukocytes through the vascular wall. This adhesion of leukocytes to vascular endothelium is a hallmark of the inflammatory process (80-82). Experimental studies show that nitric oxide reduces leukocyte adhesion to human endothelial cells (83) due to downregulation of intercellular adhesion molecule-1 and P-

selectin (84), reduction in nitric oxide therefore facilitates the inflammation process.

Increased blood viscosity is associated with microvascular dysfunction and has been shown to predict ischemic heart disease (85-87). Fibrinogen is one of the major contributors to plasma viscosity and important in the complex mechanisms of coronary microvascular dysfunction (87). Increased plasma fibrinogen concentration increases blood viscosity, and therefore increases shear stress (88) that activates endothelial cells (89, 90) and platelets (91). Fibrinogen contributes to the cardiovascular pathology not only by increasing blood viscosity but also by promoting leukocyte infiltration of the vessel wall, increase platelet aggregation, thrombus formation and vasoconstriction by stimulating endothelin-1 production (92-94). Taken together, these fibrinogen-induced mechanisms have been suggested to affect microcirculation (93).

Vascular remodelling and rarefaction

In addition to blood viscosity, endothelial dysfunction and inflammation, the progress of vascular remodelling and rarefaction also appears in microvascular dysfunction (23), emphasizing its complexity. The vascular system includes not only formation of new vessels but also a continuous adjustment of vessels and network. This “angioadaptation” is due to an interplay of vascular responses to growth factors (such as VEGFs), to the metabolic status of the tissue, and to hemodynamic forces exerted by the flowing blood. The vascular tone together with inner vessel diameter and structure determines flow resistance and perfusion. Persistent increased wall shear stress at the endothelial surface, circumferential wall stress and metabolic (especially hypoxia) signals, drives long-term vascular remodelling leading to “angioadaptation”. These changes appears within days to months and involves both angiogenesis and vessel pruning as well as changes in vessel diameter and/or wall mass (35).

Risk factors for coronary microvascular dysfunction

Traditional risk factors associated with structural and functional alterations leading to decreased coronary microvascular function includes those in obstructive coronary artery disease. Ageing is associated with increased pulse pressure, arterial remodelling and increased wall stiffness contributing to impaired coronary microvascular function (95, 96). Hypertension is also associated with remodeling of small arteries, including the coronary arteries (97-99). Furthermore, hypertension leads to arteriolar vasoconstriction and increased peripheral resistance

as well as reduction in microvessel density (9), all possible contributors of microvascular dysfunction. Also, dyslipidemia is associated with coronary microvascular dysfunction, where increased high density lipoprotein has a beneficial association, while triglycerides and low density lipoprotein is negatively associated with coronary function (100, 101). Finally, impaired vasodilation across tissues is often found in type 2 diabetes (102) and diabetes is associated with impaired coronary microvascular function (103, 104). However, these traditional risk markers are not always present in coronary microvascular dysfunction (6) and shown to correlate poorly with the decreased coronary function (7). Indeed, they have been demonstrated to only account for a low proportion in patients with coronary microvascular dysfunction (105). This has increased the focus on finding other risk markers (8, 9, 106).

Highlighting diabetes in association to microvascular dysfunction is essential considering the close relation between insulin resistance and vascular dysfunction (102). Type 2 diabetes is associated with increased risk of cardiovascular morbidity and mortality (107) where coronary microvascular dysfunction plays an important role (108). Type 2 diabetes onset is preceded by 10-20 years of insulin resistance (109). Pre-diabetes may cause functional (110, 111) as well as potentially structural vascular changes (112, 113), and is associated with microvascular dysfunction (114). In fact, insulin resistance per se, carries prognostic value for future cardiovascular events in subjects without diabetes (115). Furthermore, decreased coronary flow reserve is associated with increased insulin resistance in non-diabetic subjects without coronary angiography verified stenosis, suggesting its potential importance in coronary microvascular dysfunction (116).

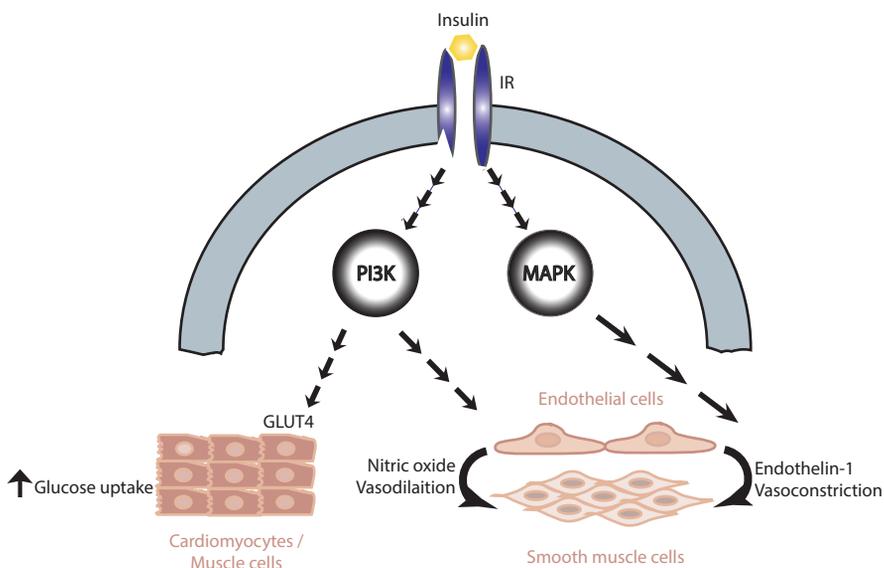


Figure 4 Insulin-dependent signalling

Schematic overview illustrating the insulin-dependent phosphatidylinositol 3 kinase (PI3K) and mitogen activated protein kinase (MAPK) pathways. Insulin binds to the insulin receptor (IR) activating both pathways. In cardiomyocytes and muscle cells, PI3K activation leads to glucose uptake mainly through glucose transporter 4 (GLUT4) as well as to production of endothelial-derived nitric oxide, favouring vasodilation. The MAPK-pathway leads to production of the vasoconstrictor Endothelin-1.

Cardiovascular actions of insulin

The insulin receptor is highly expressed in cardiomyocytes, and insulin regulates metabolism in the heart by modulating glucose transport, glycolysis, glycogen synthesis, lipid metabolism, protein synthesis, growth, contractility, and apoptosis in cardiomyocytes (117). In addition, vasodilator actions of insulin in coronary vasculature increase myocardial perfusion (118). Insulin binding to its receptor, leads to activation of parallel signalling pathways (119). In endothelial cells, the insulin activated phosphatidylinositol 3 kinase (PI3K) pathway leads to production of nitric oxide (120, 121), as well as to endothelin-1 through mitogen activated protein kinase (MAPK)-dependent insulin-pathways (122). Nitric oxide contributes to vasodilation of smooth muscle cells while endothelin-1 leads to vasoconstriction of these cells. In cardiomyocytes and skeletal muscle cells, insulin signalling mediate glucose uptake primarily through glucose transporter 4 translocation (123) (Figure 4).

The insulin-dependent signalling pathways are complex, and include several feedback loops and crosstalk between the signalling branches. Normally, the net

result favours nitric oxide production, resulting in vasodilation of smooth muscle cells, redirection of flow from non-nutritive capillaries to nutritive capillaries and increased blood flow. The capillary recruitment increases perfusion and facilitates insulin-mediated glucose uptake. Under healthy conditions, a balance between the various effects of insulin contributes to cardiovascular homeostasis (20, 123).

Insulin resistance and microvascular dysfunction

Insulin resistance is a state in which a given concentration of insulin generates a reduced biological effect. Insulin resistance before onset of type 2 diabetes is associated with decreased insulin sensitivity and/or responsiveness to its metabolic actions, leading to compensatory hyperinsulinemia trying to preserve the metabolic effects of insulin (124). Microvascular dysfunction cause impaired capillary recruitment and decreased microcirculatory blood flow to metabolically active and insulin-dependent tissue. The insulin resistance further impairs endothelial function and capillary recruitment, resulting in a negative circle (125), causing progressive endothelial dysfunction and disturbances in glucose and lipid metabolism, highlighting the reciprocal relationship between them. Consequently, vascular damage and oxidative stress to the vessel wall triggers an inflammatory response, further promoting insulin resistance and endothelial dysfunction (126).

Also, fibrinogen contributes to microvascular dysfunction as described in a previous section, and its concentrations have been shown to negatively correlate with degree of insulin sensitivity (127), further addressing the multiple actions of insulin resistance.

The gender aspect in coronary microvascular dysfunction

Significantly more women than men with suspected clinically stable ischemic heart disease have non-obstructive coronary artery disease (39, 45), but both seems to have equal risk for major adverse cardiovascular events (7, 39, 50). The presence of microvascular dysfunction in non-obstructive coronary artery disease has previously been shown more evident in women (128, 129), but recent studies demonstrate similar findings in men. Still, the mechanisms associated with coronary microvascular dysfunction appear distinct in men and women

(128). Women and men differentiate in both gender and sex related aspects. Sex differences are biological and arise from different gene expression from the sex chromosomes while gender differences arise from sociocultural processes. Both are important for development of cardiovascular disease, affecting the cardiovascular system in various ways (130).

Arterial age-related changes have been demonstrated to differ in men and women, endothelial dysfunction seems to decline after 40 years of age in men, while the vascular physiology remains stable for another decade in women (131). It is well known that men have a higher incidence of cardiovascular disease than pre-menopausal women and oestrogen is a vasoactive hormone shown to acutely improve coronary microvascular function in healthy women (132). In addition, long-term hormone treatment show beneficial effect of oestrogen on flow mediated dilation, (133, 134), and seems to involve favourable effects on both fibrinogen as well as adhesion molecule levels (133). In agreement, the risk for coronary microvascular disease seems highest among women between 45 and 65 years of age, where after the incidence of obstructive coronary artery disease is elevated (130). The decline in oestrogen levels has been associated with increased blood pressure and the prevalence of hypertension in women after menopause rises (135). Importantly, hypertension in women is often undiagnosed or inadequately treated, especially after menopause when cardiovascular risk increases (136).

Physical exercise and microvascular function

Hippocrates (460-370 BC) once wrote: "Eating alone will not keep a man well; he must also take exercise. For food and exercise...work together to produce health"(137). The WHO's Global Recommendations on Physical Activity for Health (138) as well as 2008 physical activity guidelines for Americans (139) recommend adults at least 150 minutes of moderate-intensity training spread out throughout the week. Although, the beneficial effect of physical exercise on cardiovascular status is well known, one fourth of the adult population in the European Union do not meet these recommendations, with large variation between countries (13), while only 50% of the Americans seem to fulfil these guidelines (140).

The terms 'physical activity' and 'physical exercise' refer to any bodily movement due to contraction of the skeletal musculature and is associated with the consumption of energy. Specifically, the term 'physical exercise' indicates

physical activity that is regular, structured and aimed at improving and/or maintaining physical fitness and well-being (141). Physical activity has been shown to prevent or delay the development of hypertension, increase high density lipoprotein cholesterol levels, control body weight, and lowering the risk of developing type 2 diabetes, all known risk factors for coronary artery disease (10). However, performing regular physical activity is related to reduced cardiovascular mortality in healthy individuals also after adjusting for known risk factors (142). The mechanisms associated with physical activity includes beneficial effect on endothelial function (143), reduced blood viscosity (144) and decreased platelet aggregation (145). Indeed, physical exercise has been demonstrated to increase coronary microvascular function in healthy volunteers (146) as well as in patients with stable coronary artery disease (147), highlighting its potential importance in improving coronary microvascular function and the relevance in a structured use of physical exercise in health care work.

The importance of a translational perspective

Cardiovascular clinical research can be performed from many perspectives, such as relevance in morbidity and mortality, association of specific pathology to a specific phenotype, mechanistic studies as well as treatment effects. All which are important in preventing and/or intervening cardiovascular disease. In the latter, clinical studies can be used to validate pharmaceutical targets, shown to have a beneficial effect in pre-clinical studies.

Pre-clinical studies can be used to study causality, receive deepened mechanistic understanding and perform relevant intervention studies for proof of principle. A translatable animal model should mimic the typical human disease phenotype and preferably respond to standard of care treatment in the human setting. However, an animal model is not likely to reflect a complete human disease, but rather specific aspects of the pathology. Such pathology can either be induced e.g. chemically, surgically, by diet, or develop spontaneously. An advantage with a spontaneous model developing an insulin resistant phenotype is the relative slow progress of the disease, facilitating investigation in different stages of the process. When studying the process of insulin resistance it is important to choose a model with an appropriate underlying cause of hyperglycaemia. In addition, when studying coronary microvascular function, obstructive coronary artery disease needs to be absent.

Animal models studying coronary microvascular complications in pre-diabetes and type 2 diabetes

A well-characterized model is the leptin-receptor deficient db/db mice. These mice display a severe diabetic phenotype with beta cell dysfunction (148), and diabetic complications including nephropathy (149) and endothelial dysfunction in coronary arterioles (150). The Goto–Kakizaki (GK) rat model (151) also demonstrates impaired coronary microvascular function, due to probable decreased nitric oxide bioavailability (152). This model develops hyperglycaemia as a consequence of both insulin resistance and impaired insulin secretion (149). The leptin-deficient (ob/ob) mice is an obese model that derives from a spontaneous mutation in the leptin protein (153), generating an insulin resistant model that do not develop atherosclerosis (154). This model is associated with hyperinsulinemia and insulin resistance, lacking pancreatic beta-cell dysfunction. Therefore, the ob/ob mice are characterized as a model of pre-diabetes/mild hyperglycaemia without the severe diabetic phenotype and its late complications (149). This generates a potential model of insulin resistance at a non-diabetic stage with possible early vascular dysfunction.

Aims

The overall aim of this thesis was to investigate determinators of coronary and microvascular function with specific focus on glucose homeostasis in the pre-diabetic stage.

Specific hypotheses and aims:

1. Paper I and II. We hypothesized that insulin resistance in non-diabetic patients is associated with worse clinical outcome, impaired coronary flow reserve and peripheral vascular function in patients with chest pain without myocardial perfusion defects. We aimed to study the prognostic power of coronary flow reserve as well as multiple risk factors relevant for impaired coronary microvascular function, including insulin resistance. Further, for deepened mechanistic understanding, we explored plasma protein and gene expression patterns in whole blood cells associated with insulin resistance.
2. Paper III. We hypothesized the ob/ob mice to be a potential model for microvascular dysfunction. For deepened translational understanding, we aimed to test the hypothesis that parallel early cardiac and renal microvascular dysfunction is prevalent in this non-atherosclerotic insulin resistant model, and that the dysfunction is associated with microvascular structural and functional changes related to the vascular nitric oxide pathway.
3. Paper IV. We hypothesized that coronary flow reserve and peripheral endothelial function in sedentary healthy volunteers could be improved with personalized and supervised lifestyle intervention. We aimed to study the prevalence of and potential parallel impact on impaired glucose homeostasis.

Participants and Methods

Study participants

Overview of patient characteristics included to the current thesis is shown in table I.

Table I Patient characteristics of study cohorts in paper I, II and IV

	Study population	Number (women)	Age	BMI	CFR	HOMA-IR
Paper I	Suspected myocardial ischemia with and without myocardial perfusion defects	365 202 (55%)	62±9	25.7±3.5	2.7 (2.2;3.7)	3.1 (2.3;4.3)
Paper II	Suspected myocardial ischemia without myocardial perfusion defects	202 128 (63%)	62±9	25.3±3.3	2.7 (2.2;3.3)	3.1 (2.2;4.0)
Paper IV	Healthy volunteers	36 17 (47%)	54±6	23.6±1.4	3.3 (2.8;4.0)	1.1 (0.7;1.6)

BMI; body mass index, CFR; coronary flow reserve, HOMA-IR; homeostatic model assessment for insulin resistance

Suspected myocardial ischemia (paper I and II)

Paper I and II are prospective observational studies on a low to medium risk population referred to myocardial perfusion scintigram for evaluation of chest pain and suspected myocardial ischemia. The patients were consecutively offered participation in the study at the time of their scintigram investigation, at the Department of Clinical Physiology, Sahlgrenska University Hospital. The study was performed between the years 2006 and 2008. Myocardial perfusion scintigram is a well-established method in diagnosing myocardial ischemia. The patients were examined within four weeks after the myocardial perfusion scintigram (mean two weeks), and the results were blinded to operators. The exclusion criteria were atrial fibrillation or other cardiac arrhythmia, chronic obstructive pulmonary disease, other severe disease (e.g. cancer), treatment with dipyridamol (Persantin, Asasantin), not able to assimilate information about the

study or unwillingness to participate. Patients with acute coronary syndromes were not included. For the current thesis, patients with any diabetes diagnosis, fasting glucose levels ≥ 7 mmol/l or Haemoglobin A1c (HbA_{1c}) level > 48 mmol/l were excluded. The remaining 365 patients were included to paper I for assessment of prognostic value of insulin resistance in patients with versus without myocardial perfusion defects. In this population, insulin resistance seemed to have stronger association to outcome in patients without myocardial perfusion defects. Therefore, these patients were further evaluated for coronary microvascular dysfunction and relation to insulin resistance in paper II (Figure 5).

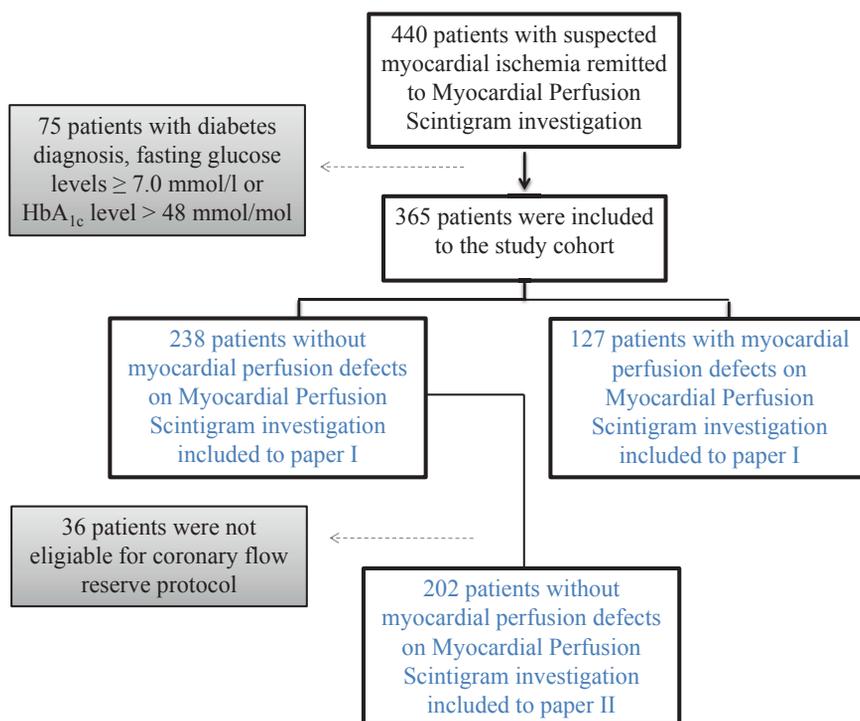


Figure 5 Flow scheme over patient populations included in paper I and II

A flow scheme visualizing patient recruiting and relationship between paper I and II.

Healthy volunteers (paper IV)

Paper IV is a randomized, longitudinal, intervention study to which we recruited 36 healthy subjects by advertising in the local newspaper and at several large working places located in the Gothenburg area. The study was performed at Department of Cardiology Research, Sahlgrenska University Hospital during the years 2014 to 2015. Inclusion criteria were 1) normal exercise electrocardiogram (ECG) during pre-screening; 2) 35-65 years of age; 3) body mass index 20-27; 4) no current or previous history of cardiovascular disease; 5) no family history of cardiovascular disease at an age below 55; 6) no ongoing regular exercise and 7) non-smoker. All participants underwent exercise ECG before study participation. Exclusion criteria were pathological findings during exercise ECG or echocardiography. Participants were randomized by a computer software into two groups matched regarding to age, gender and body mass index; Control/Standard group and Diagnosis, Analysis, Personalization and Supervision (DAPS) group.

Methods

Radionuclide myocardial perfusion scintigram (paper I and II)

In paper I and II, patients with suspected myocardial ischemia were referred to myocardial perfusion scintigram before study participation to evaluate occurrence of myocardial perfusion defects and inducible ischemia. It can be used as a diagnostic and prognostic test guiding the need for coronary angiography as well as for risk determination for future cardiovascular events and death. The test is performed using maximal exercise test or pharmacological provocation. A radionuclide is injected to determine myocardial perfusion by evaluating signs of reversible ischemia. The second day protocol is performed during rest, assessing possible irreversible ischemia areas. The two-day stress/rest examination protocol was performed according to standard clinical protocol at the Department of Clinical Physiology, Sahlgrenska University Hospital. Stress provocation was performed by exercise test or by pharmacological provocation using adenosine. Radionuclide Technetium (^{99m}Tc) sestamibi was administered and detected using gated single-photon emission computed tomography (SPECT). Images were obtained using dual-head SPECT cameras (Infinia or Hawkeye, General Electric, USA), displaying perfusion and function of left ventricle. The protocol used in the thesis is described in more detail in paper I and II. No myocardial perfusion defects were defined when both severity and extent were scored 0. In

paper I, patients with and without myocardial perfusion defects were included, whereas in paper II only patients without defects were evaluated.

Methodological considerations using myocardial perfusion scintigram

Stress myocardial perfusion scintigram is of strong prognostic value identifying hemodynamically significant obstructive coronary artery disease in patients with suspected myocardial ischemia (155). A recent study suggests myocardial perfusion scintigram to better assess functionally significant coronary artery disease compared to coronary angiography in patients with intermediate pre-test probability (156). However, since myocardial perfusion scintigram provides relative flow distribution pattern rather than absolute flow, it might still be less sensitive to detect coronary microvascular disease, including endothelial-dependent as well as independent coronary microvascular abnormalities in patients with chest pain and non-obstructive coronary artery disease (157). We therefore aimed to test the hypothesis of HOMA-IR (paper I) and coronary flow reserve (paper II) as additional tools for risk stratification of this patient population. Furthermore, myocardial perfusion scintigram might also be less suitable of detecting balanced three-vessel disease, due to the comparison of relative flow distribution pattern. Therefore, we cannot exclude having included patients with balanced three-vessel disease to the subgroup of patients displaying no myocardial perfusion defects in paper I and II. However, in paper II, due to the nature of the symptoms being majorly atypical chest pain in this group and a median time for revascularization of 2.4 years, these patients are believed to be few.

Electrocardiogram exercise test for study inclusion (paper IV)

Exercise ECG is a method for initial evaluation of demand-driven myocardial ischemia and suspected coronary disease in patients displaying no coronary flow limitation at rest (158). Before inclusion to study IV, participants performed a maximal exercise ECG test to screen for obvious signs and symptoms of inducible myocardial ischemia. A standard protocol with continuous load elevation was used. Start load was determined from age, gender and weight-reference material (159), with aim for 6-8 minutes duration of test time. Blood pressure and Borg Scale were assessed every other minute throughout the whole test.

Life style intervention

Standard lifestyle intervention program

Upon study inclusion, the standard participants were asked to maintain their normal lifestyle for three months before receiving the standard intervention program. Participants were examined before and after the control period of 12 weeks (± 2 weeks). When having completed the control period, within one week after examination the standard program achieved its design through a personal questionnaire where the individual chose one goal for physical exercise and nutrition. The pre-designed suggestions were muscle building, weight reduction/maintaining, improve your self-image, anti-stress/mood and improve overall health. Each participant was given a membership to a gym, a plan of nutrition and physical exercise to perform during three months. They received no further personalized training or feedback and were asked to perform the exercise for 60 minutes, three days a week. The participants were examined after completing the 12 weeks standard lifestyle intervention program (± 2 weeks). The three examinations at baseline, post control period and post intervention period generated a control period and a standard intervention period of 3 months, respectively (Figure 6).

DAPS personalized supervised intervention program

DAPS method is a personal training method that is escorted by two points and important differentiators compared to the standard program: the communication with the participant and the personalization of the program. The DAPS-program achieved its design through a personal questionnaire with focus on analysing the participants exercise and nutrition habits. A weekly program was coordinated by an experienced personal trainer based on personalized exercise, Mediterranean-inspired diet advice adapted to each subject, continuous contact by email and feedback for development of a new weekly program. The personalized program was to be easily accessible and conducted at home for 30 minutes, six days a week using weekly-received links to exercise programs shared on social media. The DAPS participants were examined before and after the three months intervention program (± 2 weeks) (Figure 6).

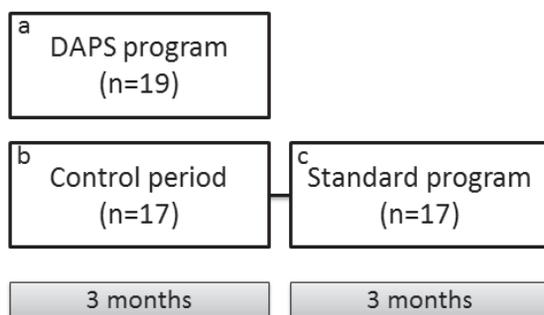


Figure 6 Study design

Schematic overview of study design. Subjects were randomized into DAPS with a three months intervention period as well as a standard group including a three months control period followed by a three months standard exercise period. The primary objective of the current study was to compare effects of the DAPS program (a) including personalized exercise training and Mediterranean-inspired diet with a time-aligned initial control period of the standard group (b). Secondary objective was to compare the DAPS program (a) with the standard program (c), mimicking a regular gym and Mediterranean-inspired nutrition program. Finally, the third objective was to compare the standard program (c) with its own control period (b). DAPS=Diagnosis, Analysis, Personalization and Supervision.

Quality of life

In the standard group, quality of life was measured at baseline, after control period and post intervention, DAPS group was evaluated pre- and post-intervention. To measure quality of life and psychological general well-being we used a generic self-administered instrument, the Psychological General Well-Being index (PGWB index). This instrument was developed for the purpose of providing a self-reporting instrument that could be used to measure subjective well-being or distress (160). The PGWB index includes 22 items, divided into six dimensions: anxiety, depressed mood, positive well-being, self-control, general health, and vitality. Scores are calculated for each dimension and summarized for an overall PGWB grade.

Cardiovascular Ultrasound

Transthoracic echocardiography (paper II and IV)

A basic transthoracic echocardiography protocol at rest was performed according to recommendations (161), using the Sequoia C256 (Acuson Siemens Mountain View, California) ultrasound system with a 4MHz probe. Hyperaemia was induced using adenosine (140 $\mu\text{mol/kg/min}$) and apical 4-chamber and 2-chamber views were stored after 5 minutes infusion. ECG was registered during the entire examination and during adenosine infusion, and blood pressure was monitored every minute. Cine loops and Doppler images were stored for off-line measurements.

All off-line measurements were performed by an experienced sonographer, blinded to the patients' clinical characteristics. Images were analysed using Image Arena 2.9.1 (TomTec Imaging Systems GmbH, Unterschleissheim, Germany) for paper II and Workstation Syngo US (version 3.5.6.34, Siemens Medical Solutions) for paper IV. Left ventricle ejection fraction and stroke volume were estimated by Simpsons biplane method using apical 4-chamber and 2-chamber views, both at rest and during hyperaemia. Heart rate was averaged from three cardiac cycles.

Cardiac output was calculated during rest (paper IV):

$$\text{Cardiac output} = \text{heart rate} * \text{stroke volume}$$

Rate pressure product was calculated during rest (paper IV):

$$\text{Rate pressure product} = \text{heart rate} * \text{systolic blood pressure}$$

Transthoracic echocardiography assessed coronary flow reserve (paper II and IV)

Ultrasound Doppler is used to study blood flow movements and velocities during the guidance of two-dimensional echo. Coronary blood flow velocity was measured in the LAD before and during adenosine infusion, by using the same ultrasound system as written above. Flow velocity was evaluated using the 4VIC transducer with 3.5 MHz colour and 1.75 MHz spectral Doppler frequency (162). For repeated measurements in paper IV, the surface anatomic position, degree of rotation of the transducer, and the LAD position relative to the left

ventricle were carefully documented at the first visit to ensure evaluation in the same segment of LAD. Flow velocity profiles were registered using pulsed wave Doppler at rest, and during 5 minutes of pharmacological stimuli with adenosine infusion to obtain maximal flow reserve capacity. Cine loops and Doppler images were stored for off-line measurements.

All off-line measurements were performed by an experienced sonographer, blinded to the patients' clinical characteristics. Coronary flow velocity profiles were analysed by using workstation Image Arena 2.9.1 (TomTec Imaging Systems GmbH, Unterschleissheim, Germany) in paper II and Syngo US (version 3.5.6.34, Siemens Medical Solutions) in paper IV. Baseline flow velocity was calculated using the mean of three profiles. Hyperaemic flow velocity was calculated as the mean of the three highest coronary blood flow profiles. Coronary flow reserve is the ratio between hyperaemic and baseline mean flow velocity values.

Methodological considerations using transthoracic echocardiography assessed coronary flow reserve

The coronary flow is mainly determined by the microcirculation, but in the presence of a significant coronary stenosis a high proximal resistance to flow is induced. Coronary flow reserve has a well-accepted cut off value of 2.0 for detecting epicardial stenosis used to predict outcome (64). Coronary flow reserve assessed by transthoracic echocardiography <2.0 has been demonstrated to predict significant LAD stenosis ($>70\%$ diameter stenosis) with a sensitivity of 90% and specificity of 93% (163). However, in normal coronary arteries, during stable haemodynamic conditions, coronary flow is mainly determined by microcirculation (164). A clear cut-off for coronary flow reserve at determining coronary microvascular dysfunction and predicting myocardial ischemia and outcome is not completely studied (164). In addition to 2.0, a value below 2.5 has been considered abnormal (165). Consequently, the cut-off of 2.0 in paper II might underdiagnose patients with coronary microvascular dysfunction.

Coronary flow velocity assessed by adenosine acts mainly at the level of the microcirculation and does not significantly alter the diameter of the coronary artery (166). Adenosine has a rapid onset and short half-life, which adds to the benefits of using it as a vasodilator. However, sometimes patients may hold contraindication such as severe bronchial asthma or poorly tolerate possible side effects and in very rare cases are adenosine non-responders. Echo-based coronary flow reserve measurement requires also an experienced operator to ensure high quality and reproducible measurements.

Carotid artery ultrasound (paper IV)

Ultrasound scanning of left and right carotid arteries was conducted according to the recommendations in “Mannheim Carotid Intima Media Thickness consensus update (2004–2006–2011) (167), based on a standardized protocol for measurement of carotid wall structure. The Acuson SC2000™ ultrasound system was used (Acuson Siemens Medical Solutions USA, Inc. Mountain View, CA 94043 USA) with an 8 MHz transducer (Acuson S2000 9L4) to assess longitudinal view with both near and far walls visible. Plaque screening was performed with and without colour Doppler. CINE-loops of the carotid bifurcation were stored for off-line analysis.

All off-line measurements were performed by an experienced sonographer, blinded to the patients’ clinical characteristics. Common carotid artery intima media thickness (IMT) as well as carotid bifurcation IMT were averaged from the left and right arteries. IMT was measured in the far wall and defined as the distance between the leading edges of lumen-intima and media-adventitia interface. Measurements of carotid bifurcation IMT were manually tracked to the thickest site in long axis view at the R-wave in the ECG. Measurements of common carotid artery IMT were obtained 1 cm proximal to the bifurcation using Workstation Syngo US (version 3.5.6.34, Siemens Medical Solutions) with automatic edge detection of mean IMT over a distance of 1 cm, at the R-wave in the ECG signal.

Peripheral vascular function assessed by EndoPAT®

To measure peripheral vascular function we used peripheral arterial tonometry (4, 67) (Figure 7). Peripheral arterial tonometry is a non-invasive technique that evaluates arterial pulse wave amplitude, which is a measure of pulsatile volume changes in the fingertip (67). EndoPAT®2000 (Itamar Medical, Caesarea, Israel) comprises of two pneumatic finger-cuffs that registers arterial pulse wave amplitude. The cuffs are placed bilaterally on each index finger and measures pressure changes resulting from finger arteriolar volume changes. The pulse wave amplitude is defined as the difference between the highest and lowest points in the pulse wave. After 5 minutes baseline registration, suprasystolic forearm occlusion is assured for 5 minutes in the non-dominant arm. The hyperaemic response is thereafter registered for 5 minutes post cuff deflation. Reactive hyperaemic index is calculated automatically as the ratio between the generated mean pulse wave amplitude signals in specified intervals within the 5 minutes post occlusion and baseline periods, in relationship to the response in the contralateral arm. To account for systemic vascular changes, multiplication with a baseline correction

factor $(0.2276 \times \ln(\text{occluded arm's baseline mean pulse wave amplitude}) - 0.2)$ is performed by the system (168).

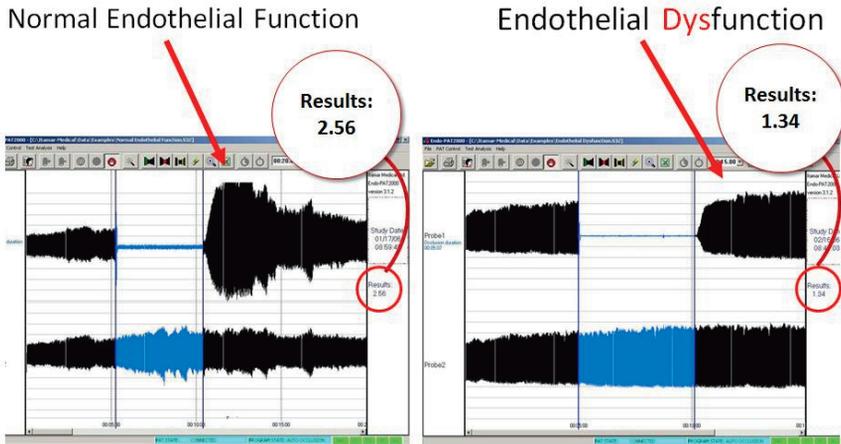


Figure 7 EndoPAT® assessed reactive hyperaemic index

Examples showing EndoPAT® assessed reactive hyperaemic index using peripheral arterial tonometry. Examination on the left hand displays normal function, while the examination on the right hand displays vascular dysfunction. Reprinted from <http://www.itamar-medical.com/>, with permission from Itamar Medical, Caesarea, Israel.

Methodological considerations using EndoPAT®

EndoPAT® was introduced by ItamarLtd. (Caesarea Israel) as an automated and less operator-dependent method to assess peripheral endothelial function (67). The correlation between reactive hyperaemic index and coronary endothelial-dependent vasodilation has been demonstrated high in patients with non-obstructive coronary artery disease (4). It has been demonstrated that approximately 50% of hyperaemia in the fingertip is blocked when nitric oxide synthase specific blocker (L-NAME) was infused prior to EndoPAT® measurement (66). However, reactive hyperaemic index is influenced also by other factors, including sympathetic nerve inflow. Indeed, directly recorded sympathetic nerve activity has been demonstrated to be inversely related to EndoPAT® generated reactive hyperaemic index (70). This may suggest that reactive hyperaemic index is a more complex measure of peripheral vascular function than endothelial function alone. Furthermore, there are several factors known to influence the endothelial function that needs to be reflected upon when performing EndoPAT®. These includes diet, smoking, medication, hormonal influence, timing of meas-

urement, stress, blood pressure hyperactivity, environmental factors and exercise (168). All these are important to consider in aiming to standardize the measurements and minimizing external influences. In addition, the interday variation of coefficient for reactive hyperaemic index has been reported to be relatively high (169, 170). Consequently, small changes in endothelial function over time potentially may be difficult to assess.

Follow-up and definitions of outcome measures (paper II and IV)

The patient cohort with suspected myocardial ischemia was followed-up for major adverse cardiovascular events during a period of five years. The follow-up was performed by a physician, blinded to all other results. Causes of death were examined by data from the Swedish National Board of Health Registry, followed by telephone interviews and reviewing of patient's medical records. Study endpoint in paper I was event-free survival in relationship to impaired glucose homeostasis and time to most severe event was analysed. Outcome was ranked as follows all-cause mortality > non-fatal myocardial infarction/non-fatal stroke > elective/emergency coronary revascularization (percutaneous coronary intervention or coronary artery bypass grafting). In paper II, cardiovascular events were evaluated in relation to coronary flow reserve. Outcome was defined as in paper I, except only cardiovascular cause of death were registered and consequently two mortalities due to cancers were excluded. The occurrence of stroke and acute myocardial infarction were collected from patient's medical records. Myocardial infarction was defined as typical symptoms and pathological troponin changes and/or ECG changes. Stroke was defined as focal or global neurological deficits lasting for more than 24 hours and verified clinically by a neurologist and/or by computed tomography brain scan.

Laboratory analyses (paper I, II and IV)

All blood samples were collected after an overnight fast.

Clinical chemistry analyses

In paper I and II the analyses were performed at AstraZeneca R&D, Gothenburg, using commercially available kits according to the manufacturers' protocols; serum triglycerides and total cholesterol (triglycerides/GB kit No. 12146029216, cholesterol kit No. 2016630, Roche Diagnostics GMBH, Mannheim, Germany).

Direct high-density lipoprotein cholesterol was measured using an enzymatic colorimetric method (kit. No. A11A01636, Horiba ABX, France). The apolipoprotein A1 and B concentrations were measured with turbidimetric technique, using polyclonal rabbit anti-human antibodies (Q 0496 and Q 0497, DakoCytomation, Glostrup, Denmark).

In paper IV, the analyses were performed at Sahlgrenska University Hospital, Department of Clinical Chemistry, Gothenburg. Serum triglycerides (TRIGL, Roche/Cobas, kit. No. 05171407190) total cholesterol (CHOL2, Roche/Cobas, kit. No. 05168538190) and high-density lipoprotein cholesterol (HDLC3 plus 3rd generation, Roche/Cobas, kit. No. 05168805190) were measured using a photometric method (Roche Diagnostics Scandinavia AB). The apolipoprotein A1 (APOBT, Roche/Cobas, kit. No. 03032574122) and apolipoprotein B (APOBT, Roche/Cobas, kit. No. 03032574122) concentrations were measured with turbidimetric technique (Roche Diagnostics Scandinavia AB). Total leukocyte count (LPK / ADVIA2120i), neutrophils (Neutrophils/ ADVIA2120i) and total platelet count (TPK / ADVIA2120i) were analysed using optical particle count (Siemens Medical Solutions Diagnostics AB). High-sensitivity C-reactive protein (CRPHS, Roche/ Cobas. Kit. No. 04628917190) was analysed using an immunoturbidimetric assay (Roche Diagnostics Scandinavia AB).

Glucose homeostasis analyses (paper I, II and IV)

In paper I and II, insulin was measured using a radioimmunoassay (Cat. No. RI-13K, Millipore Corporation, USA). In paper IV, insulin was measured by immunoassay using ElectroChemiLuminescence technology (Roche Diagnostics Scandinavia AB). Plasma glucose was measured using a photometric method (Roche Diagnostics Scandinavia AB). Plasma glucose binds irreversibly to haemoglobin in the red blood cells and thereby forms a glycosylated haemoglobin molecule known as HbA_{1c}. Red blood cells have approximately a 120 day life span, resulting in that HbA_{1c} reflects mean glycaemia for the previous two to three months (171). HbA_{1c} was measured by high-performance liquid chromatography. All analyses were performed at the department of Clinical Chemistry, Sahlgrenska University Hospital, Gothenburg.

Impaired glucose homeostasis, assessed by HOMA-IR was calculated to estimate insulin resistance using the formula: fasting serum insulin (mU/L) x fasting plasma glucose (mmol/L)/22.5 (172).

Methodological considerations using HOMA-IR

A meta-analysis including over 4000 publications, showed HOMA-IR to be more strongly associated with incident of cardiovascular events in individuals without diabetes, than fasting glucose and insulin alone (115). In addition, HOMA-IR is increasingly used as a surrogate marker for insulin resistance and correlates to euglycemic clamp assessed insulin sensitivity (172) in diabetic as well as non-diabetic patients, with no difference between age and sex (173). HOMA-IR offers an easily accessible method to address potential insulin resistance, although some issues needs to be highlighted. 1) Variation of coefficient for HOMA-IR has been reported between 7%-31% and the analysis of fasting serum insulin needs to be standardized (174). 2) HOMA-IR is not used to diagnose pre-diabetes nor diabetes. These clinical states are diagnosed using oral glucose tolerance test and/or fasting glucose levels and/or HbA_{1c} (175). 3) In addition, there is no present validated “golden standard” cut of value for HOMA-IR predicting cardiovascular events. Consequently, we used the median value as cut-off in paper I, aiming to reflect high or low insulin resistance in the present study population.

Proseek analysis (paper I)

In paper I we analysed a number of cardiovascular associated biomarkers including stem cell factor, VEGF-D, VEGF-A, Interleukin-6, and endothelial cell-specific molecule 1 also known as endocan. All biomarkers were analysed at TAATA Biocenter, Gothenburg, using the Olink Bioscience (Uppsala, Sweden) Proseek Multiplex CVD I 96x96 according to the manufacturer's instructions (176) and are presented as arbitrary units (AU) in log² values.

Cardiac injury markers (paper II)

Myoglobin, carbonic anhydrase III, fatty-acid-binding protein, glycogen phosphorylase BB, creatinine kinase MB isoenzyme and cardiac troponin-I were all measured in plasma using the cardiac array panel (Evidence® biochip array technology Randox Laboratories Limited, United Kingdom).

Ultrasensitive Troponin-I was determined using microparticle-based immunoassays and single molecule counting technology (Singulex Ltd, US) generating a LLoQ of 0.4 pg/mL, compared to typical hospital lab tests with a LLoQ at 100pg/mL).

Cytokines, myeloperoxidase, osteopontin, growth factors and cell adhesion molecules (paper II)

Soluble L-selectin, soluble E-selectin and soluble P-selectin were measured in plasma using the adhesion array panel (Evidence® biochip array technology Randox Laboratories Limited, United Kingdom). VEGF-A and epidermal growth factor in plasma were measured using the cytokine array panel (Evidence® biochip array technology Randox Laboratories Limited, United Kingdom). Circulating Interleukin-18 levels were analysed with an enzyme-linked immunosorbent assay (Medical and Biological Laboratories, Nagoya, Japan). Plasma myeloperoxidase was analysed by an enzyme-linked immunosorbent assay (MerckodiaAB, Uppsala, Sweden). Plasma Osteopontin was measured using a sandwich enzyme-linked immunoassay (R&D systems).

L-arginine, symmetric dimethylarginine and asymmetric dimethylarginine (paper II)

L-arginine, symmetric dimethylarginine and ADMA in plasma were quantified using isotope dilution mass spectrometry analysis (liquid chromatography coupled to tandem mass spectrometry (LC-MS-MS)).

Liquid chromatography was performed by hydrophilic interaction chromatography (HILIC), which enabled a simplified and automated sample preparation protocol that involved protein precipitation subsequently followed by direct injection of the supernatant onto the LC-MS system. The detailed description of the sample preparation is outlined in paper III.

Fibrinogen (paper II and IV)

In paper II, plasma fibrinogen was determined at the Karolinska University Laboratory, University Hospital Solna, Stockholm, by an immunonephelometric method using Polyclonal Rabbit Anti-Human antibody (Q0122, Dako Sweden) and analysed on Beckman Coulter Immage 800 Immunochemistry System. In paper IV, plasma fibrinogen was analysed by the Clauss clotting method using the STA-R Evolution system Fibrinogen (Triolab AB, Mölndal) at the department of Clinical Chemistry, Sahlgrenska University Hospital, Gothenburg.

Gene expression analysis (paper I)

Gene expression profiling reflects which genes that are expressed at a certain time point. Affymetrix Gene Arrays use whole-transcript arrays that include probes measuring both messenger (mRNA) and long intergenic non-coding RNA transcripts (lincRNA). This aim to provide a complete expression profile of mRNA as well as the intermediary lincRNA transcripts that impact the mRNA expression profile.

We performed gene expression profiling in 54 consecutive patients without myocardial perfusion defects with the purpose to study potentially regulated gene pathways involved in inflammation and angiogenesis. Extraction of RNA was done on whole blood samples, collected using Paxgene tubes in conjunction to the imaging procedure. Affymetrix Gene Arrays (Human Gene 1.0 ST array) was used to interrogate 28869 transcript clusters and CEL files were imported to Partek Genomics Suite version 6.5 (Partek Inc., MO, USA). The data was \log_2 -transformed and Robust Multi-array Average normalization was performed using ArrayStudio (Omicsoft, version 3.2.0 and 3.5.0). The normalized gene expression values were then fitted with a linear regression model using the Bioconductor R package 'limma' (linear models for microarray data) version 3.22.7. Using an empirical Bayesian approach, the 'limma' package infers differential expression in individual genes from the microarray data (177).

Partial least squares – discriminant analysis, functional and pathway analysis

Partial least squares (PLS) is an efficient statistical regression technique that is suited for the analysis of genomic data for modelling of regulation network (178). The microarray data from the 54 patient samples were analysed using the application of partial least squares – discriminant analysis (PLS-DA) by using the MATLAB® software v. 8.3.0.532 (R2012a, The MathWorks, Inc.) and the MATLAB® PLS Toolbox v. 7.5.2 (Eigenvector Research, Inc.). We applied PLS-DA scores and weights plot to identify markers, from the 'limma' gene expression analysis results, correlated to patients with high HOMA-IR and low reactive hyperaemic index assessed by EndoPAT®. Cut-off for high HOMA-IR and low reactive hyperaemic index were based on median values.

Pre-clinical Ultrasound methods

Anaesthesia and preparations

Mice were anaesthetized using Isoflurane (Abbot Scandinavia AB, Solna, Sweden). During anaesthesia, normal body temperature was maintained using a thermo-regulating lamp. Anaesthesia impacts circulation and respiration, but is a necessary approach for ultrasound examination and adenosine infusion in mice. Isoflurane has been demonstrated to give reproducible cardiac measurements in mice and is recommended for studying cardiac function in longitudinal studies (179). Isoflurane has a rapid onset and short half-life, which enables good control of anaesthesia time and depth. These advantages are of particular importance in longitudinal studies to minimize stress levels in mice. Moreover, our current pre-clinical ultrasound protocol has been validated during isoflurane anaesthesia, which has also been shown to give rise to stable heart rate (180, 181). However, higher doses of isoflurane have been shown to perform vasodilating effects on coronary arteries (182). For this reason, mice were kept on low levels (1.0-1.5%) during ultrasound examination. The total time of anaesthesia and protocol performance was approximately 25 minutes/mouse and projections were captured in the same order in all mice. Mice were kept on a ventilated and heated bench. The chest was shaved using an electrical razor and hair removal cream.

Transthoracic Echocardiography

Transthoracic echocardiography was performed at 10, 16 and 21 weeks of age in all mice using a high-frequency ultrasound imaging system (Vevo 2100 Visual Sonics, Inc, Toronto, Ontario, Canada) with a 40-MHz central frequency transducer. The maximal theoretical resolution of the transducer is 40 μm and we used a framerate of approximately 233 Hz/s in long axis view and 309 Hz/s in short-axis view. Cine loops were stored for off-line measurements. Measurements of left ventricle dimensions were performed in standard B-Mode images in short axis views at the papillary level. The calculations has been validated previously in mice (183).

*Fractional shortening = ((LVEDD - LVESD) / LVEDD) * 100*

LVEDD = left ventricle end-diastolic diameter

LVESD = left ventricle end-systolic diameter

Left ventricle mass = (LVEDD + AW + PW)³ - (LVEDD)³

LVEDD = left ventricle end-diastolic diameter

AW = anterior wall thickness

PW = posterior wall thickness

Heart rate was calculated as average from three consecutive cardiac cycles in Doppler mode.

Transthoracic Echocardiography assessed coronary flow velocity reserve

A catheter (0.4 x 10mm, Becton Dickinson Infusion Therapy, Helsingborg, Sweden) was inserted into the tail vein for intravenous infusion of adenosine (140 µg/kg/min) (ITEM Development AB, Stocksund, Sweden) administration, using an infusion pump. This dose of adenosine has been validated previously to induce maximal coronary hyperaemia without influencing systemic hemodynamic in mice (181). Maximal hyperaemic response is obtained within 2-3 minutes post adenosine infusion. Resting and hyperaemic flow velocity were measured in the same part of the proximal left coronary artery in a modified parasternal long-axis view using the colour-Doppler guided mode (Figure 9). This modified view generates a typical parallel angle between the artery and Doppler beam, facilitating Doppler measurements without angle correction. Cine loops and Doppler images were stored for off-line measurements.

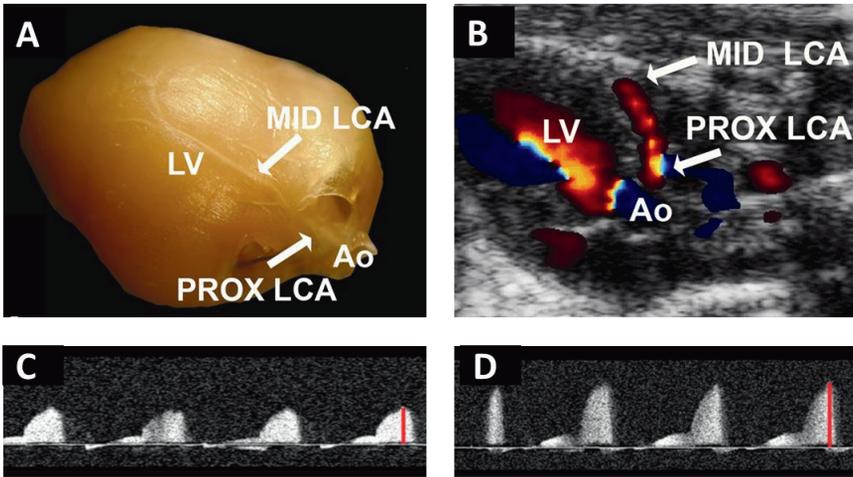


Figure 9 Murine transthoracic Doppler echocardiography assessed coronary flow reserve
 (A) Illustrates a formalin-fixed mouse heart with the left coronary artery. The corresponding image visualized with colour Doppler echocardiography is shown in (B). Coronary flow velocity profiles from the proximal left coronary artery during rest (C) and hyperaemic (D) conditions. Ao; aorta, LCA; left coronary artery, LV; left ventricle, MID; middle, PROX; proximal. Reprinted and modified with permission from Wolters Kluwer Health, Inc. Grönros J. et. al. Proximal to middle left coronary artery flow velocity ratio, as assessed using colour Doppler echocardiography, predicts coronary artery atherosclerosis in mice. *Arterioscler Thromb Vasc Biol.* 2006 May;26(5):1126-31 (184).

Coronary flow reserve is calculated as follows:

hyperaemic coronary flow velocity / basal coronary flow velocity

and has shown to correspond well to the actual volumetric counterpart, coronary flow reserve, CFR (185).

Renal ultrasound

Microvascular dysfunction is not only a coronary or peripheral vascular disease, but might be evident also in other organs such as the kidney. The parallel progression of cardiac and renal vascular dysfunction in type 2 diabetes, called cardio-renal syndrome, is a growing area of interest and importance. Clinically, coronary dysfunction is related to early renal dysfunction in terms of microalbuminuria in type 2 diabetic patients without obstructive coronary artery disease (186) as well as to decreased renal function at an early stage (187). These clinical observations indeed further support microvascular impairment to be a com-

mon mechanism underlying both cardiac and renal vascular complications in type 2 diabetes.

Pulsatility index and resistive index, derived from renal Doppler flow velocity profiles (188), can be used as tools for diagnosing renal artery stenosis, as well as markers for renal vascular function in e.g. diabetic nephropathy in humans (189). In addition, resistivity index has been measured using Doppler ultrasound in a mouse model of cardiorenal syndrome (190). Thus, in this study, we aimed to use established Doppler ultrasound techniques to document parallel changes in cardiac and renal vascular function.

In combination with the cardiac echocardiography examination, before adenosine infusion, we performed examination of the right kidney at 21 weeks of age. Standard B-mode examination of the kidney was performed in a long-axis view using the same ultrasound imaging system as described above, with a framerate of approximately 200 Hz/s. Following colour-Doppler mapping of the renal vascular tree using a colour-Doppler frequency of 32 MHz, a cursor was placed at the central segmental artery and renal flow velocity was measured using pulsed wave Doppler with an angle correction of 0-5 degrees (Figure 10). For repeated measurements, the examined segment was precisely visualized and degree of angle correction documented at the first time point, to ensure evaluation in the same renal segment.

The renal resistance parameters were determined as follows:

$$\text{Resistivity Index} = (PSV - EDV) / PSV$$

$$\text{Pulsatility Index} = (PSV - EDV) / MV$$

PSV = peak systolic velocity

EDV = end-diastolic velocity

MV = mean velocity (time averaged velocity)

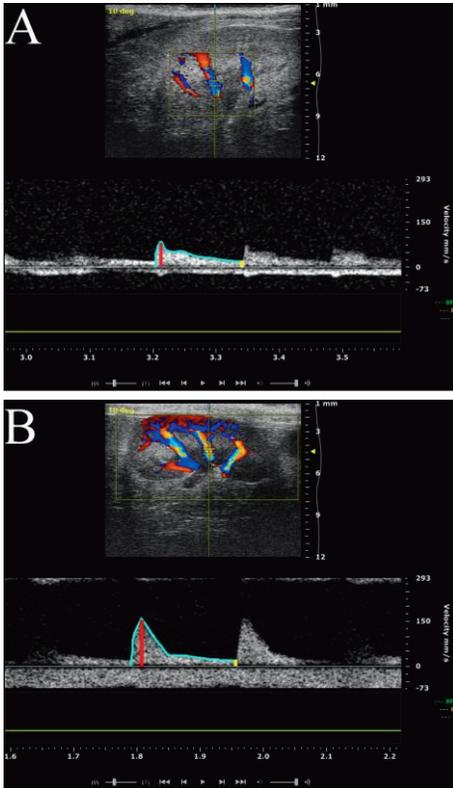


Figure 10 Colour Doppler images for measurement of renal flow velocities

Recordings of intrarenal flow velocities measurement in segmental renal arteries were performed with colour Doppler ultrasound in lean and leptin-deficient (*ob/ob*) mice at 21 weeks of age. The pulsatility flow profile was used for measurement of peak systolic velocity (PSV) (red line), mean velocity (MV) (blue line) and lowest diastolic velocity (LDV) (yellow line) for calculation of pulsatility index and resistive index. A: Renal flow velocity profile in lean mice. B: Renal flow velocity profile in *ob/ob* mice. Reprinted with permission from PLoS One. Westergren HU. et. al. Impaired Coronary and Renal Vascular Function in Spontaneously Type 2 Diabetic Leptin-Deficient Mice. PLoS One. 2015 Jun 22;10(6):e0130648 (191).

Off-line measurements

All off-line measurements were performed by an experienced sonographer using workstation Vevo 2100 (version 1.6.0, Visual Sonics). Coronary flow profiles were calculated as the mean from two peak diastolic flow velocity profiles. Renal flow velocities were obtained by delineating the entire flow profiles (mean velocity, MV), the peak velocity (peak systolic flow velocity, PSV) and the lowest velocity (end-diastolic velocity). All measurements were averaged from two representative profiles. Kidney length was measured off-line in long axis view (Figure 11).

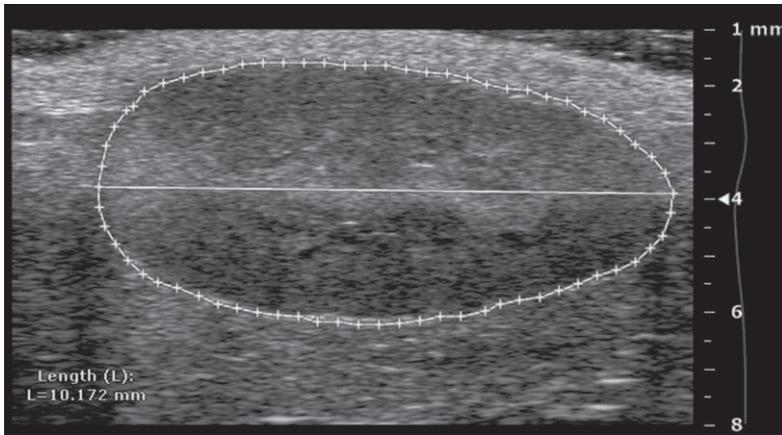


Figure 11 B-mode ultrasound measurement of kidney length in long-axis view

Off-line measurements of kidney length. Reprinted with permission from PLoS One. Westergren HU. et al. Impaired Coronary and Renal Vascular Function in Spontaneously Type 2 Diabetic Leptin-Deficient Mice. PLoS One. 2015 Jun 22;10(6):e0130648 (191).

Methodological considerations using pre-clinical high-resolution ultrasound

Anaesthesia is known to affect hemodynamic parameters, but isoflurane has been demonstrated to induce less cardiac depression compared to other agents, as well as being associated with reproducible measurements in mice (179). In our protocol, all imaging was performed in this condition, with lowest possible isoflurane concentration (1.0-1.5%). Furthermore, we captured all projections in the same order, trying to minimize time-associated effects on cardiac parameters.

Measurements of cardiac parameters such as fractional shortening and left ventricle mass have majorly been evaluated in M-mode in previous studies as recommended by guidelines from American Society of Echocardiography (192). However, the guidelines might not apply with high-resolution ultrasound assessed cardiac parameters as are currently possible in rodents. Our study used high-resolution ultrasound, which facilitates for cine loops captured in B-mode with frame rate of 309 frames per second in short axis view and 200 frames per second in long axis view in cardiac and kidney imaging, respectively. Furthermore, assessment of left ventricle mass in mice using high-resolution ultrasound and B-mode has been demonstrated feasible and as good if not possibly better than M-mode (193).

Pre-clinical laboratory analyses

Measurement of haptoglobin, cholesterol and triglycerides

Plasma levels of haptoglobin were measured using an enzymatic colorimetric method (Kit No TP 801) from Tridelta Development LTD, Ireland. Total cholesterol was measured using an enzymatic colorimetric method (Kit No A11A01634) from Horiba ABX, France. Plasma triglycerides were measured using an enzymatic colorimetric method (Kit No 12146029 triglycerides /GB) from Roche Diagnostics GmbH, Germany. The content of triglycerides in liver and heart tissues were determined after homogenisation in isopropanol. Triglycerides in the supernatant were measured using an enzymatic colorimetric method (Kit No A11A01640) from Horiba ABX, France.

L-arginine and asymmetric dimethylarginine

Analysed as written on page 35.

Measurement of glucose homeostasis

The measurement of HbA_{1c} was performed from tail vein blood at week 10, 16 and 21 using A1CNow+ (Bayer Healthcare, Austria). Blood glucose concentration was determined from tail vein using an ACCU-CHEK[®] Compact Plus analyser (Roche Diagnostics Scandinavia AB). Blood insulin levels were measured using an ELISA kit (Ultra-Sensitive Mouse Insulin ELISA kit, Chrystal Chem Inc., IL, USA). Both glucose and insulin were measured at the age of 21 weeks, after 4 hour fasting in the awake mouse.

Insulin resistance-index was used to validate insulin resistance in our mice to reflect HOMA-IR in humans (174). Insulin resistance-index was calculated with the following formula: fasting blood insulin (ng/mL) x fasting blood glucose (mmol/L).

Measurement of Albumin and Creatinine in urine

Urine Albumin was measured using a commercial ELISA kit (Cat No E-90AL) from ICL, USA. Urine Creatinine was measured using an enzymatic colorimetric method (Kit No A11A01933) from Horiba ABX, France. All mentioned bi-

omarkers were measured at the terminal endpoint of 21 weeks of age. Albuminuria was evaluated calculating spot urine albumin/creatinine ratio ($\mu\text{g}/\text{mg}$).

Vascular area fraction

Histological staining of endothelial cells was performed using Lectin I, detailed description is found in paper III. Lectin-I is a family of glycoproteins that binds specific carbohydrate moieties of endothelial surface glycoproteins. Lectin I is preferable due to easy, strong, distinct staining of vessels in different sizes, resulting in a reliable staining pattern of blood vessels.

Computerized image analysis is a fast and reproducible method to quantify blood vessels in histological sections. Stained tissue sections were digitized using a Zeiss Mirax slide scanner (3D Histech, Budapest, Hungary). The resulting virtual slides were imported into Visiopharm Integrator System software (version 3.6.5, Visiopharm, Hørsholm, Denmark). Computerized image analysis was used to quantify transversely sectioned blood vessels with defined characteristics: cross sectional areas between 20 and 200 μm^2 (to avoid larger vessels, longitudinally sectioned vessels and background staining). The resulting segmentation was validated by an imaging expert and are reported as the mean area fraction of the identified vessels compared to the total tissue area examined (including the vessels) for each animal.

Statistics

All analyses were performed in SPSS, (version 21.0, Chicago Inc, USA). A p-value <0.05 was considered statistically significant. Test of skewness was used to assess normal distribution for the numerical variables, data are presented with mean \pm standard deviation or median with interquartile ranges (IQR) for normal and non-normally distributed parameters, respectively. Detailed descriptions of analyses are found in respective paper.

Paper I and II

Spearman's correlation coefficients were used to examine relationships between different variables.

Differences between groups among continuous variables were analysed using **Student's t-test** or its non-parametric alternative **Mann-Whitney U test**, for normally distributed and non-normally distributed parameters, respectively. Categorical data was analysed by **Pearson chi-square test**.

Univariate linear regression model was used to examine relationship between different variables and reactive hyperaemic index (paper I) and coronary flow reserve (paper II).

Multivariable linear regression model was used to adjust for potential confounders. Possible co-linearity between the independent variables was tested and a coefficient >0.7 was considered significant. Relevant independent parameters associated to the dependent parameter with a p-value <0.25 entered the model.

Kaplan-Meier curves were used to display survival rates.

Cox proportional-hazards regression was used to assess the association between HOMA-IR (paper I) and coronary flow reserve (paper II) and events. Adjusted hazard ratios (HR) with corresponding 95% confidence intervals (CI) were estimated in both univariate and multivariable models. Independent parameters were selected due to their known significant clinical value and/or association to the dependent parameter with a p-value <0.25 .

Principal component analysis (paper II)

Principal component analysis is a projection method used to bring out strong patterns in a dataset. It allows for a simultaneous comparison and illustration of the inter-variable relationship and contribution of all variables to an endpoint, in a non-biased manner. To rank and illustrate which variables predicted coronary flow reserve in this cohort, a supervised principal component analysis was performed using Simca 13 (Umetrics, Umeå, Sweden). Before analysis, all data was scaled to unit variance and mean centered. In addition, variables with a max/mean-ratio >10 , were log-transformed to increase equal leverage of all variables. This generated a data matrix consisting of 69 variables in 202 patients. The dataset was subjected to an orthogonal projection to latent structures by partial least square analysis (OPLS), in which the variability not related (orthogonal) to what is predicted (coronary flow reserve) is filtered out (194). In the model generated by the OPLS, each variable gets a loading score, the amplitude of which quantifies the contribution of the variable to what is predicted in the

model. The OPLS analysis was performed to identify and rank 66 variables predicting coronary flow reserve (basal and hyperaemic flow excluded to avoid dominance of these variables in the model). The model described (R²) 21% of the coronary flow reserve data, with negative predictive (Q²) value of -0.014 after a Jack knife cross validation as implemented in the SIMCA software. Given the biased gender distribution in outcome, an OPLS discriminant analysis (OLPS-DA) was performed to illustrate major differences between the genders.

Paper IV

When appropriate, data were log-transformed before analysis.

Coefficient of variation was calculated as standard deviation(x-y)/mean(x,y) × 100%.

Linear regression analysis was used for within group comparisons with change from baseline as dependent variables and baseline values as covariates.

Analysis of covariance was used to compare DAPS intervention to control period as well as to Standard intervention. We compared the variables at visit 2, using the values at visit 1 as covariates.

Paired t-test was used to compare effects of three months intervention in the standard group with its own control period using delta control-data versus delta intervention-data.

Ordinal data were analysed by means of **Wilcoxon signed-rank test**.

Power calculations (paper I, II and IV)

Paper I: We calculated the sample size based on the Cox PH one-sided superiority formula. With an overall event rate 20%, an alpha level 5% and 80% power, we need approximately 260 patients to estimate a HR of 2.0.

Paper II: We calculated the sample size based on the Cox PH one-sided superiority formula. With an overall event rate 15%, an alpha level 5% and 80% power, we need approximately 214 patients to estimate a HR of 3.0.

Paper IV: The sample size was calculated based on a 10% improvement in coronary flow reserve during DAPS intervention, with an alpha level 5% and 80% power.

Paper III

The number of animals/group was based on previous in house experiments.

As data appeared clearly non-normally distributed **Mann-Whitney U-test** was used to detect statistical differences between lean and ob/ob mice. Before analysis, we assumed an increased difference due to time in bodyweight, HbA_{1c}, coronary hyperaemic flow velocity, coronary flow velocity reserve, fractional shortening, and left ventricle mass between strains. Therefore, a **stepwise Mann-Whitney U-test** on the 5%-significance level was used to compare changes between strains, starting at 21 weeks of age.

Results and Discussion

Insulin resistance, coronary microvascular dysfunction and outcome (paper I and II)

Impaired glucose homeostasis in the non-diabetic state may be of importance in progression of microvascular dysfunction, i.e. in patients with chest pain without obstructive coronary artery disease. We therefore investigated the additive value of HOMA-IR to myocardial perfusion scintigram results. In paper I, the study cohort included 365 non-diabetic patients with and without myocardial perfusion defects, displaying an overall event rate of 78 (21%). In paper II, 202 patients without myocardial perfusion defects were included, of these 25 patients (12%) displayed major adverse cardiovascular events. Indeed, in our non-diabetic population, we demonstrate insulin resistance assessed by HOMA-IR to independently predict event-free survival during a follow-up period of 5 years in patients without myocardial perfusion defects (Figure 12A), independently of previous history of coronary artery disease (HR:5.0, $p=0.014$). Furthermore, coronary flow reserve <2.0 independently predicted cardiovascular outcome (Figure 12B) in patients without myocardial perfusion defects, also regardless of previous history of coronary artery disease (HR:6.6, $p=0.001$). This indicates that patients with chest pain but without myocardial perfusion defects may still display coronary microvascular dysfunction possibly associated with increased insulin resistance.

In the current population of 365 patients (paper I) with chest pain, most patients (238 patients, 65% of the study cohort) displayed no myocardial perfusion defect. Of these, 202 patients performed coronary flow reserve investigation (paper II) and 29 patients (14%) appeared to have pathological coronary flow reserve <2.0 . Interestingly, as many as 81 patients (40%) had coronary flow reserve <2.5 , indicating coronary microvascular dysfunction. Traditional risk factors associated with structural and functional alterations leading to decreased coronary microvascular function includes those in obstructive coronary artery disease. However, these traditional risk markers are not always present in coronary microvascular dysfunction (6) and are shown to correlate poorly with the decreased coronary function (7). Interestingly, decreased coronary flow reserve is associated with increased insulin resistance in non-diabetic subjects without

coronary angiography verified stenosis, highlighting its potential importance in coronary microvascular dysfunction (116). In agreement, insulin resistance per se, alone carries prognostic value for future cardiovascular events in subjects without diabetes (115).

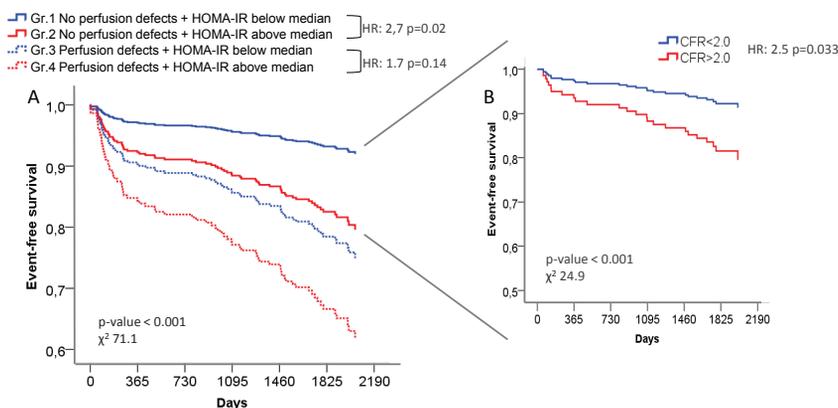


Figure 12 Multivariable adjusted COX regression analyses

In a COX regression analysis adjusting for age, gender, high density lipid cholesterol, systolic blood pressure and body mass index, (A) HOMA-IR provides independent prognostic information in non-diabetic patients with no myocardial perfusion defects (n=238), but not in patients with myocardial perfusion defects (n=127). In a COX regression analysis (B) of 202 patients with successful coronary flow reserve investigations from the 238 patients without myocardial perfusion defects, coronary flow reserve provides independent prognostic information after adjusting for age and gender. CFR; coronary flow reserve, Gr; group, HR, hazard ratio, HOMA-IR; homeostatic model assessment for insulin resistance. Figure 12A is reprinted with permission from Cardiovascular Diabetology. Westergren HU. et. al. Insulin resistance, endothelial function, angiogenic factors and clinical outcome in non-diabetic patients with chest pain without myocardial perfusion defects. *Cardiovasc Diabetol.* 2016 Feb 19;15(1):36 (195).

The gender aspect (paper II)

Insulin resistance at a non-diabetic level is related to functional (110, 111) as well as potentially structural vascular changes (112, 113), and is associated with microvascular dysfunction (114). We therefore applied principal component analysis to identify and rank 66 cardiovascular parameters predicting decreased coronary flow reserve in patients without myocardial perfusion defects, again HOMA-IR seemed of significant importance. Interestingly, the men appeared more insulin resistant than the women, 3.6 (IQR:2.6;6.3) and 2.8 (IQR:2.1;4.8), respectively (p<0.001). We therefore performed multivariable linear regression models adjusting for five relevant parameters in a gender specific manner. Indeed, in a multivariable linear regression analysis, the association between

HOMA-IR and coronary flow reserve was independent in men ($\beta=-0.132$, $p=0.041$), while in women increased systolic blood pressure ($\beta=-0.009$, $p=0.011$) was independently associated to decreased coronary microvascular function, here visualized in correlation curves for women and men respectively (Figure 13A and 13B).

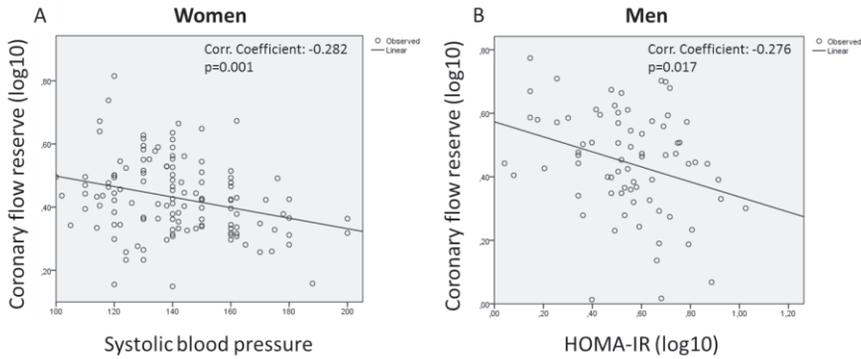


Figure 13 Correlation curves

Correlation curves between coronary flow reserve and A) systolic blood pressure in women (n=128) and coronary flow reserve and B) HOMA-IR in men (n=74) in patients with suspected myocardial ischemia but without myocardial perfusion defects. HOMA-IR; homeostatic model assessment for insulin resistance.

In alignment with our results, hypertension is known to influence the underlying mechanisms in microvascular dysfunction (5) and induce coronary structural and functional alterations (3, 8). One of the hallmarks of hypertension is the increase of vascular resistance in all major organ systems, affecting mostly small arteries and arterioles (196). In fact, human hypertension is associated with a narrowing of the internal lumen and an increase in media wall thickness. This remodelling has been demonstrated to correlate with decreased coronary flow reserve (98). Indeed, hypertension in women is often undiagnosed or inadequately treated (136) emphasizing the value of assessing systolic blood pressure in addition to hypertension diagnosis in female coronary microvascular disease. Facilitating the male observation, decreased coronary flow reserve is associated with increased insulin resistance in non-diabetic subjects without angiography verified stenosis, highlighting its potential importance in coronary microvascular dysfunction (116).

Insulin resistance and associated mechanisms potentially underlying the observed microvascular dysfunction (paper I, II, III and IV)

The pathophysiological mechanisms underlying microvascular dysfunction are complex and involve multifactorial mechanisms, all interacting with each other (3, 23). Endothelial dysfunction and insulin resistance share common pathology. This includes increased oxidative stress (123) as well as low-grade inflammation, such as increased levels of chemokines, cytokines, and adhesion molecules (197). Also, fibrinogen contributes to microvascular dysfunction, and its concentrations have been shown to negatively correlate with degree of insulin sensitivity in non-diabetic patients (127). Insulin resistance is associated with reduced nitric oxide bioavailability, shown to contribute to microvessel rarefaction (198) and a decreased capillary surface has been observed in insulin resistant patients (199). Consequently, impairment in insulin-stimulated capillary recruitment may play an important role in further progress of insulin resistance (200). Possible links between microvascular function and underlying mechanisms has been aimed to be potentially addressed in all papers include in this thesis.

Insulin resistance and endothelial dysfunction

Insulin resistance and endothelial function are closely related. In the insulin resistant state, reduced PI3K-nitric oxide pathway and an intact MAPK-endothelin-1 pathway typically accompany endothelial insulin resistance. To maintain euglycemia, hyperinsulinemia is triggered, overdriving the unaffected MAPK-dependent pathway. This will lead to an imbalance between the pathways, favouring vasoconstriction (122, 123, 201). In paper I, we demonstrate continuous HOMA-IR to be negatively associated with digital reactive hyperaemic index (β : -0.014, $p=0.004$), considered to reflect peripheral endothelial function. Also, patients with HOMA-IR in the upper compared to lower median value displayed a significant inverse association with reactive hyperaemic index (β : -0.273, $p=0.002$), also after multivariable adjustment (β : -0.230, $p=0.022$) (paper I). The correlation between reactive hyperaemic index and coronary endothelial-dependent vasodilation has been demonstrated high in patients with non-obstructive coronary artery disease (4). These observations together with our results indicate insulin resistance to be associated with decreased endothelial function in patients with chest pain but without myocardial perfusion defects.

Furthermore, in the translational study in insulin resistant ob/ob mice (paper III), we demonstrate decreased coronary flow reserve (Figure 14A) at 16 and 21 weeks of age. These mice lack atherosclerotic plaques (154), indicating impaired microvascular dysfunction. In agreement, the ob/ob mice demonstrated decreased levels of plasma L-arginine compared to lean controls assessed at 21 weeks of age (Figure 14B).

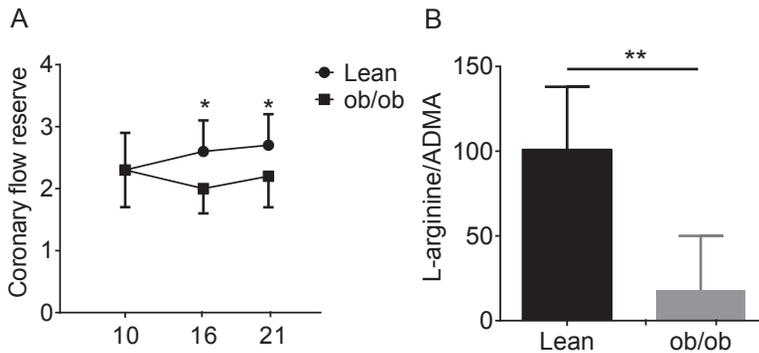


Figure 14 Decreased coronary flow reserve and L-arginine/ADMA ratio in ob/ob mice

A) Coronary flow reserve was measured in lean (n=11-12) and leptin-deficient (ob/ob, n=10-12) mice at 10, 16 and 21 weeks of age using the ultrasound colour Doppler technique. B) L-arginine and asymmetrical dimethylarginine (ADMA) was analysed in plasma at 21 weeks of age and L-arginine/ADMA ratio was calculated. Values are displayed as mean \pm standard deviation. *: $p < 0.05$; **: $p < 0.001$.

L-arginine is the substrate for both nitric oxide synthase, which converts arginine to nitric oxide and citrulline, as well as for arginase that converts L-arginine to urea and ornithine. Arginase competes with nitric oxide synthase for their common substrate L-arginine and thereby might contribute to the decreased nitric oxide-availability in pathological conditions such as type 2 diabetes (202). This might lead to a lack of nitric oxide-availability and thereby reduced endothelial artery function. In line with our observations, Saraiva et.al. demonstrated decreased cardiac nitric oxide production and increased oxidative stress in ob/ob mice (203). These mechanisms might also contribute to the observed coronary microvascular dysfunction, potentially reflecting a relevant aspect of the human pathology.

Furthermore, cardiovascular and renovascular diseases are known to be closely related. Patients with non-obstructive coronary artery disease and chronic kidney disease have lower coronary flow reserve than those with normal glo-

merular filtration rate (187) and low coronary flow reserve is of prognostic value for cardiovascular outcome in patients with chronic kidney disease (204). Indeed, at 21 weeks of age the ob/ob mice display increased renal vascular resistance profiles compared to lean mice (Figure 15A and B).

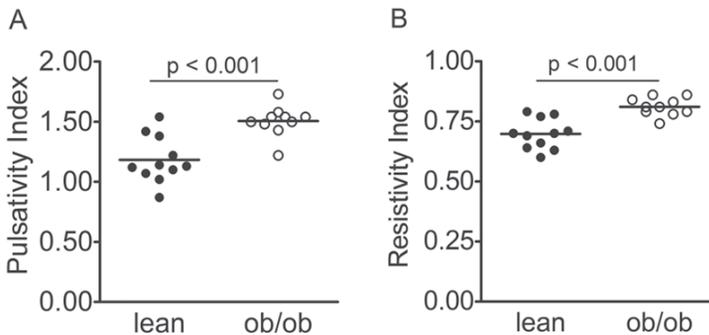


Figure 15 Increased renal pulsatility index and resistivity index in ob/ob mice at 21 weeks of age
Renal flow velocity was measured in lean (n=11) and leptin-deficient (ob/ob, n=10) mice at 21 weeks of age using the ultrasound colour Doppler technique. A: Renal pulsatility index, B: Renal resistivity index. Values are presented as individual data points and means. Reprinted with permission from PLoS One. Westergren HU. et. al. Impaired Coronary and Renal Vascular Function in Spontaneously Type 2 Diabetic Leptin-Deficient Mice. PLoS One. 2015 Jun 22;10(6):e0130648 (191).

In the ob/ob mice, this observation is probably explained by initial renal hypertrophy as indicated by increased renal tissue area ($p=0.002$) and ultrasound-assessed kidney length ($p=0.027$) compared to lean mice. Furthermore, a small increase in spot urine albumin/creatinine ratio was found in ob/ob: $59 \pm 17 \mu\text{g}/\text{mg}$ versus lean mice $18 \pm 3 \mu\text{g}/\text{mg}$, $p < 0.001$. Interestingly, elevated resistance in renal blood flow in type 2 diabetic patients has been shown to be a marker of early renal vascular alteration, even before the onset of microalbuminuria (205). In line with this data, the ob/ob mouse model might be suitable for further mechanistic studies of endothelial dysfunction as well as for changes in renal blood flow as an early marker of renovascular changes.

Insulin resistance and blood viscosity and vascular inflammation

Systemic inflammation is known to be prevalent in the pre-diabetic state (206). In agreement, in paper I we demonstrate increased insulin resistance in terms of

HOMA-IR to be associated with increased levels of Interleukin-6, corr. coeff=0.200, $p=0.002$ and Interleukin-6 to be increased in patients with HOMA-IR above compared to below median value (3.8 ± 1.1 and 3.4 ± 0.9 AU, respectively, $p=0.008$). This cytokine has been shown to be related with both decreased coronary and peripheral microvascular function (207, 208), possibly contributing to the observed decreased microvascular dysfunction in our patient population.

Blood viscosity is a contributor to microvascular function and shown to predict ischemic heart disease (85, 86). Fibrinogen is one of the major contributors to the viscosity of plasma and important in the complex mechanism of coronary microvascular dysfunction (87, 93). Indeed, we demonstrate lifestyle intervention to decrease fibrinogen levels in healthy volunteers as well as to increase coronary flow reserve (Figure 16A and B).

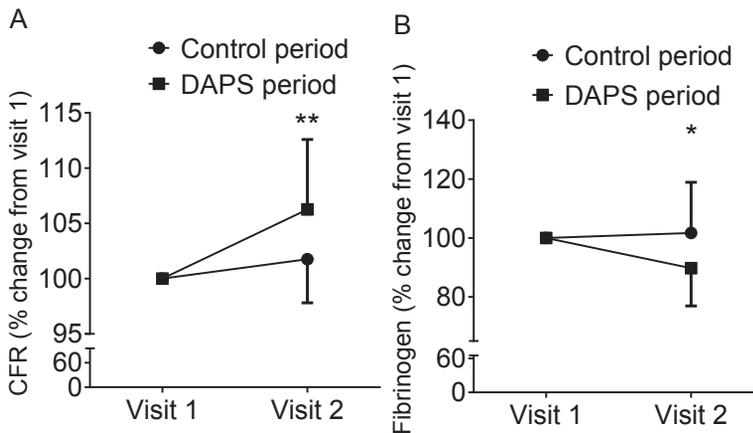


Figure 16 Increased coronary flow reserve and decreased fibrinogen after 12 weeks life style intervention

In healthy volunteers, 12 weeks of personalized life style intervention (DAPS) significantly increased A) coronary flow reserve (CFR) ($n=19$) as well as B) decreased plasma fibrinogen ($n=14$). Values are presented as % change from visit 1. No significant change was observed during the control period. * $p<0.05$, ** <0.001 .

Furthermore, fibrinogen correlates to HOMA-IR in our study cohort of 202 patients with chest pain, without myocardial perfusion defects, corr. coeff=0.296, $p<0.001$. Also, fibrinogen levels are increased in patients with HOMA-IR above median as compared to below, 3.0 (IQR:2.4;3.5) g/L and 3.3 (IQR:2.9;3.8) g/L, respectively, $p<0.001$. These results may suggest that fibrinogen may be a link between microvascular dysfunction and insulin resistance at a non-diabetic level.

Vascular remodelling and rarefaction

In addition to endothelial dysfunction, inflammation and blood viscosity, the progress of vascular remodelling and rarefaction also appears in microvascular dysfunction (23). Microvascular dysfunction cause impaired capillary recruitment and decreased microcirculatory blood flow to metabolically active and insulin-dependent tissue. Insulin resistance further impairs endothelial function and capillary recruitment, resulting in a negative circle (125). Ischemia is known to stimulate angiogenesis, and conditions of only minimal ischemia stimulate development of collateral vessels (209). Stem cell factor as well as VEGFs are important in vascular angiogenesis. Stem cell factor is a hypoxia-induced growth factor that binds to its receptor c-kit and promotes survival, proliferation, mobilization, and adhesion of all c-kit-expressing cells, which includes hematopoietic stem cells, endothelial progenitor cells and cardiac stem cells (210). Furthermore, c-kit has been found on smooth muscle progenitor cells in the artery wall, indicating a functional role of stem cell factor in the vessels (211). Interestingly, decreased stem cell factor levels were recently found in diabetic patients and were related to incidence of cardiovascular events, showing a predictive value in the same range as established risk factors (212). The growth factor VEGF-D has been shown to be important for vascular angiogenesis and lymphangiogenesis, as well as stimulating endothelial production of nitric oxide (213, 214).

Indeed, in paper I, VEGF-D was inversely correlated to HOMA-IR (corr. coeff=-0.213, p=0.001) and was significantly lower in patients with HOMA-IR above compared to lower median value (6.3 ± 0.3 and 6.1 ± 0.4 AU, below versus above median, respectively, p=0.04). Furthermore, high HOMA-IR was inversely correlated with stem cell factor (corr. coeff=-0.219, p=0.001) and patients with HOMA-IR above median had reduced level of stem cell factor (8.1 ± 0.4 and 8.0 ± 0.3 AU, below versus above median, respectively, p=0.001). Interestingly, decreased stem cell factor correlated significantly to decreased VEGF-D levels (corr. coeff=0.221, p<0.001). In line with our data, He et al. showed that reduced PI3K signalling is likely responsible for the reduction in VEGF induced vascularization in the myocardium at both basal and ischemic states (112). Furthermore, Bonner et al. showed that deletion of VEGF in murine cardiac muscle induced capillary rarefaction and promoted insulin resistance (215). Taken together, these studies indicate insulin resistance to be associated with downregulation of VEGF, causing vascular rarefaction, which may accelerate further development of insulin resistance due to insufficient delivery of insulin. Ischemia-induced hypoxia is also a trigger for increased transcription and translation of VEGFs, necessary for the angiogenic sprouting of capillaries (209). In paper I, an inhibited eukaryotic initiation factor 2 (EIF2) pathway was associated with

increased insulin resistance and endothelial dysfunction. Since VEGF mRNA translation is dependent on the EIF2 pathway, especially during hypoxia (216), an impaired EIF2 pathway could at least in part be responsible for the low VEGF-D protein levels associated with high HOMA-IR. Taken together, the reduced levels of VEGF-D and stem cell factor could be mechanisms underlying endothelial dysfunction associated with insulin resistance in our cohort.

Personalized life-style intervention improves coronary microvascular function in healthy volunteers (paper IV)

The favourable impact of physical activity on cardiovascular health has been long studied and today a sedentary life style is considered one of the major modifiable risk factors for cardiovascular disease (217). Performing regular physical activity is related to reduced cardiovascular mortality in healthy individuals also after adjusting for known risk factors (142). The mechanisms associated with physical activity includes beneficial effect on endothelial function (143) and reduced blood viscosity (144). Indeed, physical exercise has been demonstrated to increase coronary microvascular function in healthy volunteers (146) as well as in patients with stable coronary artery disease (147). In the guidelines from the European Society of Cardiology for prevention of cardiovascular disease (version 2012) not only regular physical exercise but also a healthy diet are considered important cornerstones in preventing cardiovascular disease (10). Mediterranean diet is one of the most studied in cardiovascular prevention, showing beneficial effects on cardiovascular incidence as well as the components of metabolic syndrome (218).

The current study included 36 sedentary healthy volunteers with normal coronary flow reserve. The participants received Mediterranean-inspired diet instructions combined with regular physical exercise (standard group) or a personalized and supervised exercise program (DAPS group) to be performed for three months. The standard group had an initial control period with no instructions and the variance of coefficient of coronary flow reserve assessed by transthoracic Doppler echocardiography measurement for this period was 4.0%. Mean change of coronary flow reserve during the intervention period with a personalized supervised health program was statistical significant increased in DAPS (6.1% (CI:3.7,8.5%), $p<0.001$) but unchanged in the control group (1.5% (CI:-0.9,4.1%), $p=0.198$). Also left ventricle ejection fraction was improved in

the DAPS group (6.2% (CI:1.9,10.7%), $p=0.007$) but unaltered in controls (-3.6% (CI:-11.9,5.5%), $p=0.381$). The significant increase of coronary flow reserve and left ventricle ejection fraction in DAPS remained with 5.0% and 10.2%, respectively when corrected for controls, $p=0.005$ and 0.022 , respectively. In addition, coronary flow reserve and hyperaemic left ventricle ejection fraction remained significantly increased with 4.0% and 8.9%, respectively in DAPS also after adjusted for the standard program, $p=0.018$ and $p=0.017$, respectively. Furthermore, the DAPS program generated a decrease in body mass index (DAPS: -3.9% (CI:-6.0,-1.7%), $p=0.002$), waist/hip ratio (DAPS: -3.1% (CI:-4.7,-1.4%) as well as fat percentage (DAPS: -5.1% (CI:-7.8,-2.4%), $p=0.001$). These remained significant in the DAPS group with a decrease of -3.6%, -3.0% and -5.2%, respectively when corrected for controls. These results emphasize a personalized and supervised health intervention (DAPS) to amplify the increase in coronary flow reserve compared to the standard program. The observed effect may involve both increased exercise capacity as indicated by increased cardiac reserve as well as decreased body mass index, waist/hip ratio and estimated fat percentage. In agreement, Kiviniemi et.al. showed visceral adipose tissue including waist/hip ratio to be associated with coronary flow reserve in healthy lean young men (219). The favourable effects of exercise training and weight loss can potentially be through both endothelial-independent and dependent functions of the coronary vasculature (220) leading to improved adenosine-induced maximum flow response. In agreement, the effect of adenosine is fulfilled both by metabolic dilatation at a pre-capillary level as well as by flow-mediated nitric oxide-dependent vasodilatation (221). Exercise training improves endothelial function by increasing nitric oxide production and bioavailability, contributing to a better vasodilator capacity (220). Indeed, in the current study improved coronary flow velocity reserve could be due to both reduced resting cardiac load as well as improved microvascular function.

Interestingly, we found no effect of either intervention program on HOMA-IR. However, plasma fibrinogen decreased after DAPS intervention with 11.1% (CI:-18.4,-3.2%), $p=0.011$, while fibrinogen remained unchanged in controls with 0.5% (CI:-8.0,9.8%), $p=0.915$. During the personalized supervised health program in the DAPS group, fibrinogen remained significantly increased with 12.1%, when corrected for controls. Lifestyle changes have been shown to prevent type 2 diabetes (222) and reduce cardiovascular mortality (223) in populations with impaired glucose tolerance test. The lack of effect on HOMA-IR is probably partly explained by the indicated high insulin sensitivity in this population (HOMA-IR median: 1.1 (IQR:0.7;1.6)). However, regular physical activity

is known to reduce fibrinogen levels (144) as observed in the present study, possibly contributing to the increased coronary flow reserve.

Limitations of the current thesis

1. Specific limitations are found in respective paper. Paper I and II are observational studies in which problems with possible confounding factors has been addressed using multivariable methods, although possible unadjusted and/or unmeasured confounders can still result in biased estimates. Furthermore, even though performing analysis on complete datasets for HOMA-IR and coronary flow reserve, other parameters included missing values. However, further reduction in sample size reduces statistical power. *Strengthening the Reporting of Observational Studies* (STROBE) are guidelines for writing manuscripts on observational studies, including how to report missing values. Both manuscript I and II have been written according to these guidelines.
2. The included patient cohort of non-diabetic patients without myocardial perfusion defects are a relatively healthy population with low risk-probability. This further result in few events during the follow-up period and consequently small multivariable Cox-regression models.
3. The intervention study on healthy volunteers was designed at studying the effect of lifestyle changes on primarily coronary flow reserve. In addition, we hypothesized HOMA-IR to be improved after 12 weeks of DAPS intervention. However, the volunteers recruited to the current study displayed unexpectedly low HOMA-IR levels and the hypothesis could therefore not be fairly tested. In this healthy population, euglycemic clamp or oral glucose tolerance test might have added further information.

Conclusions

An integrated overview of the results is visualized in Figure 17.

- Coronary microvascular dysfunction seems prevalent in non-diabetic patients with suspected myocardial ischemia but without myocardial perfusion defects. A decreased coronary flow reserve as well as increased HOMA-IR independently predicts outcome. In addition, insulin resistance is associated with decreased coronary flow reserve in this population.
- There seems to be gender differences in risk factors associated with impaired coronary microvascular function. Insulin resistance might be of significance in men contributing to decreased coronary flow reserve, while the value of systolic blood pressure appears to be important in women.
- In this non-diabetic state, insulin resistance seems to be associated with decreased peripheral endothelial function as well as a low-grade inflammation and impaired angiogenesis, all potentially contributing to microvascular dysfunction.
- In the insulin resistant ob/ob mouse model, we observed a decreased coronary flow reserve and increased renal vascular resistance. These findings could probably be explained by decreased L-arginine as well as initial renal hypertrophy.
- Personalized lifestyle intervention with continuous feedback during three months improves coronary microvascular function in healthy volunteers. This is probably explained by increased cardiac performance and decreased fibrinogen levels.

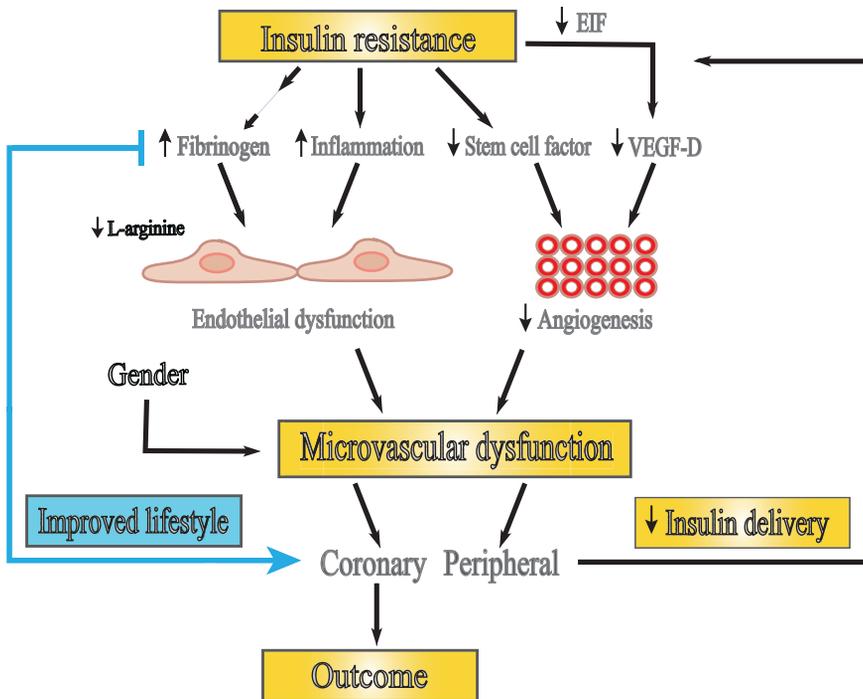


Figure 17 Integrated overview of the conclusions generated from the current thesis

This thesis shows insulin resistance to be apparent in non-diabetic patients with chest pain without myocardial perfusion defects and to be associated with endothelial dysfunction and decreased angiogenesis. These mechanisms might further contribute to the observed coronary as well as peripheral microvascular dysfunction. Both are likely to be related to increased risk for cardiovascular events and to further contribute to decreased insulin delivery to the tissue, leading to increased insulin resistance, respectively. There seems to be a gender aspect in risk factors for prediction of coronary microvascular dysfunction in this patient population. Finally, the ob/ob mouse model has decreased L-arginine levels, probably underlying the detected decreased coronary microvascular function. EIF; eukaryotic initiation factor, VEGF-D; vascular endothelial growth factor D.

Future Perspectives

- My thesis shows insulin resistance to be associated with cardiovascular outcome and microvascular dysfunction. Future intervention studies would aim to improve insulin sensitivity in non-diabetic patients with coronary microvascular dysfunction, and in a gender specific manner study cardiovascular outcome. A sodium-glucose co-transporter 2 inhibitor could be an option for such a study.
- An important translational perspective is to perform an intervention study in the insulin resistant ob/ob mice using the same treatment as above and to measure coronary microvascular function alongside renal flow resistance.
- Risk stratification in this patient population seems to be improved using HOMA-IR as a biomarker. This incorporates both glucose and insulin, which adds more information regarding insulin resistance than glucose alone. To advance the results further, focused population- and gender specific research regarding cut off for HOMA-IR and increased risk for microvascular dysfunction in non-diabetic populations could potentially guide clinical care and treatment in patients with suspected myocardial ischemia.
- Paper III indicates microvascular dysfunction to be prevalent in both kidney and heart in the ob/ob mice. It would be interesting to investigate the correlation between impaired coronary flow reserve and estimated glomerular filtration rate in the studied patient population. This would add further knowledge regarding the cardiorenal syndrome at an early stage.

Acknowledgements

Learning is easier with an open mind and great people around you
–*Helena Utkovic Westergren*

- I would like to thank all that have contributed to this thesis, and I would like to give special thanks to the following.

I am truly thankful to **Li-ming Gan** for your generosity throughout my thesis work. Your energy is never ending and I am grateful for your patience and enthusiastically sharing your great expertise, being always positive and taking your time. Most of all I am thankful for the invaluable learning, complementing teamwork, encouragement, your ability to always see solutions and pushing me to perform outside of my comfort zone.

I am also thankful to my co-supervisors; **Sara Svedlund** for teaching me, guiding me and sharing your professional knowledge within healthcare, medicine, research, amazing language skills, statistics and so much more. Most of all, I am thankful for a great time, helping me grow and being my friend. **Julia Grönros**, for being an amazing coach with the greatest pedagogical skills, for your enthusiasm to teach and share your never ending knowledge and always guiding and encouraging me. For all invaluable lab-memories, but most of all for teaching me how to make boundaries, whilst making me believe in myself. **Regina Fritsche-Danielson** for your encouragement, guidance, skilled input and wise advice throughout my work.

Jan Oscarsson, thank you for your generosity and amazing expertise, for your enthusiasm to educate and willingness to share your broad knowledge. Your input within the glucose homeostasis area has been invaluable. Many thanks for always taking your time, fast response, humbleness and great support.

I must also thank **Li-ming Gan** and **Jan Oscarsson** for unifying the cardiovascular research and glucose homeostasis area within my thesis and for the unforgettable meetings with you two together sharing your knowledge. Thank you for your dedication.

I am also grateful to my group colleagues: **Juuso Blomster** for your valuable discussions and expertise in cardiology inputted to my work. For helping me with my manuscripts and always being generous. **Therese Dijnér** for your ultrasound expertise, efficiency, carefulness and organization skills. For the great time we

had together and being a fantastic team member. **Tove Brodin** for your dedication, admirable measuring efficiency, willingness to learn, sharp mind and humbleness. My former group colleague **Charlotte Eklund** for your ultrasound expertise, accuracy and valuable introduction to the research area.

My deepest of thanks to **Sofie, Olivia, Jonas, Miriam, Åsa, Lina, Eva-Lena, Carola, Tina, Margareta, Lotta, Anki** and **Annika** at the Cardiology Research Unit, Sahlgrenska University Hospital, for welcoming me and giving your invaluable help throughout my thesis work, for introducing me to GCP and sharing your professional expertise in performing clinical studies. Special thanks to **Sofie** for all the laughs, your thoughtfulness and professionalism and for increasing the standard of my clinical work at the Research Unit.

Co-author **Marianne Månsson**, many thanks for your admirable statistical expertise and patience when instructing me! For your generosity and invaluable input to my work.

All friends and colleagues at Bioscience, AstraZeneca R&D Gothenburg. Special thanks to **Margareta Behrendt** and former colleague **Mikael Brusberg** for sharing your ultrasound competence, carefulness and invaluable discussions. My former colleague and co-author **Suvi Heinonen** for your expert advice in diabetic pre-clinical models and molecular biology. **Andrea Ahnmark** for your great kindness and skills and always taking your time to help me. **Bengt Kull** for your great kindness and logistic robot skills, together with **all of you** that helped labeling and aliquoting many thousand tubes; that was a great team work, crossfunction- and crossproject work and I am still impressed and warmly thankful for the engagement from you all! To my friends and amazing colleagues **Anna Lindgren, Malin Palmér, Linda Hägerstrand, Daniel Karlsson** and **Johannes Wikström** for your admirable skills, the best of laughs and making AstraZeneca a great place to work. Special thanks to **Anna Lindgren** for always encouraging me and being a great friend. **My group** for welcoming me to the kidney area, helping me to unite renal and cardiovascular imaging and always being supportive. **Damilola Adingupu** for your generosity sharing your great microvascular, clinical and language expertise. Your courage facing new challenges, enthusiasm and positive mind. You always cheer me up! Warmest of thanks to my co-author **Anette Ericsson** together with **Nick Oakes** for invaluable input within the diabetic and chronic kidney disease area combined with impressive pre-clinical knowledge. My grateful thanks to co-authors **Ann-Cathrine Jönsson-Rylander** and **Erika Rehnström** for all your help, generosity and expert advice in biomarker analysis and histology evaluation and for your support throughout this thesis. Thanks to co-authors **Tasso Miliotis, Karin Jennbacken** and **Alan Sabirsh** for great collaborations and invaluable analyses within your expertise areas. I would also like to thank **Katarina Rydén-Markinhulta, Ann-Katrin Andersson, Anne-Christine Andréasson, Anne-Cristine Carlsson** and **Lena Amrot-Fors** for professional and skilful help, and for valuable discussions.

To **Early Clinical Development**, AstraZeneca R&D Gothenburg for welcoming me and letting me share your booked-up meeting rooms for five years!

Josefin Olausson for being my “third cycle level” course mate and patiently guiding me through the end of my thesis.

My **mother** and **father** for giving me the deepest of roots, strongest of stability and always encouraging me to try my wings. For all support making this thesis possible! To my **sister** and **brother** for speaking the same language, thinking in the same direction and forever being my friends.

Håkan and **Lena** for your thoughtfulness and always being supportive, making this thesis work easier for me.

Kjell-Ove and the whole **Utkovic-tribe**, for being the best family, having the most beautiful values, for always being around and for with you nothing is impossible!

To my beloved **mormor**! You were with me almost all the way ♥ Thank you for enthusiastically showing interest in my work, supporting me and at the same time being accurate telling me when to take a break.

To my husband **Rickard** for your great patience and wholeheartedly having encouraged and supported me with a never-ending understanding. To our children **Thea**, **Elina** and **Leon** for giving life balance. For teaching me so much and for giving me the possibility to give you deep roots and strong wings.

This work was supported by grants from the Agreement concerning research and education of doctors (ALF/LUA grant), Swedish federal government.

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