Studies on microcirculation in insulin resistance

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ABSTRACT

The overall aim of this thesis was to investigate the microcirculation in insulin resistance, with focus on the expression of endothelin-1, through a translational approach.

Specific aims: 1) Investigate if circulating endothelin-1 levels predicts incident coronary heart disease events. 2) To assess if sex differences modify endothelin-1 as a predictor of type 2 diabetes. 3) To investigate if microvascular insulin resistance impairs insulin delivery to the subcutaneous adipose tissue and skeletal muscle. 4) To investigate if acute administration of the PDE-5 inhibitor tadalafil induces positive vascular, metabolic and anti-inflammatory effects in type 2 diabetes. 5) To further elucidate the molecular action of tadalafil in tumour necrosis factor-α (TNF-α) stimulated human endothelial cells.

Principal findings: The population-based cohort in Vara-Skövde was investigated for paper I-II. During baseline cardiovascular risk factors and endothelin-1 were assessed and incident coronary heart disease (CHD) was followed-up during a 10-year period *(paper I)*. Endothelin-1 levels had a predictive value for incident CHD in women, but not in men. A randomly selected subgroup was investigated in a follow-up after 10 years, and impaired glucose tolerance (IGT) and T2D was documented for *paper II*. Here, higher quartiles of endothelin-1 at baseline were associated with IGT/T2D at follow-up in women. *Paper III* investigates microvascular aspects of insulin resistance using microdialyis; participants with T2D and age-matched healthy controls were studied after an oral glucose load. Participants with T2D had decreased delivery of insulin to adipose tissue, and a blunted subcutaneous adipose tissue blood flow compared with controls. In *paper IV*, T2D participants received either placebo or tadalafil (20 mg) before a mixed meal in a randomized controlled trial. Tadalafil increased forearm blood flow, glucose uptake and capillary recruitment, and blunted a postprandial increase of endothelin-1. In *paper* V, the effects of tadalafil were studied in an experimental setting using TNF-α stimulated endothelial cells. Tadalafil treatment decreased expression of c-Jun N-terminal kinase (JNK) phosphorylation as well as reduced gene expression and secretion of endothelin-1.

Conclusions: This thesis shows that (i) endothelial dysfunction precedes IGT/T2D and CHD, and that endothelin-1 may pose as a risk factor for women, (ii) delivery of insulin from the circulation to subcutaneous adipose tissue is impaired in participants with T2D, and that participants with T2D exhibit a blunted postprandial blood flow response, (iii) acute administration of tadalafil induces positive vascular and metabolic effects in the postprandial state in T2D, and tadalafil decrease gene expression of endothelin-1 in cultured endothelial cells by decreasing activation of JNK.

Keywords: endothelin-1, coronary heart disease, type 2 diabetes, phosphodiesterase-5 inhibition, tadalafil, insulin resistance, endothelial dysfunction, c-Jun N-terminal kinase, nitric oxide, microdialysis.
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SAMMANFATTNING PÅ SVENSKA

Antalet personer med typ 2 diabetes (åldersdiabetes) ökar i världen. Denna ökning tros bero på dagens livsstil med mycket stillasittande och att fler personer idag är överviktiga. Hjärtsjukdom är en av de vanligaste dödsorsakerna till följd av diabetes. Typ 2 diabetes definieras som en okänslighet mot insulin som är ett hormon som bildas i bukspottskörteln och som gör att kroppen kan ta upp socker från blodet. När man får en okänslighet mot insulin, bildar kroppen större mängder av insulin efter en måltid för att upprätthålla sockerupptaget. Insulinet är även viktigt för att styra hur kärlen beter sig, genom att stimulera de celler som sitter på insidan av kärlen, endotelcellerna, att bilda olika ämnen. Två av dessa ämnen är speciellt viktiga, kväveoxid (NO) och endothelin-1. Dessa ämnen är principiellt varandras motsatser, NO gör så att kärlen slappnar av medan endothelin-1 gör så att kärlen drar sig samman. Vid typ 2 diabetes är signalen som gör att NO bildas av insulin skadad, medan signalen som bildar endothelin-1 fortfarande fungerar. Detta gör att framför allt de små kärlen blir mer sammandragna och man får inte lika bra blodflöde i muskel- och fettvävnad, vilket medför att sockret inte når sin målvävnad.

I denna avhandling har vi tittat på nivåer av endothelin-1 i blodet hos 2816 personer som bor i Vara och Skövde, dessa har sedan följts upp för att se vilka som fick hjärtsjukdom under en 10-års period. Ungefär en tredjedel kom tillbaka på ett nytt besök där de undersöktes utefter insulinkänslighet och ifall de fått typ 2 diabetes. Denna studie visade att de kvinnor som hade höga nivåer av endothelin-1 vid grundundersökningen hade större risk för hjärtsjukdom, samt en större risk för att utveckla en okänslighet mot insulin och typ 2 diabetes. Det syntes dock ingen ökad risk hos männen, vilket fick oss att dra slutsatsen att det finns en skillnad mellan könen gällande effekterna av endothelin-1 och utvecklingen av dessa sjukdomar.
I en annan studie jämfördes personer med typ 2 diabetes och friska kontroller genom att mäta insulin-nivåerna i underhudsfettet och i en underarmsmuskel. Där såg vi att insulinet inte kom fram till underhudsfettet från blodet hos personer med typ 2 diabetes lika snabbt som hos friska, och att deras kärlåterhämtning var sämre. Detta ger stöd åt teorin att kärlens endotelceller aktivt transporterar insulinet till sin målvävnad.

Tadalafil är ett läkemedel som efterliknar effekterna av NO genom att öka ämnet cGMP inuti cellerna. Tadalafil har tidigare visats hjälpa endotelcellernas funktion hos personer med impotens, och därför ville vi undersöka ifall denna positiva effekt även sker hos personer med typ 2 diabetes. För att göra detta gav vi forskningspersonerna antingen tadalafil eller placebo och mätte sockerupptag och blodflöde i en muskel i underarmen. Vi mätte även endothelin-1 och inflammationsmarkörer i blodet hos forskningspersonerna. Vi fann att de personer som fick tadalafil hade ett förbättrat sockerupptag och ökat blodflöde i de små kärlen i muskeln efter måltiden, samt att den ökning av endothelin-1 efter måltid som sågs hos den grupp som fick placebo inte sågs i gruppen som fick tadalafil.
För att ytterligare fördjupa vår kunskap om de mekanismer som tadalafil påverkar inuti cellen odlades endotelceller på laboratoriet. Genom att behandla cellerna med tadalafil samtidigt som man stimulerade dem med en inflammatorisk markör kunde vi mäta deras uttryck av gener och proteiner. Även här minskades produktion och utsöndring av endothelin-1, dessutom minskades genuttryck av inflammationsmarkörer. Vi kunde även identifiera ett protein som minskas av tadalafil, c-Jun N-terminal kinase.

Sammanfattningsvis förevisar detta vikten av ett fungerande endotel vid utvecklingen av diabetes och hjärtsjukdom, framför allt hos kvinnor. Dessutom kan läkemedlet tadalafil påverka endotelets funktion och leder troligtvis till ett förbättrat sockerupptag, sänkta nivåer av endothelin-1 och en ökning av andelen små kärl som genomblöds i vävnaden.

LIST OF PAPERS

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

I. Bledar Daka*, Josefin Olausson*, Charlotte A Larsson, Margareta I Hellgren, Lennart Råstam, Per-Anders Jansson, Ulf Lindblad. Circulating concentrations of endothelin-1 predict coronary heart disease in women but not in men – a longitudinal observational study in the Vara-Skövde Cohort. *Accepted for publication in BMC Cardiovascular Disorders 2015.*

II. Josefin Olausson*, Bledar Daka*, Margareta I Hellgren, Charlotte A Larsson, Max Petzold, Ulf Lindblad, Per-Anders Jansson. Endothelin-1 as a predictor of impaired glucose tolerance and type 2 diabetes – A longitudinal study in the Vara-Skövde Cohort. *Submitted.*

III. Josefin Olausson, Reza Mobini, Per Fogelstrand, Karin Mossberg, Emanuel Fryk, Lena Strindberg, Lillemor Mattsson Hultén, Per-Anders Jansson. Delivery of insulin to subcutaneous adipose tissue and skeletal muscle in type 2 diabetes patients and healthy controls – A microdialysis study. *Manuscript*

IV. Lovisa Sjögren, Josefin Olausson, Lena Strindberg, Reza Mobini, Per Fogelstrand, Lillemor Mattsson Hultén, Per-Anders Jansson. Postprandial effects of the phosphodiesterase-5 inhibitor tadalafil in people with well-controlled type 2 diabetes mellitus: A randomised controlled trial. *Accepted for publication in Diabetic Medicine 2015.*

V. Josefin Olausson*, Lovisa Sjögren*, Reza Mobini, Emanuel Fryk, Per Fogelstrand, Lillemor Mattsson Hultén, Per-Anders Jansson. Tadalafil decreases expression of endothelin-1 in TNF-α-activated human endothelial cells – possible role of the c-Jun N-terminal Kinase pathway. *Manuscript.*

TABLE OF CONTENT

ABBREVIATIONS

ACE	Angiotensin converting enzyme
AP-1	Activator protein-1
Apo	Apolipoprotein
ATBF	Adipose tissue blood flow
BMI	Body mass index
cGMP	Cyclic guanosine monophosphate
CHD	Coronary heart disease
c-PTIO	2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3oxide potassium salt
CRP	C-reactive protein
DPP	Dipeptidyl peptidase
EBM	Endothelial basal medium
ECE	Endothelin-converting enzyme
eNOS	Endothelial nitric oxide synthase
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular regulated kinase
ET_A	Endothelin receptor A
ET_B	Endothelin receptor B
FFA	Free fatty acids
FSH	Follicle stimulating hormone
GLP	Glucagon-like peptide
GLUT4	Glucose transporter 4
HbA_{1c}	Glycated haemoglobin
HDL	High-density lipoprotein
HOMA-IR	Homeostatic model assessment of insulin resistance
HUVEC	Human umbilical vein endothelial cell
IAUC	Incremental area under the curve
ICAM-1	Intercellular adhesion molecule-1
I/C	Interstitial/Circulating
ICD	International statistical classification of diseases and related health problems
IFG	Impaired fasting glucose

IGT	Impaired glucose tolerance
IRS	Insulin receptor substrate
IκBα	Inhibitory kappa B alpha
IKK	IκB kinase
JNK	c-Jun N-terminal kinase
LDL	Low-density lipoprotein
MAPK	Mitogen activated protein kinase
NF-1	Nuclear factor-1
NFκB	Nuclear factor kappa B
NO	Nitric oxide
ODQ	1H-[1,2,4]Oxadiazolo[4,3-a]quinxolain-1-one
OGTT	Oral glucose tolerance test
$ONOO^{\bullet}$	Peroxynitrite
PI3K	Phosphoinositide 3 kinase
PDE-5	Phosphodiesterase-5
PKG	Protein kinase G
RHI	Reactive hyperemia index
ROS	Reactive oxidative species
sGC	Soluble guanylate cyclase
Shc	SH2-domain containing protein
siRNA	Small interfering RNA
T2D	Type 2 diabetes mellitus
TNF-α	Tumour necrosis factor alpha
VCAM-1	Vascular cellular adhesion molecule-1
WHR	Waist-hip ratio
^{133}Xe	^{133}Xenon

1 INTRODUCTION

Insulin resistance is a common denominator for numerous pathologies. It is foremost described in the area of cardiovascular diseases, however mechanisms causing insulin resistance are complicated and several are still not completely elucidated. The microcirculation is heavily implicated in the progress of insulin resistance, and a close relationship has been described between microvascular dysfunction and insulin resistance. This thesis focuses on the potential predictive value of a marker for microvascular dysfunction, endothelin-1, investigates the transendothelial transport of insulin, and explores the effect of a drug, tadalafil, which might have positive effects on the microcirculation.

1.1 Type 2 diabetes

Type 2 diabetes mellitus (T2D) is considered one of the major health concerns in the western world, with an increasing prevalence [1]. Diabetes is the fifth most common cause of death globally [2], and cardiovascular disease is the principal cause of death among the complications of T2D [3]. The growing prevalence of diabetes, with an estimation of 285 million persons affected worldwide, has been attributed to a more sedentary lifestyle in combination with an excessive dietary intake leading to an increasingly obese population [4]. Additional risk factors for T2D include smoking, hypertension and a genetic predisposition for diabetes [4, 5]. T2D is characterised by a peripheral insulin resistance defined by an impaired insulin-mediated whole body glucose-uptake and, in later stages, impaired insulin secretion [6, 7]. The pathophysiology of T2D involves several tissues in the body, in the pancreas the glucagon production by α-cells is increased due to impaired glucose sensing, and β-cell loss of function leads to decreased insulin production [8, 9]. The brain is involved through a compromised appetite regulation and insulin resistance through impaired insulin signalling in hypothalamus [10], the liver by increased glucose production and triglyceride secretion and the kidney by an increased glucose reabsorption [8, 9]. Further, skeletal muscle and the adipose tissue displays decreased glucose uptake, and the adipose tissue has an increased lipolysis. The gastrointestinal tract has an impaired incretin function [11], and the intestinal microbiome has during recent years emerged as an important factor in the pathophysiology of T2D [12]. The cardiovascular system is also affected in T2D, with a compromised micro- and macrocirculation (Fig. 1).

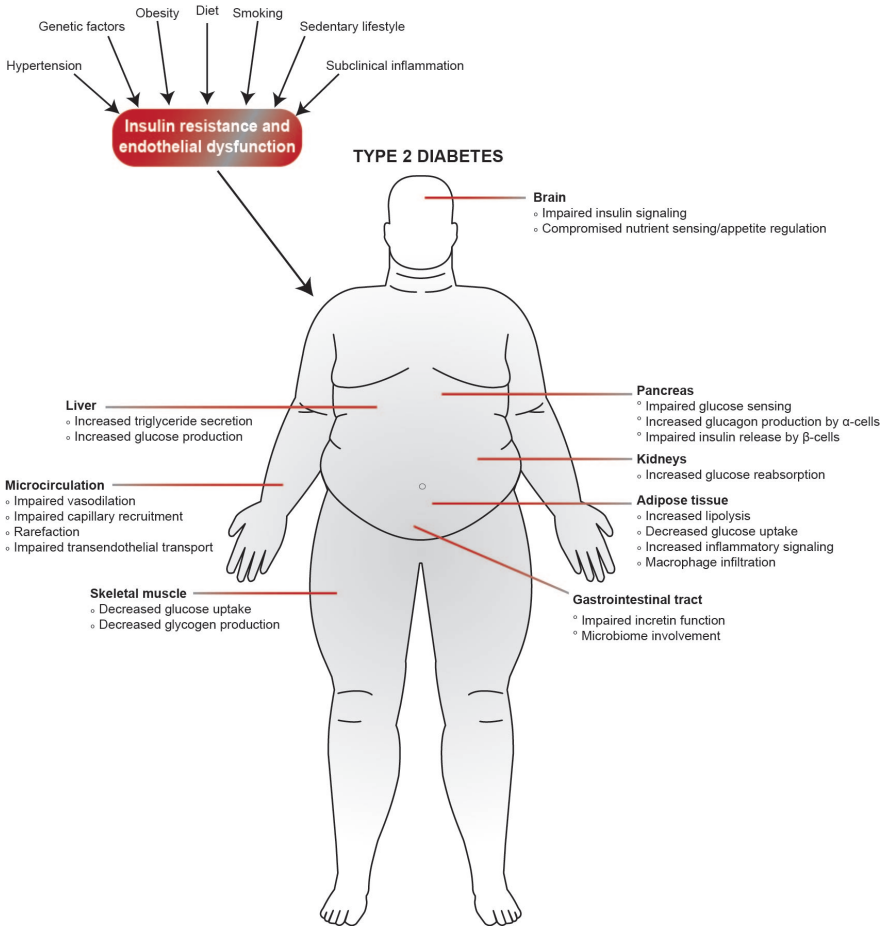

Figure 1. A schematic overview of various risk factors and pathophysiology involved in type 2 diabetes

1.2 Coronary heart disease

Coronary heart disease (CHD) most frequently causes death among the cardiovascular diseases [13]. CHD refers to different conditions of failing circulation of the heart and includes coronary atherosclerosis, myocardial infarction and angina pectoris [14], among others. The risk of CHD is doubled in subjects with T2D [15], and other conditions implicated in CHD are hypertension [16] and obesity [17]. Endothelial dysfunction has been suggested to play a central role in atherosclerosis [18, 19]. Endothelial dysfunction has also been shown to increase the risk for cardiovascular events synergistically with T2D, impaired glucose metabolism or insulin resistance [20]. However, CHD is a multifactorial condition and several other factors are implicated in the pathophysiology, many of them related to lifestyle. In contrast to T2D, morbidity of CHD has been unchanged during the last 10 years, but mortality rates have decreased overall [13].

1.3 Microcirculation

The role of the microcirculation is to adapt perfusion in tissues to local metabolic demands. The microvascular bed is roughly divided into three types of vessels; mid- and small sized arterioles, capillaries and venules (Fig. 2a), with different functionality corresponding to specific structures and responsibilities. Arterioles are responsible for vascular resistance, venules for collecting blood after passing tissue and capillaries, lacking smooth muscle cells, are responsible for delivery and exchange of molecules in tissue [21]. Heterogeneity in microcirculation allows for adaptations depending on tissue and stimulus, and changing permeability, blood flow and capillary recruitment (the number of blood-perfused capillaries) are all essential parts of a healthy microcirculation [22, 23].

The innermost layer in all vessels consists of a single layer of endothelial cells [24] (Fig. 2b). The endothelium has a gate-keeping role sustaining the integrity of both the circulation and the tissues [24]. Furthermore, the endothelium mediates inflammatory responses [25], and contributes to vascular tone [26], among other functions. The endothelium contributes to the regulation of blood flow and blood pressure by releasing vasodilator and vasoconstrictor substances [27]. The predominant vasodilator released from endothelial cells is nitric oxide (NO) [28, 29], which is generated through conversion of L-arginine to L-citrulline by the

enzyme NO synthase (NOS) [30]. NO is synthesized by endothelial cells in response to shear stress [31], as well as to insulin [32]. In addition to vasodilation, NO regulates proliferation and inflammation, as well as having oxidative effects [33, 34]. One of the vasoconstrictors produced by the endothelium is the peptide endothelin-1 [35], also induced by insulin [36]. In healthy endothelium, there is a balance between expression of NO and endothelin-1 [37], with low production of endothelin-1 preserving the bioavailability of NO, favouring vasorelaxation [38].

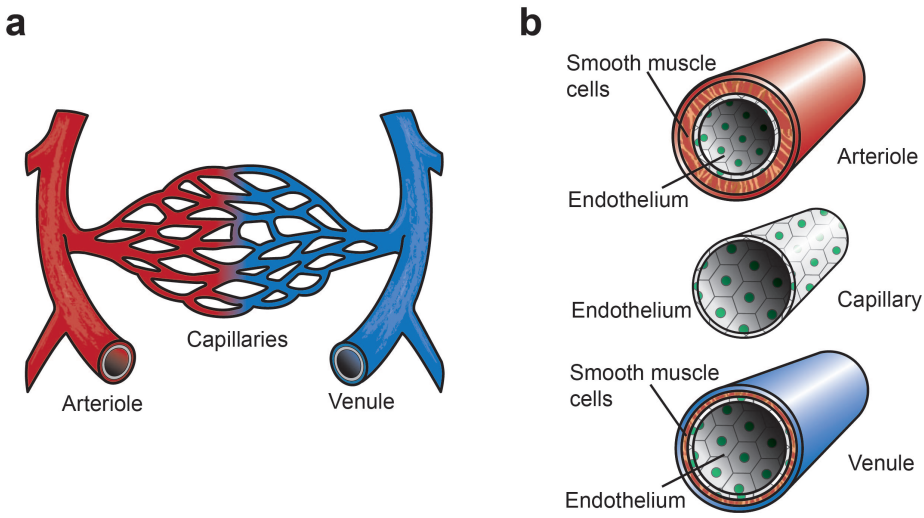

Figure 2. Overview of a microvascular unit (a) and the differences between the anatomies of the vessels (b).

1.4 Insulin resistance and endothelial dysfunction

Insulin resistance demonstrates before overt T2D, and is characterized by decreased sensitivity and/or responsiveness to the metabolic actions of insulin. In an effort to maintain the metabolic effects of insulin, insulin secretion is increased causing hyperinsulinemia [6]. Endothelial dysfunction is proposed to antecede insulin resistance [39], since microvascular dysfunction is a feature seen in obesity, a known risk factor for insulin resistance. Insulin resistance and

endothelial dysfunction is closely related [40], and shares many of the underlying mechanisms, such as glucotoxicity, lipotoxicity and inflammation [41, 42], creating a reciprocal relationship between them. Thus, metabolic and cardiovascular diseases are closely linked together. One of the most consistent observations of vascular function in insulin resistance and T2D is an impaired endothelium-dependent vasodilation across tissues [43], further implicating the prospect of common underlying mechanisms. Moreover, impaired capillary recruitment, decreased blood flow and decreased capillary density (rarefaction) are other ways microvascular dysfunction manifests in insulin resistance (Fig. 3).

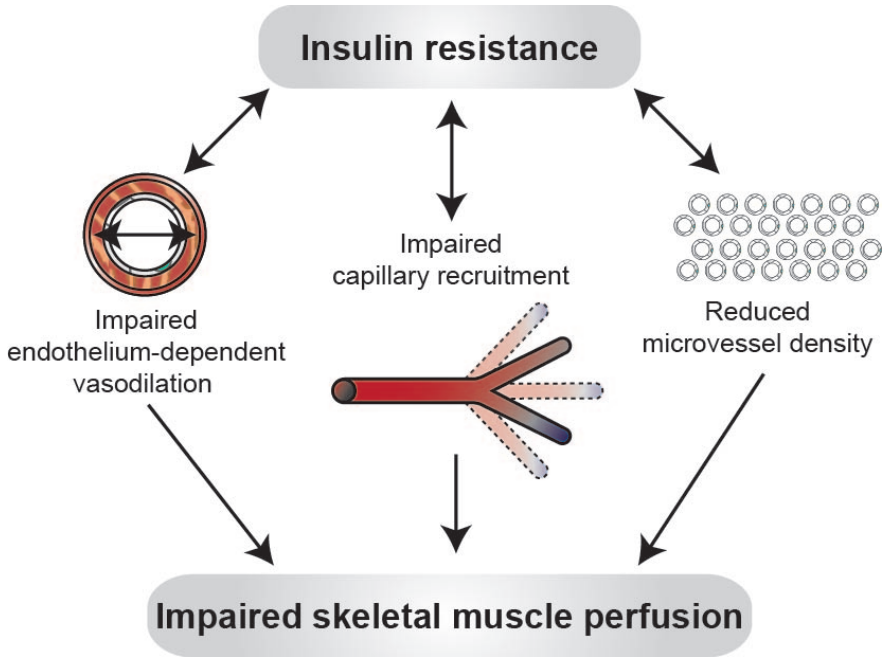

Figure 3. Overview of the relationship between insulin resistance and functions of microcirculation with the outcome of decreased skeletal muscle perfusion

The reduced ability of insulin to mediate glucose uptake probably has multifactorial underlying mechanisms. Skeletal muscle blood flow and perfusion have been shown to mediate skeletal muscle glucose uptake [44, 45]. Further, it has been proposed that the importance of the hemodynamic actions of insulin is associated with an increased capillary surface area for glucose exchange [46, 47],

which is decreased in insulin resistance [48]. Insulin results in muscle capillary recruitment, which occurs independently of blood flow [49, 50], suggesting a specific action of insulin to induce muscle perfusion. Additionally, structural and functional rarefaction contribute to impaired capillary recruitment in hypertension [51], which might be an explanatory mechanism of the relationship between hypertension and the compromised vascular and metabolic actions of insulin [52]. Further, decreased vascular NO bioavailability has been implicated as a central stimulator of microvessel rarefaction in obese rats [53].
Decreased NO bioavailability is highly involved in endothelial dysfunction [54, 55]. Studies have shown that insulin production of NO is impaired in the insulin resistant state, while in contrast the insulin stimulated production of endothelin-1 remains intact, causing an imbalance between these two molecules [56, 57]. NO and endothelin-1 has been shown to impair each other's production in cell culture [58, 59], further exhibiting the close relationship between these antagonistic molecules.

1.4.1 Endothelin-1

Endothelin-1 is a 21 amino acid cyclic peptide [60], which acts by binding to endothelin receptors on vascular muscle and endothelial cells [61]. In health endothelin-1 is primarily produced by endothelial cells, however, in pathologies it can be produced by several different cell types [38]. Studies have shown increased circulatory endothelin-1 levels in insulin resistance and T2D [62, 63], in contrast, others did not observe an increase of endothelin-1 in T2D [64]. Preproendothelin is synthesized through transcriptional activation of the preproendothelin gene, which is regulated by nuclear factor-1 (NF-1), activator protein-1 (AP-1) family members, Smad, and GATA-2 [65, 66]. Preproendothelin is cleaved to form big endothelin-1 which then is further cleaved by endothelin converting enzymes (ECE) to form mature endothelin-1 (Fig. 4) [67].

Endothelin-1 is mainly secreted on the basolateral side of endothelial cells [68], where it acts on vascular smooth muscle cells causing vasoconstriction. Two subtypes of receptors mediate the effects of endothelin-1, ET_A and ET_B [61, 69, 70]. In the vasculature, ET_A is mainly located on vascular smooth muscle cells and ET_B is primarily located on both vascular smooth muscle and endothelial cells [71], but both receptors are also expressed across other cell types [71]. ET_A and ET_B on vascular smooth muscle cells mediate the constrictive effects of endothelin-1, while endothelial ET_B on endothelial cells induces vasodilation by

release of prostacyclin and NO [35]. Selective blockade of ET_A abolished, while selective blockade of ET_B enhanced, the vasoconstrictive effect of endothelin-1 [72], and NO inhibition attenuated the effect of ET_A blockade [73]. Further, blockade of ET_A improved skin capillary circulation in T2D [74]. Dual receptor antagonism enhanced insulin sensitivity and glucose uptake in obese subjects, and improved endothelial function in T2D and hypertension [75-77]. Finally, chronic endothelin-1 treatment led to insulin resistance in rats [78]. In a Swedish cohort with elderly subjects, circulating endothelin-1 levels did not correlate with endothelium-dependent vasodilation. However, a genotype score constructed from genes involved in the endothelin-1 pathway was significantly correlated to endothelium-dependent vasodilation as well as endothelium-independent vasodilation in forearm resistance vessels in this population [79].

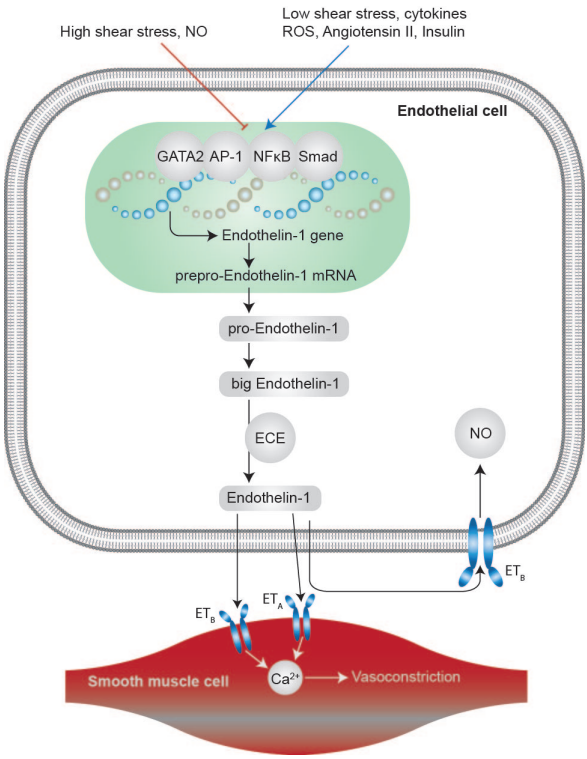

Figure 4. Production of endohelin-1. Released endothelin-1 binds ET_A and ET_B on smooth muscle cells leading to vasoconstriction. ET_B is also present on the endothelial cell where it promotes NO production leading to vasodilation.

1.4.2 Obesity and inflammation

As previously mentioned, obesity is one of the most important risk factors for T2D [80-82]. Obese study subjects show reduced endothelium-dependent vasodilation [83], and reduced transcapillary transport of insulin to skeletal muscle and adipose tissue [84, 85]. A strong association between obesity and disturbances in metabolism has been described, with differences between men and women [82, 86]. However, it should also be noted that insulin resistance is not displayed in all obese [87], and that T2D and cardiovascular risks may be different in metabolically healthy obese.

A chronic low-grade inflammation is described in obesity as well as in T2D [43, 88-90]. Higher circulating levels of inflammatory markers, such as tumour necrosis factor-α (TNF-α), C-reactive protein (CRP) and cytokines have been suggested to contribute to the pathogenesis of T2D [91], and are manifested in overt T2D [92]. Further, TNF-α has been described to impair capillary recruitment and glucose uptake [93, 94]. Adipose tissue in obese mice and humans overexpress TNF-α, which could contribute to insulin resistance [95, 96]. Cross-talk occurs between perivascular adipose tissue and blood vessels [97, 98] and TNF-α released from perivascular adipose tissue, has been shown to contribute to the imbalance between NO and endothelin-1 [99].

1.5 Insulin signalling and action

The response of endothelial cells to insulin is rapid, and is initiated by insulin binding to the specific insulin receptor leading to phosphorylation of insulin receptor substrates (IRS) [100] and SH2-domain containing protein (Shc), which functions as docking proteins for downstream signalling molecules [42]. Parallel signalling pathways leads to the upregulation of endothelin-1 and NO by insulin [37] (Fig. 5).

The phosphatidylinositol 3-kinase (PI3K) – Akt – endothelial NOS (eNOS) pathway leads to production of NO [101, 102], while the Ras-mitogen-activated protein kinase (MAPK) pathway leads to production of endothelin-1 [103, 104]. The pathways are complex, and includes several feedback loops and crosstalk between the signalling branches [42]. TNF-α induces vasoconstrictor-effects and decrease vasodilator-effects through c-Jun N-terminal kinase (JNK) [105].

Additionally, TNF-α mediated IκB kinase (IKK) [106], among others, can disrupt insulin signalling through serine/threonine phosphorylation of IRS-1.

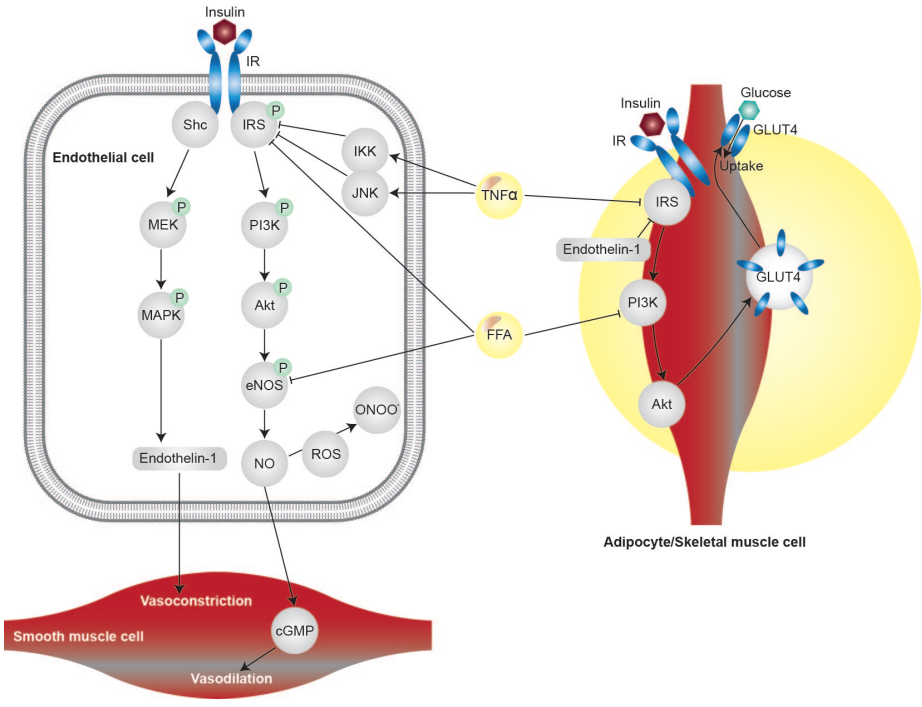

Figure 5. Insulin signalling in endothelial cells, effects and crosstalk between tissues. Insulin binds to the insulin receptor (IR) which activates two parallel signalling pathways leading to NO or endothelin-1. NO leads to vasodilation in smooth muscle cells while endothelin-1 leads to vasoconstriction. Insulin signalling in target cells, e.g. adipocytes and skeletal muscle cells leads to translocation of GLUT4 enabling glucose uptake. TNFα from adipose tissue lead to activation of IKK and JNK, which serine/threonine phosphorylates IRS-1 thereby impeding the PI3K pathway. Free fatty acids (FFA) inhibit the PI3K-pathway in both endothelial cells and skeletal muscle cells. ROS interacts with NO and forms $ONOO^{\cdot}$ which leads to an imbalance between NO and endothelin-1. Endothelin-1 impairs IRS-1 in adipocytes and decrease glucose uptake.

Insulin signalling in skeletal muscle and adipose tissue leads to glucose uptake by translocation of glucose transporter 4 (GLUT4) through PI3K [107] (Fig. 5). The cytoplasmic tyrosine kinase portion of the insulin receptor directly interacts with caveolin-1, and both caveolae and caveolin-1 augments the insulin signal and modulates GLUT4 function, thus playing an important role in energy metabolism [108]. Further, impaired insulin signalling in endothelial cells decreased insulin-mediated glucose uptake in skeletal muscle [109]. During oxidative stress, reactive oxygen species (ROS) form in excess (e.g. superoxide or hydrogen peroxide). Superoxide can interact with NO forming peroxynitrite, a highly reactive radical compound, which impedes the insulin signalling by nitrosylation of tyrosine residues on key proteins such as IRS-1 [110]. Further, endothelin-1 is capable of impairing IRS-1 and Shc in cultured adipocytes [111], as well as forming superoxide in arteries [112]. Endothelin-1 also decrease glucose uptake in skeletal muscle *in vivo* and *in vitro*, which is reversed by dual endothelin receptor blockade [113, 114].

Thus, dysregulated cross talk between tissues and different cell types are an important factor in the pathology of insulin resistance. This is further illuminated by the notion that free fatty acids inhibits eNOS in endothelium and decrease PI3K activation in skeletal muscle [115, 116], and that depletion of GLUT4 in adipose tissue leads to insulin resistance in skeletal muscle and liver [117]. Moreover, gene and protein expression of IRS-1 in adipocytes is low in subjects at risk of T2D [118] further connecting deficiencies of the insulin signalling to the pathology of T2D.

1.5.1 Transendothelial transport

The endothelial barrier has been postulated to play a role in the development of insulin resistance [119, 120]. Whether transport of insulin is a saturable or non-saturable process is debated [121-123]. However, studies have demonstrated that a functioning insulin signalling is required for insulin uptake and transport by endothelial cells [124, 125]. Further, NO promotes insulin uptake and transport in endothelial cells [126]. Insulin in cultured endothelial cells co-localized with the insulin receptor and caveolin-1 [127, 128], and caveolae has been implicated in the transport of insulin [129]. Further, importance of transendothelial transport has been indicated *in vivo* [130, 131]. Nonetheless, separating insulin-mediated transport from insulin-mediated capillary recruitment *in vivo* is challenging [132, 133], and further studies are needed to investigate the involvement of the insulin receptor.

1.5.2 Postprandial vascular response

Patients with insulin resistance and T2D have postprandial hyperglycaemia [134], and an inflammatory response has been reported in the postprandial state [135, 136]. Furthermore, impaired microvascular recruitment and a subsequent reduction of glucose disposal to skeletal muscle are seen after a meal in obesity and T2D [137, 138]. A high-fat meal impaired endothelium-dependent vasodilation [139, 140] in healthy subjects. These postprandial impairments could be explained by an increased oxidative stress following the meal [141, 142], indicating a formation of peroxynitrite. A postprandial increase of triglycerides is seen in obese subjects with metabolic syndrome [143] and potentiates superoxide production by leukocytes [144]. Nocturnal and postprandial free fatty acid release is increased in T2D [145], displaying the unsuppressed lipolysis in insulin resistance.

1.6 Phosphodiesterase-5 inhibition

NO activates soluble guanylate cyclase (sGC) which leads to increased production of cyclic guanosine monophosphate (cGMP) [146] and activation of protein kinase G (PKG) (Fig. 6). Phosphodiesterases (PDEs) are catabolic enzymes grouped into 11 isoenzymes depending on their substrate affinity and selectivity [147]. One of the most studied PDEs is PDE5, which hydrolyses cGMP into its inactive form.

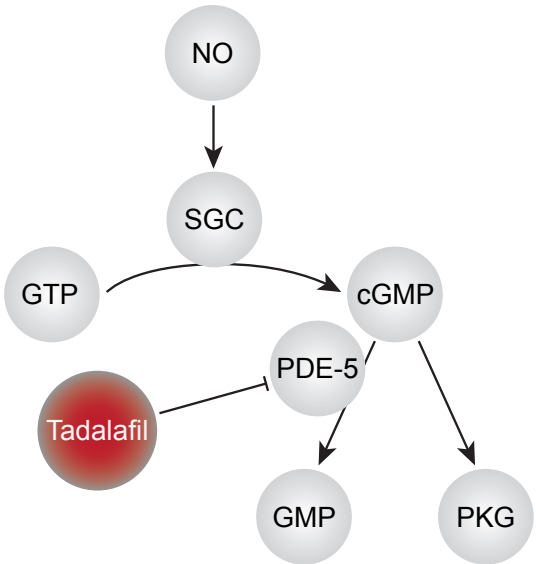

Figure 6. Site of intracellular mechanisms of tadalafil. NO activates sGC, which produces cGMP and activates PKG. PDE-5 hydrolyses cGMP into its inactive form GMP. Tadalafil inhibits PDE-5 leading to a maintained cGMP signalling.

Pharmacological inhibitors of PDE5 have been developed to sustain the NO mediated pathway. Three inhibitors are approved for treatment of erectile dysfunction and pulmonary hypertension - sildenafil, vardenafil and tadalafil. Sildenafil was the first selective PDE5 inhibitor, and was primary developed for treatment of angina pectoris, although its off-target effects led to the change of

focus to erectile dysfunction [148], while tadalafil was developed later [149]. Interestingly, they have emerged as potential pharmaceuticals in treatment of diabetes and cardiovascular diseases. Tadalafil has certain advantages to sildenafil and vardenafil, it has a longer half time of 17.5 h, short time to maximum concentration (2 h) [150], and absorption is not affected by food intake [151, 152]. Additionally, tadalafil is more selective towards PDE5 compared to PDE6 (780:1), as a result visual side effect are rare [153].

Chronic administration of tadalafil has been shown to improve endothelial function and flow-mediated dilation in subjects with erectile dysfunction [154-157]. Chronic treatment with tadalafil has been reported to decrease circulating levels of endothelin-1 and/or adhesion molecules [156-158] in men with erectile dysfunction, and in systemic sclerosis [159]. Further, tadalafil decreased oxidative stress in cardiomyocytes and circulating inflammatory markers in diabetic mouse models [160, 161]. In contrast, other chronic studies in humans did not observe a decrease of adhesion molecules [162] or an effect on insulin resistance [163]. A study showed increased muscle capillary recruitment and increased glucose conversion to lactate in adipose tissue after acute administration of tadalafil in the fasting state [164]. Similarly, glucose uptake and capillary recruitment in muscle was increased in T2D after acute administration of tadalafil in the fasting state [165], while this was not observed in obese women after an oral glucose load [166]. PDE5 inhibition using sildenafil increased insulin sensitivity in an eNOS knockdown mouse model and improved insulin action in cell culture [167, 168]. PDE5 in endothelial cells is localized in proximity to caveolae, and is involved in modulation of eNOS, suggesting a possible function in insulin action [169].

1.7 The gender aspect

Men and women differs physiologically in terms of body composition and adipose tissue distribution, and abdominal obesity has been shown to be more involved in the development of metabolic disease [170, 171]. Differences between genders have been reported in T2D and cardiovascular diseases, both regarding risk, treatment and outcome. Elderly men have more controlled T2D than elderly women [172]. Women also have more comorbidities and reported lower score on health status and functioning, which could affect their self-care [173].

Endothelial function declines in men after 40 years, while function in women remains stable for 10 more years [174]. Decreased levels of male reproductive hormones in men was associated with higher mortality from cardiovascular disease [175], and increased levels of testosterone in women was associated to a higher prevalence of insulin resistance and CHD [176]. Further, oestrogen appear to have positive effects on endothelial function in both genders, while androgens seem to have more deleterious effects, depending on age and gender of subjects [177]. Hormone replacement therapy has been suggested in some studies to protect against CHD [178], and in other studies contradictory been proposed to increase incidence of CHD [179]. These contrasting results have been suggested to depend on an age-dependent increase of superoxide rather than changed signal mechanisms of oestrogen [180].

1.8 Endothelin-1 as a biomarker and type 2 diabetes heredity

As described above, insulin resistance and obesity can predict the development of future T2D, and endothelial dysfunction is likewise highly implicated in the pathology of T2D and cardiovascular diseases. Increased levels of endothelin-1 was observed in T2D [62], the metabolic syndrome [181] and impaired glucose tolerance (IGT) [182], but whether endothelin-1 could predict T2D remains elusive.

Circulating endothelin-1 is increased in subjects with advanced atherosclerosis and coronary artery disease progression [183]. An observational study showed a predictive role of high circulating endothelin-1 levels during the first indications of atherosclerosis for future cardiovascular events [184]. Additionally, high endothelin-1 levels were associated with CHD in women of all ages, while this observation only was seen in elderly men [185]. This suggests that endothelin-1 might be a predictor of CHD in women.
In a study investigating first-degree relatives of T2D, endothelial dysfunction was associated with insulin resistance [186]. Additionally, microvascular reactivity was impaired in first-degree relatives of T2D [187], and healthy first-degree relatives had insulin resistance and postprandial lipid intolerance [188]. Further, during controlled hyperinsulinemia first-degree relatives had larger subcutaneous fat cells and an increased net lactate release per fat cell [189]. Normoglyceamic offspring of parents with diabetes have defects in insulin

resistance and glucose disposal, more than 10 year prior to the onset of T2D. Thus, defects in insulin mediated and insulin-independent glucose uptake might precede and predict T2D [190]. Genetic risk scores had a weak but significant association in subjects with parental T2D, and due to the weak association, the authors suggest that shared environmental factors might be a source behind the higher risk of T2D among relatives [191].

2 AIMS

The overall aim of this thesis was to investigate the microcirculation in insulin resistance, with focus on the expression of endothelin-1, through a translational approach.

Paper I To investigate whether circulating endothelin-1 levels predict incident coronary heart disease.

Paper II To investigate whether circulating endothelin-1 levels predict impaired glucose tolerance and type 2 diabetes.

Paper III To investigate whether microcirculation is associated with insulin delivery to subcutaneous adipose tissue and skeletal muscle after an glucose load in type 2 diabetes patients.

Paper IV To investigate whether acute administration of the PDE-5 inhibitor tadalafil could induce positive vascular, metabolic and anti-inflammatory effects after a mixed meal in type 2 diabetes patients.

Paper V To elucidate the molecular action of tadalafil, and investigate the effects of tadalafil in TNF-α stimulated human endothelial cells.

3 PARTICIPANTS AND METHODS

3.1 Paper I and II

3.1.1 Participants

Between 2002-2005 a population-based cohort was established in the municipalities of Vara and Skövde. A random sample (n=2816) stratified by gender and five-year age groups was created based on all residents between 30-74 years. Criteria for inclusion were: 1. Answering questionnaires. 2. Donating venous blood samples. 3. Completing the physical examination. Participants between 30-50 years were purposely over-sampled by three-fold compared to participants over 50 years. Through 2011-2014 around 2/3 of the baseline population were summoned for a follow-up visit in the same order as at the baseline (n=1834). Of these, 490 declined participation, 35 had moved from the region, 85 had died and 10 were not included for other reasons, leaving 1334 participants in the follow-up.

In all, 2745 participants were included in paper I, and 1099 participants were included in paper II from the Vara-Skövde cohort. A summary of the participants is shown in Table 1.

3.1.2 Study procedure

Participants arrived in the morning after overnight fasting. They signed an informed consent form and venous blood was sampled. Anthropometry was studied with participants in light clothing. In participants without known T2D an oral glucose tolerance test (OGTT) was performed using 75 g glucose to determine their status [192]. During the two-hour wait, participants filled out questionnaires regarding lifestyle. Further, the study nurses collected information about medical history and medication. At the follow-up examination after ten years, the same procedures were repeated.

3.1.2.1 Diagnostic procedures

Standard laboratory tests were performed for serum cholesterol, triglycerides, high-density lipoprotein (HDL)-cholesterol, low-density lipoprotein (LDL)-cholesterol, apolipoproteinB/A1 (apoB/A1), and high sensitivity CRP (hs-CRP). Estradiol was measured at Unilabs at Skaraborg Hospital in Skövde, and total

testosterone was measured using UniCelTM DxI 800 Beckman Access® Immunoassay System Main Instrument DxI-1 (Beckman Coulter, Brea, USA).

Blood pressure was measured twice, a minute apart, with the arm adjusted to the heart level, with participants in a resting state. Hypertension was defined in agreement with guidelines [193] and known hypertension was considered as on-going treatment against high blood pressure. Participants without known hypertension were followed up with further blood pressure examinations if the initial diastolic blood pressure was ≥90 mmHg or if systolic blood pressure was ≥140 mmHg. Subjects with three consecutive high readings were diagnosed as being hypertensive.

Coronary heart disease was defined by ICD10 codes as non-fatal myocardial infarction (code I21), percutaneous coronary intervention and/or coronary bypass grafting, or fatal CHD (code I21-23 and I25) [194].

Diagnosis of T2D was confirmed with either two fasting plasma glucose levels ≥7.0 mmol/l or after one 2-hour plasma glucose value ≥11.1 mmol/l after the OGTT and diabetes symptoms [192]. Impaired fasting glucose (IFG) was diagnosed when participants had a fasting plasma glucose level of 6.0-6.9 mmol/l and IGT when 2-hour plasma glucose was 7.8-11.0 mmol/l post OGTT.

Circulating endothelin-1 concentrations was assessed at baseline using Quantiglo Chemiluminescent ELISA for human endothelin-1 from R&D systems (Minneapolis, USA). Serum insulin was analysed using ELISA from DAKO Diagnostics Ltd (Glostrup, Denmark).

Table 1. Characteristics of the participants included in this thesis

	Participants	Sex (M/F)	Age (years)	BMI (kg/m^2)	HOMA-IR
Paper I	CHD	52/20	62±11	27±4	2.3±1.7
	Control	1298/1375	47±11	27±5	1.6±1.5
Paper II	IGT/T2D	56/48	53±11	29±4	1.8±1.0
	Control	480/515	46±11	26±4	1.3±0.8
Paper III	T2D	3/4	59±4	33±2	2.4±1.1
	Control	4/2	59±6	24±1	1.2±1.1
Paper IV	T2D tadalafil	8/2	58±9	30±4	2.3±1.2
	T2D placebo	6/4	63±5	31±4	2.7±2.3

3.2 Paper III and IV

3.2.1 Participants

In total, 27 participants with T2D and six lean controls were included in two different clinical trials (Table 1). Participants with T2D were excluded if they displayed significant cardiovascular disease, concomitant disease or complications. All included women were postmenopausal. Treatments with β-blockade, glucagon-like peptide-1 (GLP-1) agonists, insulin, dipeptidyl peptidase (DPP)-inhibitors or glitazones led to exclusion in both trials. Additionally, in paper IV, treatment with oestrogens, nitrates, angiotensin converting enzyme (ACE)-inhibitors and glucocorticoids led to exclusion. All participants signed informed consents prior to the start of examinations.

In paper III participants were included if they met the following criteria: Age 50-70 years for men, and 55-70 years for women; BMI 30-40 kg/m^2 for T2D and 18-25 kg/m^2 for lean controls. Participants with T2D were eligible if they were in a good metabolic control with diabetes duration < 5 years. Seven participants with T2D and six age-matched controls were recruited. Participants with treatment for diabetes or hypertension were asked not to take their medication during 10 days prior to their visits, around five days prior to their visits plasma glucose and blood pressure was examined for safety reasons.

In paper IV participants were included if they had a diabetes duration between 3 months and 15 years. Further inclusion criteria were: Age 40-75 years for men, and 50-75 years for women, BMI 25-40 kg/m^2 and HbA_{1c} < 57 mmol/mol. Twenty participants were included in this study. Participants were asked not to take their treatment against diabetes or hypertension for 3 or 7 days, respectively, prior to the study.

3.2.2 Study procedure

In paper III, intramuscular and subcutaneous microdialysis was performed after an overnight fast to investigate interstitial insulin concentrations before and during three hours after an oral glucose load (75 g). Participants were asked to drink the glucose solution during 1-2 minutes. Arterialized venous blood was repeatedly sampled and ^{133}Xenon clearance was used to assess subcutaneous blood flow (Fig. 7). On a second visit, a subcutaneous adipose tissue needle biopsy was performed and endothelial function was assessed with peripheral arterial tonometry using the EndoPAT2000 device.

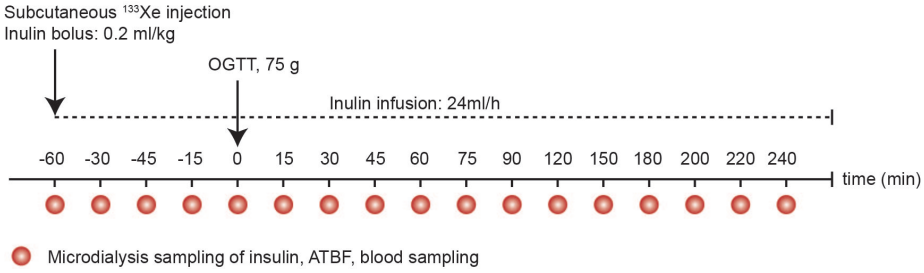

Figure 7. Overview of study protocol for paper III.

In Paper IV, intramuscular microdialysis was performed after an overnight fast to investigate interstitial glucose concentrations after acute administration of tadalafil (20 mg) or placebo in combination with a mixed meal (Fig. 8). Computer-generated randomization of the participants was done 1:1. The randomization was double-blinded, and neither participants nor investigators were aware of which treatment the participants received. The mixed meal was ingested 30 minutes after the participants received their tablet. Total energy content was 786 kcal (3291 kJ); 46% fat, 48% carbohydrates and 7% protein.

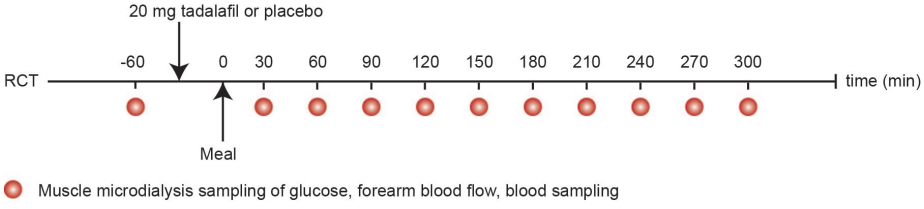

Figure 8. Overview of the study design for the randomised controlled trial (RCT) in paper IV.

3.2.2.1 Analytical procedures

Circulating insulin was analysed using Mercodia insulin ELISA. Insulin in dialysates was determined using Mercodia ultrasensitive insulin ELISA (Mercodia, Uppsala, Sweden). Plasma glucose was determined with Hemoque® Glucose 201+ (Hemoque, Ängelholm, Sweden) (paper III).

In paper IV glucose and urea in dialysates and plasma was determined using a colorimetric method (glucose) and a UV method (urea) on a CMA600 Microdialysis analyser (CMA Microdialysis, Stockholm, Sweden). Arterial Endothelin-1 levels in circulation was determined with Quantiglo Chemiluminescent ELISA for human endothelin-1 from R&D systems. Arterial intercellular adhesion molecule-1 (ICAM-1), vascular cellular adhesion molecule-1 (VCAM-1) and E-selectin was analysed using Human Vascular Injury II kit and Human E-selectin kit from Meso Scale Diagnostics LLC (Rockville, USA). Circulating concentrations of free fatty acids and triglycerides was analysed using spectrophotometric methods at the accredited clinical laboratory at Sahlgrenska University Hospital.

3.2.3 Microdialysis technique

The microdialysis technique is applied for determination of concentrations of interstitial molecules *in vivo*. In paper III microdialysis was used to measure interstitial levels of insulin in subcutaneous adipose tissue and in skeletal muscle. In paper IV microdialysis was applied to assess interstitial levels of glucose in skeletal muscle. This is made possible by using thin catheters with a semipermeable membrane. These are inserted into the tissue of interest, and continuously perfused using a microinjection pump allowing for molecules to diffuse and the dialysate is collected and analysed. The relative recovery of a molecule, defined as the exchange between the perfusion fluid and surrounding interstitial fluid, is dependent on several factors; the length of the catheter, molecular cut-off of the membrane, flow rate, temperature, physical and chemical properties of the molecule and the composition of the perfusion fluid [195]. The recovery factor is the ratio between the concentration in the dialysate and the concentration in the interstitial fluid outside the catheter. Interstitial concentrations can therefore be calculated by estimations of the dialysate concentration and the recovery factor.

3.2.3.1 Insulin

Interstitial insulin was measured in periumbilical subcutaneous adipose tissue using two Asahi-catheters with a 3 MDa molecular cut-off [196], and in brachioradialis muscle using two custom-made CMA catheters with dialysis membrane 12 mm x 0.5 mm and a 0.1 MDa molecular cut-off. The catheters were perfused with isotonic saline with 1% albumin and 1.5 mmol/l glucose at a rate of 2.5 µl/min in subcutaneous tissue and 1.5 µl/min in skeletal muscle. The external reference technique was used to calibrate the catheters [119, 197]. This

technique builds on the theory that even though recovery of molecules is different *in vitro* compared to *in vivo* the relationship between the recovery of two different molecules could be the same both *in vitro* and *in vivo*. Thus, inulin, a polysaccharide, similar to insulin in molecular weight, was injected intravenously as a bolus dose in the beginning of the study day and then continuously infused during four hours, and measured in dialysate during the following hour. Inulin equilibrates completely between plasma and interstitial fluid [198], and can therefore be used to calculate recovery.

$$\text{Recovery of insulin in vivo} = \left(\frac{\text{recovery of insulin in vitro}}{\text{recovery of inulin in vitro}}\right) \times \text{recovery of inulin in vivo}$$

Interstitial concentrations of insulin (I-insulin) in subcutaneous adipose tissue and skeletal muscle could then be calculated as:

$$\text{I - insulin} = \frac{\text{[insulin in dialysate]}}{\text{recovery of insulin in vivo}}$$

3.2.3.2 Glucose

Interstitial glucose in the brachioradialis muscle was measured using two CMA catheters 16 mm x 0.5 mm, 20 kDa molecular cut-off, perfused with isotonic saline supplemented with 1.5 mmol/l glucose and 0.5 mmol/l urea at a rate of 2.5 µl/min. Urea was used as a endogenous reference to calibrate the catheters [199]. The relative recovery of urea was estimated from concentrations of urea in dialysate and plasma, and the recovery of glucose could then be calculated by the relationship to urea *in vitro* [199].

3.2.4 Blood flow measurements

In paper III subcutaneous adipose tissue blood flow (ATBF) was measured using ^{133}Xenon (^{133}Xe) clearance technique [200]. 4-6 MBq of ^{133}Xe (Mallincrodt, Petten, The Netherlands) in gaseous form was injected during 1 minute in the periumbilical subcutaneous adipose tissue at two sites. After 60 minutes of equilibration, the activity at the injection sites was registered in 30-second intervals using GMS411 Mediscint detectors (John Caunt Scientific, Lancashire, England). Subcutaneous blood flow was calculated by using the following formula:

$$ATBF = -\lambda \times \frac{\ln(C_A) - \ln(C_B)}{n_A - n_B} \times \frac{60s \times 100g}{T_S}$$

λ is the tissue to blood partition coefficient for ^{133}Xe at equilibrium (approximated to 10 ml/g in both groups) [201], ln is the natural logarithm of counts detected from each of the sites, n is the corresponding sample number and T_S is interval of detection (here 30 sec).

In paper IV forearm blood flow was studied applying venous occlusion plethysmography [202]. A mercury in-silastic strain gauge was placed around the upper third of the forearm and venous outflow was interrupted using a cuff, increasing the volume of the tissue due to unaffected arterial inflow. The strain gauge registers the volume change as ml/100 ml tissue/min.

3.2.4.1 The forearm model

Studies of local metabolism in the brachioradialis muscle were made possible investigating the arterio-venous (A-V) differences of metabolites over the forearm [203, 204]. In paper IV a catheter was inserted retrogradely into one of the branches of the antecubical vein, while another was inserted into the radial artery. Blood was sampled simultaneously from the deep vein and the artery. Forearm glucose uptake was calculated according to Fick's Principle [205]:

$$FGU = (A - V) \times forearm\ blood\ flow$$

Additionally, the capillary recruitment was calculated using an equation combining arterio-venous and microdialysis measurements [47, 48]:

$$V - A = (I - A) \times (1 - e^{PS/Q})$$

Where V and A is venous and arterial plasma concentration, respectively, I is the interstitial concentration, *e* equals the base of the natural logarithm (2.71828), PS is the permeability surface area for glucose and Q is the plasma flow rate, calculated by multiplying the forearm blood flow by 100 minus the haematocrit as a percentage.

3.2.5 Assessment of endothelial function

In paper III, peripheral endothelial function was determined by assessing peripheral arterial tonometry [206, 207] with the EndoPAT2000 device from Itamar Medical (Caesarea, Israel). Designed finger probes were placed bilaterally on each index finger to assess digital volume changes before, during and after complete occlusion of the circulation in the test arm. In short, a blood pressure cuff was placed on the upper part of the dominant arm, while the other arm was

used as a control. After 6 minutes of baseline recordings, occlusion was applied using the cuff during 5 minutes. Then the pressure was released and post-occlusion recordings were measured during 5 minutes. The device automatically calculated reactive hyperaemia index (RHI) and a ratio between baseline recordings and post-occlusion recordings.

3.3 Paper V

3.3.1 Cell culture and Experimental Design

Human umbilical vein endothelial cells (HUVEC) were used as an *in vitro* model for studying molecular effects of tadalafil.

HUVECs were cultured in endothelial basal medium-2 supplemented with 5% fetal bovine serum, human epidermal growth factor, GA-1000 (Gentamicin, Amphotericin-B), vascular endothelial growth factor, Arg3 insulin-like growth factor-1, ascorbic acid, heparin and hydrocortisone at 37°C in an atmosphere of 5% CO_2.

Experiments were performed in HUVECs in passage 4-5 cultured to near confluence and incubated in basal medium without supplements for three hours. HUVEC were pre-treated with either 1 μM tadalafil, or left untreated during 1 hour prior to addition of 4 ng/ml TNF-α. In additional experiments, inhibitors or analogues for specific key molecules were used to modify either the effect of tadalafil or simulate similar effects as tadalafil. Briefly, in separate sets of experiments, the following were used, PKG was blocked using DT-3, NO was inhibited with c-PTIO, sGC was blocked with ODQ, and SP600125 was used to inhibit JNK. 8-Bromo-cGMP or DEA-NONOate replaced tadalafil in further sets of experiments. By applying RNA interference PDE-5 expression was knocked down. Finally, after tadalafil treatment and TNF-α stimulation, nuclear extracts were isolated and the AP-1 family members were investigated.

3.3.2 Methods

Gene expression was determined after 12 hours of TNF-α stimulation, by real-time PCR. Real-time PCR amplify and simultaneously detects specific cDNA sequences using primers specific for sequences of interest, generated from total RNA preparations by reverse transcription. By using an endogenous reference gene (here, 18S), the relative expression of the specific gene is calculated, and fold change compared to TNF-α stimulated cells were analysed.

Intracellular protein expression was determined after 15 minutes of TNF-α stimulation using western blot. Briefly, proteins were extracted and concentrations were determined. Proteins were separated according to size on 4-12% Bis-Tris gels and transferred to nitrocellulose membranes. Membranes were incubated with primary antibodies of interest followed by horseradish peroxidase

conjugated secondary antibodies. Protein bands were detected using chemiluminescence and ratio between phosphorylated and total protein from the same membranes were calculated.

Secreted proteins were quantified with solid-phase sandwich ELISA applying chemiluminescence detection from R&D systems or Meso Scale Discovery. Cell medium was collected after 12 hours of incubation with TNF-α, cell debris was removed by centrifugation, and the supernatant was saved for ELISA quantification.

HUVECs were transfected with PDE-5 small interfering RNA (siRNA). siRNA promote degradation of the targeted mRNA, ultimately leading to loss of protein expression. Briefly, two different siRNA targeting PDE-5 was used, and cells were transfected during four hours prior to serum-starvation followed by TNF-α stimulation for 15 minutes.

To determine activation of the AP-1 family, nuclear extracts were isolated after 15 minutes of TNF-α stimulation. Binding activity of AP-1 family members were analysed using an ELISA containing an immobilized oligonucleotide covering a TPA-response element (TRE; 5'-TGAGTCA-3'), epitopes on the AP-1 family members bound to the oligonucleotide were detected with primary antibodies.

3.4 Statistical analysis

In paper I-V data is presented as mean±SD in tables, and mean±SEM in figures. All analyses were two-sided and $p < 0.05$ was considered statistically significant. SPSS Base Systems for Macintosh 22.0 was used for analysing data in paper I-II, and GraphPad Prism 6.0 for Macintosh was used for analysis of data in paper III-V. Additional data was analysed using SAS v9.2 in paper IV.

3.4.1.1 Paper I

Linear regression models were used to investigate association between endothelin-1 and continuous variables while mean differences of continuous variables were analysed by general linear models. Kaplan-Meier survival curves were applied for tertiles of endothelin-1 and Cox proportional hazard regressions were used to investigate associations between endothelin-1 and CHD. Theoretical multivariate models and stratification were used to evaluate interactions and role of possible confounders.

3.4.1.2 Paper II

Logistic regression models were applied to analyse the association between quartiles of endothelin-1 and odds ratio of IGT/T2D, using the lowest quartile as reference. Multivariate models and stratification were used to investigate interactions and the role of possible confounders. General linear models were used to assess differences of clinical variables in participants. Linear regression was applied for associations between clinical variables and quartiles of endothelin-1.

3.4.1.3 Paper III

Mann-Whitney U-test was used for comparisons between T2D and lean controls. Friedman's analysis with Dunn's multiple comparison tests or Wilcoxon signed rank test was applied for comparison between different time-points and baseline. Non-parametric Spearman's ranked test was used for correlations.

3.4.1.4 Paper IV

Fischer's exact test was used for dichotomous variables and Mann-Whitney U-test was used for continuous variables. Friedman's analysis with Dunn's multiple comparison tests was applied to investigate changes from baseline, and Wilcoxon signed rank test was applied for comparisons between different time-points.

3.4.1.5 Paper V

Data from three or more independent experiments were presented as per cent increase or decrease compared to TNF-α stimulated cells. Differences between treatments were analysed using repeated measures ANOVA or Student's t-test.

4 SUMMARY OF RESULTS

4.1 Paper I – Circulating endothelin-1 levels predict coronary heart disease

4.1.1 Study design

At baseline, 1811 participants from Vara (81% participation rate) and 1005 participants from Skövde (70% participation rate) were studied. In all, 2816 participants (76% participation rate) were included in the Vara-Skövde cohort. In this study, 50 participants were excluded due to known CHD at baseline and 21 participants were excluded due to endothelin-1 levels below detection limit. Thus, 2745 participants were included into the analysis of incident CHD.

Follow-up time of this study was 8.1±1.3 years, and records of CHD events were retrieved by data linkage with the Swedish Cause of Death and Hospital Discharge Registers. During the follow-up time 52 events of incident CHD was found in men, and 20 events in women, giving a total of 72 events of incident CHD. Of these, 19 events were fatal (14 in men, 5 in women). Event rate was 2.6 per 1000 person years.

4.1.2 Main results

Age, waist-hip ratio (WHR), apoB/A1 and blood pressure were significantly higher at baseline in men and women with incident CHD in the Vara-Skövde cohort compared to participants without incident CHD. Homeostatic model assessments for insulin resistance (HOMA-IR), hs-CRP and total s-cholesterol were only increased in men. Further, hypertension and T2D were more frequent in both sexes with incident CHD.

Baseline circulating endothelin-1 concentrations was significantly higher in women with incident CHD than in women without CHD, a difference that was not observed in men. Further, circulating endothelin-1 levels increased with age in both sexes up until 40-49 years, although while concentrations remained constant in women with higher age, they declined in men. Thus, linear regression analysis showed an association between age and endothelin-1 in women. Moreover, association with clinical variables and endothelin-1 showed differences between the sexes and an interaction-term evaluating the relationship between endothelin-1 and sex was significant. Kaplan-Meier survival curves showed that women with the highest tertile of endothelin-1 levels had significantly shorter CHD event-free time compared to the two lower quartiles. In women, there was a robust association between higher

circulating endothelin-1 concentrations and incident CHD after adjustment for age, HOMA-IR, apoB/A1 and smoking. In contrast, no association was seen in men between endothelin-1 and incident CHD.

4.2 Paper II – Endothelin-1 as a predictor of type 2 diabetes

4.2.1 Study design

During the follow-up 1334 participants were studied (73% participation rate). Out of these, 152 participants were excluded because of known IGT/T2D at baseline and 67 were excluded due to impaired fasting glucose at baseline. Furthermore, 5 participants were excluded, as their IGT/T2D status at follow-up was missing, and 11 participants due to endothelin-1 levels below detection limit. In total, 1099 participants were included in the analysis of developing IGT/T2D.

Follow-up time was 9.7±1.4 years and at the follow-up examination 104 cases of IGT/T2D (56 in men and 48 in women) were identified. Of these, 12 men and 7 women were diagnosed with T2D between the two visits, and the remaining 85 participants (44 men and 41 women) were identified at the follow-up.

4.2.2 Main results

Age, HOMA-IR, WHR, blood pressure, BMI, s-HDL-cholesterol and s-triglycerides at baseline were higher in men and women who had IGT/T2D at follow-up. Furthermore, apoB/A1 was higher in men with IGT/T2D when comparing to men with normal glucose tolerance.

Circulating endothelin-1 levels at baseline was not significantly higher in either sex comparing IGT/T2D although a trend was observed in women. Therefore, endothelin-1 levels were allocated into quartiles based on circulating concentrations. Again, in this smaller population in the Vara-Skövde cohort, differences between men and women were seen when investigating association between clinical variables and quartiles of endothelin-1. Logistic regression showed a significant test for trend, as well as higher odds ratio for IGT/T2D in higher quartiles of endothelin-1 for women compared to the lowest quartile. These results withstood adjustment with smoking, low leisure time physical activity, apoB/A1, systolic blood pressure, hs-CRP, first-degree heredity of T2D and WHR. There was no association between quartiles of endothelin-1 and IGT/T2D in men.

4.3 Paper III – Delayed delivery of insulin to adipose tissue in type 2 diabetes

4.3.1 Study design

Seven participants with T2D and six lean controls were included and studied during two separate visits. During the first visit participants were studied during an oral glucose load. Microdialysis in skeletal muscle and subcutaneous adipose tissue, together with subcutaneous adipose tissue blood flow was monitored repeatedly and blood was sampled repeatedly during this visit. During the second visit a subcutaneous adipose tissue needle biopsy was performed and peripheral arterial tonometry was assessed.

4.3.2 Main results

Interstitial insulin in subcutaneous adipose tissue increased in lean controls 60 minutes after the glucose load, while this increase was delayed in participants with T2D. The ratio between interstitial and circulating insulin (I/C) in adipose tissue was significantly lower in T2D participants than in lean controls. This pattern was also seen in skeletal muscle. Additionally, the increase of ATBF was blunted in T2D compared to lean controls. At 90 minutes after the glucose load correlated ATBF with the I/C ratio and postprandial ATBF was inversely correlated to fat cell size. Furthermore, when assessing endothelial function by ratio between post-occlusion/pre-occlusion signal ratio lean controls showed a more rapid response and higher ratio in comparison to participants with T2D.

4.4 Paper IV – Postprandial effects of the PDE-5 inhibitor tadalafil

4.4.1 Study design

In this study, 20 participants with T2D was included and randomised to either placebo or tadalafil (20 mg) in a double-blind manner. The participants received either placebo or tadalafil 30 minutes before they ingested a mixed meal. Interstitial glucose in muscle microdialysis, A-V differences, and forearm blood flow was monitored during the study day.

4.4.2 Main results

Participants were well matched for clinical characteristics except for longer diabetes duration in the group randomized to tadalafil. Acute administration of tadalafil increased IAUC for forearm blood flow, forearm glucose uptake and muscle capillary recruitment in the postprandial state compared to placebo. Furthermore, postprandial circulating endothelin-1 was increased in the placebo group (vs. 60 minutes after the meal), but not in the group receiving tadalafil. Circulating levels of insulin, glucose, free fatty acids and triglycerides were not different between the two groups.

4.5 Paper V – Effects of tadalafil in TNF-α stimulated endothelial cells

4.5.1 Study design

In this study the effects of tadalafil was investigated using TNF-α stimulated HUVEC. Key molecules were studied by modifying the signalling pathways, and the effect of PDE-5 inhibition was further studied with knockdown using RNA interference.

4.5.2 Main results

Tadalafil decreased phosphorylation of JNK after 15 minutes of TNF-α stimulation. A cGMP analogue, 8-bromo-cGMP reduced JNK phosphorylation in a similar manner, while pre-treatment with a PKG blocker reversed the effect of tadalafil. However, a NO-donor and a NO-scavenger did not affect JNK phosphorylation. Further, a blocker against sGC did not significantly change JNK phosphorylation. Nuclear extracts was isolated and the AP-1 transcription factor family was studied, showing that tadalafil significantly decreased TNF-α induced c-Jun activation. Furthermore, knockdown of PDE-5 using RNA interference decreased phosphorylation of JNK comparable to tadalafil.

In addition, tadalafil reduced mRNA expression of endothelin-1, ICAM-1, VCAM-1 and E-selectin, and decreased endothelin-1 secretion. A JNK inhibitor reduced mRNA expression and secretion of endothelin-1, ICAM-1 and E-selectin while VCAM-1 was unaffected (Fig. 9).

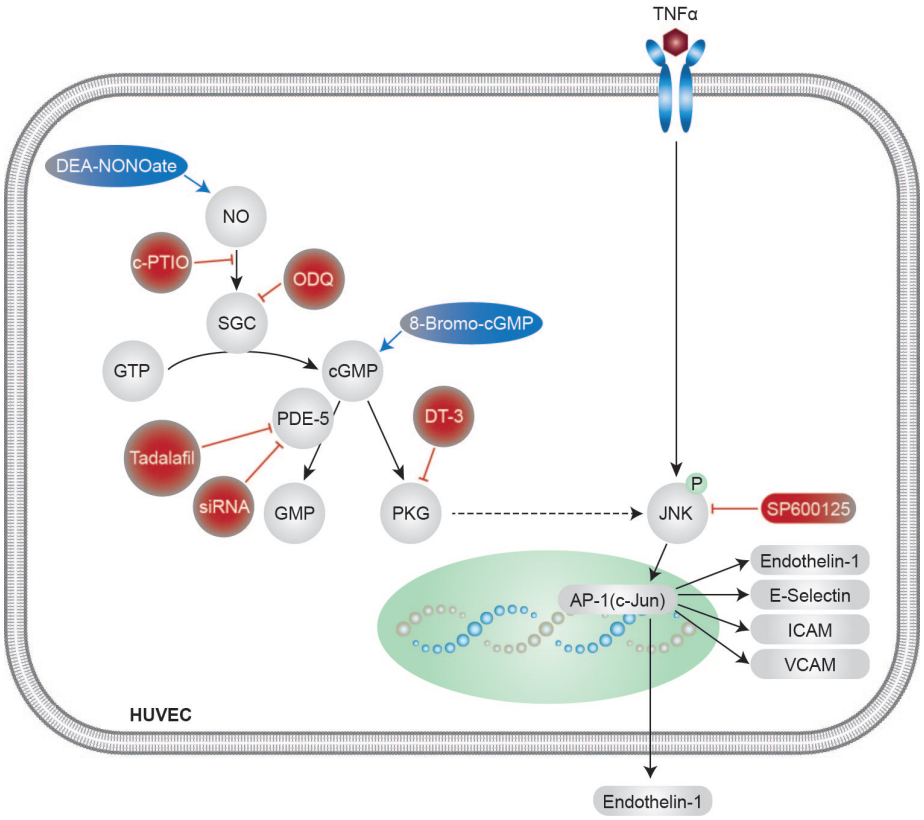

Figure 9. Schematic overview of findings in paper V. Treatment with tadalafil decreased TNF-α stimulated mRNA expression of endothelin-1, E-Selectin, ICAM and VCAM, and decreased secretion of endothelin-1 into the cell medium. RNA interference with siRNA led to a similar decrease as tadalafil on JNK phosphorylation. 8-bromo-cGMP mimicked the effect of tadalafil on JNK, while DT-3 blocked tadalafils effect. Either DEA-NONOate, c-PTIO or ODQ had a significant effect on JNK phosphorylation. SP600125 reduced mRNA expression and secretion of endothelin-1, E-selectin and ICAM.

5 DISCUSSION

5.1 Major findings

5.1.1 Endothelin-1 as a predictor of coronary heart disease and type 2 diabetes

In the population-based Vara-Skövde cohort studied in paper I, increased levels of circulating endothelin-1 were associated with incident CHD in women. This association was independent from other risk factors for CHD as well as circulating levels of estradiol. No association between circulating endothelin-1 levels and incident CHD was demonstrated in men.

Thus, the principal observation proposes sex-differences in the association between endothelin-1 and outcome in this population, which was supported by a significant interaction term. In addition, linear regression exploring the association between circulating endothelin-1 levels and clinical variables and lifestyle traits showed striking differences between men and women. Endothelin-1 levels in men correlated to HOMA-IR, which supports previous results, where endothelin-1 levels are higher in T2D than in controls [187]. Further, endothelin-1 levels in women positively correlated with age, which is in line with previous publications [208]. This correlation was visualized by a maintained endothelin-1 level from 40 years and older in women (Paper I, Fig. 2) while endothelin-1 levels decline after a peak between ages 40-50 in men (Paper I, Fig. 2). Interestingly, previous studies show that endothelial function gradually decline in healthy men after age 40 while endothelial function women have a steeper decline after age 50 [174], which the authors suggests depends on menopause.

While estradiol did not correlate to endothelin-1 in women in this cohort, studies have described that exogenous oestrogen decrease endothelin-1 production in cell culture [209], and hormone replacement therapy increase the ratio between NO and endothelin-1 in women [210]. Women with hormone replacement therapy had lower endothelin-1 levels in the Rancho-Bernardo study, however, the association between endothelin-1 and CHD were similar between women with hormone replacement therapy, and women without [185]. While menopause was not ascertained in this study, 19 of the 20 women with incident CHD were 55 years or older when they got their event, and could therefore be assumed to be postmenopausal. Although hormone replacement therapy was slightly more common in women with incident CHD compared to women without CHD, this was not significant. This suggests that

oestrogen may not have a main responsibility in alterations of the association between endothelin-1 and CHD in this cohort. Furthermore, the Rancho-Bernardo cross-sectional study did not show differences in expression of endothelin-1 depending on sex [185]. However, the association between endothelin-1 levels and CHD were only significant in men older than 70 years, while the association in women was significant independent of age [185]. Although the association in men was not confirmed in our study, this could be explained by the lower age of the participants in our cohort.
The main observation in this study, that endothelin-1 predict incident CHD in women, was not affected by adjustment for clinical variables that differed between the groups, further indicating that endothelin-1 may be an independent predictor of CHD in women.

In paper II, a subpopulation of the Vara-Skövde cohort was investigated prospectively, and we show that endothelin-1 is associated with IGT/T2D in women. The highest quartile of endothelin-1 levels in women showed 4-fold higher odds of being diagnosed with IGT/T2D during follow-up compared with the lowest quartile. This association remained after adjustment for several risk factors for IGT/T2D.

In this study, a decision to not include participants with impaired fasting glucose was taken, since endogenous endothelin-1 appears to have more effects on insulin action than on glucose production. Accordingly, a previous study showed that endothelin-1 limits insulin action, including glucose uptake [211] and another study showed that exogenous endothelin-1 infusion reduces splanchnic glucose exchange while arterial glucose concentrations were unchanged [212].
Surprisingly, baseline HOMA-IR was not associated to quartiles of endothelin-1 in either sex, while HOMA-IR previously have been positively correlated to endothelin-1 using simple regression analysis in a smaller cohort [213]. Additionally, quartiles of endothelin-1 were associated with total cholesterol, LDL-cholesterol and inversely associated to apoB/A1 in men. This is in line with earlier studies showing an increase of ET_B by LDL-cholesterol through extracellular signal regulated kinase (ERK1/2) and p38 MAPK [214]. In contrast, exposure of apoB impaired endothelium-dependent vasodilation [215] and apoB/A1 has previously been shown to be inversely related to endothelium-dependent vasodilation but not flow-mediated dilation in an elderly population [216]. Thus, the significant inverse association between apoB/A1 and endothelin-1 in men was surprising, but was also seen in the larger population of the Vara-Skövde cohort presented in paper I. Thus, an interaction between lipoproteins and endothelial function might confound the effects of endothelin-1 in men; however, adjusting for apoB/A1 did not change the

association of endothelin-1 quartiles and outcome. In women, no associations were seen between lipids and quartiles of endothelin-1, but a positive association was seen between CRP and quartiles of endothelin-1 levels in women.

A subclinical inflammation has together with endothelial dysfunction been associated with T2D [217]; and CRP measured at baseline was borderline significantly increased in women with IGT/T2D at follow-up in this study. In endothelial cells *in vitro* CRP have been demonstrated to increase endothelin-1 production and secretion [218]. Further an endothelin-1 receptor antagonist, bosentan, inhibited the effect of CRP on adhesion molecules in endothelial cells, indicating that endothelin-1 could mediate the inflammatory effects of CRP [218]. There was no significant correlation between endothelin-1 quartiles and BMI and WHR in this cohort; however, there was a significant association for both variables to incident IGT/T2D. A link between subclinical inflammation in perivascular fat and endothelial dysfunction have been suggested, through inhibition of NO production by inflammatory markers secreted from adipocytes, leaving insulin-mediated production of endothelin-1 unopposed [98].

Earlier studies in smaller cohorts have shown inconsistencies whether endothelin-1 levels are different depending on sex [219, 220]. In the Vara-Skövde cohort, endothelin-1 levels was similar between healthy men and women, however, in both papers I and II, baseline endothelin-1 were increased in women while the baseline endothelin-1 levels in men did not differ depending on outcome.

5.1.2 Delivery of insulin to adipose tissue and skeletal muscle

In paper III, transendothelial transport of insulin was impaired in subcutaneous adipose tissue in T2D compared to lean healthy controls after an oral glucose load. The ratio between interstitial and circulating insulin was lower in T2D. This finding supports the notion of an increased gradient of insulin between circulation and the subcutaneous interstitial fluid and further, the presence of an endothelial barrier in insulin resistance.

Endothelial function in the current study was assessed by peripheral arterial tonometry. The difference of RHI was not statistically different between the two groups. However a reduced and delayed recovery post-occlusion was shown in T2D, additionally linking the role of endothelial dysfunction to the delay of insulin delivery. Previously, microvascular endothelial cells from subcutaneous adipose

tissue in T2D had increased endothelin-1 expression and impaired activation of insulin signalling [103]. This further implicates the role of the endothelium in insulin resistance [221].

Further, an association between ATBF and the subcutaneous I/C ratio of insulin was observed in this study. In agreement, ATBF was increased in lean controls after the glucose load, while no increase was detected in T2D participants. This implicates that the increase of blood flow in subcutaneous adipose tissue may enable insulin delivery to adipose tissue. This supports the findings in obese women [85]. Impaired ATBF has been suggested as a characteristic of insulin resistance with lower ATBF in T2D compared to obese participants [222] and in the postprandial state in first-degree relatives [223]. Thus, a close relationship between ATBF and insulin resistance has been described [224]. However, insulin has been shown to not affect ATBF directly [225], but rather in an indirect manner, possibly through β-adrenergic activation of the sympathetic nervous system in the postprandial state [226]. Further NO seems to affect the fasting levels of ATBF, but did not affect the postprandial increase of ATBF [226, 227]. Consequently, a well-functioning ATBF for the delivery of insulin is important, and a blunted increase of the postprandial blood flow may contribute to the endothelial barrier for insulin.

In addition, ATBF was inversely associated with subcutaneous adipocyte size in this study, in accordance with earlier studies showing an inverse association between ATBF and subcutaneous adipocyte size [228]. Size of adipose tissue areas has similarly been shown to associate with ATBF [229], as well as adipocyte cell size in dogs [230]. Large adipocytes might secrete cytokines such as TNF-α, which in turn could induce microvascular insulin resistance by disturbing insulin signalling [231]. Further, previous studies have also shown that intact insulin signalling in the endothelial cells is required for insulin transport over the endothelium [124].

The existence of an insulin gradient between the interstitial fluid and circulation, i.e. an endothelial barrier for insulin, is debated. Some studies reported no significance for a gradient on metabolic effects of insulin [232] and that the transendothelial transport of insulin is non-saturable [122]. In contrast, others show the importance of an endothelial barrier, and the rate-limiting effects of transendothelial transport of insulin [130, 131, 233]. An additional study showed impaired delivery of insulin to both adipose tissue and skeletal muscle in obese women [85]. Here, no significant difference in insulin delivery to skeletal muscle was seen, however a clear trend was indicated.

Taken together, the delivery of insulin might be an important mechanism to maintain normal glucose uptake, and endothelial dysfunction appears to be an important

contributor to an impeded insulin-mediated glucose uptake [109, 131]. The results in paper III support the hypothesis of an endothelial barrier between the interstitial fluid and circulation. However, further studies to investigate the specific mechanisms behind insulin delivery and endothelial dysfunction are needed, particularly in adipose tissue, which is not as well explored as the skeletal muscle.

5.2 Role of tadalafil *in vivo* and *in vitro*

In paper IV, a placebo-controlled, double blind, randomized study was conducted to investigate the postprandial effects of the PDE-5 inhibitor tadalafil in men and women with well-controlled T2D. The main observations in this study was that acute treatment with tadalafil 20 mg significantly improved capillary recruitment, forearm blood flow and glucose uptake in participants. In addition, tadalafil blunted the increase of endothelin-1 that was seen in the postprandial state in the placebo group.

Our observation on the positive effects of tadalafil on capillary recruitment and glucose uptake are in line with previous study from our group investigating the acute effects of tadalafil 20 mg in the fasting state [165]. However, in the previous study the increase of forearm blood flow seen here was not demonstrated, which might indicate that tadalafil does not affect this parameter in the fasting state. Additionally, another study using a lower dose of tadalafil, 10 mg, in the fasting state showed no effect on forearm blood flow or muscle glucose uptake, but an increase in capillary recruitment [164], while a study investigating the lower dose of tadalafil after an oral glucose load did not demonstrate a change in either variable [166]. Thus, the dose of tadalafil used seems to be important when investigating the metabolic and microvascular effects of tadalafil.

In the study presented in paper IV, circulating levels of insulin and glucose increased in the postprandial state as expected. However, an acute dose of tadalafil was not sufficient to alter these levels in comparison to placebo. It has earlier been shown that chronic treatment of tadalafil in obese subjects or the metabolic syndrome may increase insulin secretion through positive effects on beta-cell function [163, 234], however they did not see an effect on insulin sensitivity. It is thus possible that tadalafil improves glucose metabolism through an increase of microcirculation both in islets and insulin sensitive tissues such as muscle [165, 234]. The findings in this study, with positive postprandial microvascular effects of tadalafil, are important since impaired capillary recruitment after a mixed meal have been observed in subjects with obesity, insulin resistance and T2D [137, 138]. Further, postprandial

blood flow is impaired in all stages of T2D pathophysiology [235]. The finding that tadalafil increased glucose uptake without altering postprandial circulating glucose concentrations might be explained by increased function of the microcirculation in skeletal muscle by PDE-5 inhibition [236], leading to an increased muscle perfusion, which is indicated in this study by an increased capillary recruitment. In this study, hepatic glucose production was not assessed and therefore we cannot rule out an effect of tadalafil on hepatic glucose production in the postprandial state. The effect of tadalafil on hepatic glucose production in T2D needs to be addressed in future studies.

Here, we observed an increase of circulating endothelin-1 levels in the placebo treated group in the postprandial state. This increase was not seen in the group receiving tadalafil. In line with these results, chronic studies of tadalafil have been shown to improve endothelial function and decrease endothelin-1 in subjects with erectile dysfunction [156, 157]. This could be of relevance since endothelin-1 has been shown to inhibit insulin signalling in cells [237], and decrease insulin sensitivity and glucose uptake in a rat model [78]. Additionally, endothelin-1 decreases glucose uptake in humans, and this effect could be blocked by infusion of dual endothelin-1 receptor antagonists [113, 114]. A study investigating the chronic effects of tadalafil in T2D subjects could further elucidate the possibility of tadalafil to modulate circulating endothelin-1 levels.

In this study, serum levels of adhesion molecules did not increase in either the placebo group or in the group receiving tadalafil in the postprandial state, and it could therefore not be determined whether tadalafil has an effect on these inflammatory markers. However, an earlier study has shown that the inflammatory response to a meal is higher in T2D than in healthy subjects [136]. Further some chronic studies with tadalafil have shown a decrease of VCAM in addition to endothelin-1 [156], while others only showed decreased endothelin-1 levels after chronic administration of tadalafil [158].

In paper V, the molecular effects of tadalafil were investigated in TNF-α stimulated human endothelial cells. The main findings in this experimental study were that tadalafil attenuated endothelin-1 gene expression and secretion as well as decreased phosphorylation of JNK in TNF-α stimulated HUVEC. This indicates a potential molecular signalling pathway by which tadalafil exerts its effect in endothelial cells.

The decreased phosphorylation of JNK by tadalafil was paralleled by a siRNA-mediated knockdown of PDE-5, which denotes that PDE-5 inhibition could have an

effect on JNK activation in these cells. TNF-α has been shown to impair insulin mediated vasodilation by activation of JNK [105], and vasoconstriction induced by endothelin-1 can be abrogated by inhibition of JNK [238]. In this study, amplifying cGMP with 8-Bromo-cGMP mimicked the effect of tadalafil on JNK phosphorylation, and blocking PKG ablated the effect of tadalafil on JNK. This suggests a potential pathway by which the tadalafil effect is exerted.

Interestingly, treatment with a NO-donor or NO-scavenger did not affect JNK phosphorylation, indicating that the effect of PDE5 inhibition on JNK is mediated directly through the cGMP-PKG signal rather than through a feedback loop to NO in our model. Earlier studies have indicated that peroxynitrite activates JNK in arterial endothelial cells [239], and that tadalafil attenuates oxidative stress in a T2D mice model [161]. Therefore, it would be interesting to further explore the effects of oxidative stress in our setting, as well as study tadalafil on endothelial cells isolated from the microcirculation, possibly oxidative stress is more apparent on the arterial side of the circulation [240]. When blocking sGC, similar results were observed as when inhibiting NO, further indicating that the effect of tadalafil on cGMP signalling is exerted downstream of cGMP.
Further, long-term incubation of HUVEC with insulin decreased Akt and eNOS and increased VCAM, showing that prolonged hyperinsulinemia could potentially contribute to the detrimental effects of endothelial dysfunction in T2D [241], by linking high insulin-mediated eNOS downregulation with a pro-atherogenic endothelium reflected by an augmentation of VCAM surface expression. Moreover, in a study investigating the PDE-5 inhibitor sildenafil in insulin resistant HUVEC, sildenafil was able to restore phosphorylation of Akt and eNOS, leading to an increase of NO secretion [168], which the authors suggests is possibly due to sildenafil-mediated increase of intracellular Ca^{2+} levels and attenuation of oxidative stress. Taken together with our findings that PDE-5 inhibition decreases JNK activation and secretion of endothelin-1 in cells stimulated by TNF-α in absence of insulin, one can speculate that tadalafil might have a positive effect on the insulin-signalling pathway leading to an increased NO production.

Here, tadalafil significantly decreased mRNA expression and secretion of endothelin-1 but had no effect on secretion of adhesion molecules. NO has previously been shown to decrease TNF-α stimulated VCAM through NFκB independently of cGMP in earlier studies [242], which could explain why tadalafil or the JNK inhibitor did not decrease secreted VCAM. In high-fat diet fed mice, PDE-5 inhibition using sildenafil downregulated ICAM [243]. Additionally, a JNK inhibitor followed a similar pattern as tadalafil in decreasing endothelin-1 in paper V, Thus, this finding supports that the effect of tadalafil on endothelin-1 is mediated by JNK. Additionally,

tadalafil decreased activation of c-Jun, further showing an involvement of JNK, since it is described to be the dominant kinase responsible for c-Jun phosphorylation after TNF-α stimulation [244]. Further, c-Jun has been shown to have a positive regulative role on endothelin-1 gene transcription in bovine endothelial cells [245].

Concluding the results from paper IV and V, tadalafil are more involved in maintaining vascular, metabolic and endothelial function, than in the inflammatory response. Tadalafil may thus be a potential treatment to maintain the balance between NO and endothelin-1.

5.3 Strengths and limitations of the study

In paper I and II, a limitation is the low number with CHD events and IGT/T2D, and the results need to be confirmed in a larger population. The lack of information regarding menopause in women, and NO bioavailability are also limitations. Strengths of the studies include the high participation rate, the unselected study population and the prospective design that allowed us to investigate the direction of the observed associations. Furthermore, the inclusion of participants aged 30-74 years gave us the opportunity to investigate the impact of endothelin-1 on incident CHD prospectively in a population less confounded by comorbidity.

In paper III and IV, the low number of participants is a limitation, which could lead to a risk of type II error, i.e. accepting a false null hypothesis. It could also be argued that a hyperinsulineamic clamp would lead to a more stable environment to study these hypothesizes, however, we wanted to have a more physiologically relevant situation, and therefore preferred an oral glucose ingestion, and a mixed meal, respectively. One of the strength in paper III is brief diabetes duration of the T2D participants, allowing us to detect early defects in the pathophysiology. Our inclusion criteria in both paper III and IV was different regarding age between the genders, this was to ascertain a postmenopausal state in women. In paper IV, there were more men than women included, which may confound the results. Further the randomization led to longer diabetes duration in the group receiving tadalafil, which could mask some of the positive effects of tadalafil.

6 SUMMARY AND CONCLUSION

The results in this thesis demonstrates that (Fig. 10):

- Circulating levels of endothelin-1 may predict incident CHD in women, an association that remained after adjustments for several possible confounders. This predictive value of endothelin-1 is not seen in men.
- Women in the highest endothelin-1 quartile have a four-fold higher odds ratio for developing IGT/T2D after 9.7 years compared with women in the lowest quartile.
- Delivery of insulin to subcutaneous adipose tissue and subcutaneous blood flow are both impaired after an oral glucose load in participants with T2D compared with lean controls.
- Acute administration of tadalafil provides positive metabolic and vascular effects in T2D in the postprandial state and postprandial increase of endothelin-1 displayed in the placebo group is not observed in the group receiving tadalafil
- Tadalafil exerts its effect in endothelial cells by decreasing activation of JNK through a maintained cGMP-PKG signal. In addition, treatment with tadalafil decreases gene expression and secretion of endothelin-1.

Thus, this study shows that endothelial dysfunction precedes IGT/T2D and CHD, and that endothelin-1 may pose as a risk factor for women. The impaired insulin delivery in subcutaneous adipose tissue in T2D suggests an involvement of the microcirculation, and an impaired transendothelial transport. Additionally, tadalafil may be a potential treatment for promoting microcirculation and peripheral glucose uptake in T2D. Intracellular effects in cultured endothelial cells suggest a mechanism by which tadalafil might restore the balance between NO and endothelin-1 in T2D patients who are characterized by microvascular insulin resistance.

7 FUTURE PERSPECTIVES

In this thesis we described noteworthy sex differences, which affect development of T2D and cardiovascular diseases. Women seem more susceptible to microvascular dysfunction. Future studies focusing on the dissimilarities between men and women in insulin resistance as well as in the response of treatment are needed. Specified treatment depending on sex could be an option in the future.

To further advance the results from this thesis, a chronic study investigating the effects of tadalafil is planned and approved by the Ethical Committee at the University of Gothenburg. Participants will receive either tadalafil 20 mg or placebo during 6 weeks and after a washout period switch treatment. At the end of each treatment period, microvascular effects of tadalafil in T2D will be evaluated using microdialysis for measuring insulin in adipose tissue, and glucose in skeletal muscle during a hyperinsulinemic euglycemic glucose clamp. Further, blood flow in adipose tissue and forearm will be measured. Microvascular endothelial cells will be isolated and investigated. Thus, the aim with this study is to investigate the effects of tadalafil on different aspects of insulin resistance.

It would be interesting to investigate the effects of tadalafil in cell culture after insulin stimulation, to investigate the effects of tadalafil on the insulin signalling pathways. This is to determine whether the positive effects seen in human studies could be due to an enhanced PI3K pathway, since tadalafil in the current setting decreases endothelin-1. The hypothesis thereby being that tadalafil could have positive effects on the NO/endothelin-1 balance.

Additionally, insulin uptake in microvascular endothelial cells isolated from adipose tissue is under investigation, and the effects of endothelin-1 on insulin uptake and signalling in these cells are planned. The results in paper III indicates the importance of an endothelial barrier with an impaired transendothelial transport of insulin, and future studies in our group will further focus on the underlying mechanisms of the endothelial barrier.

Thus, future studies are designed to further investigate the function of the microcirculation in the insulin resistant state (Fig. 10).

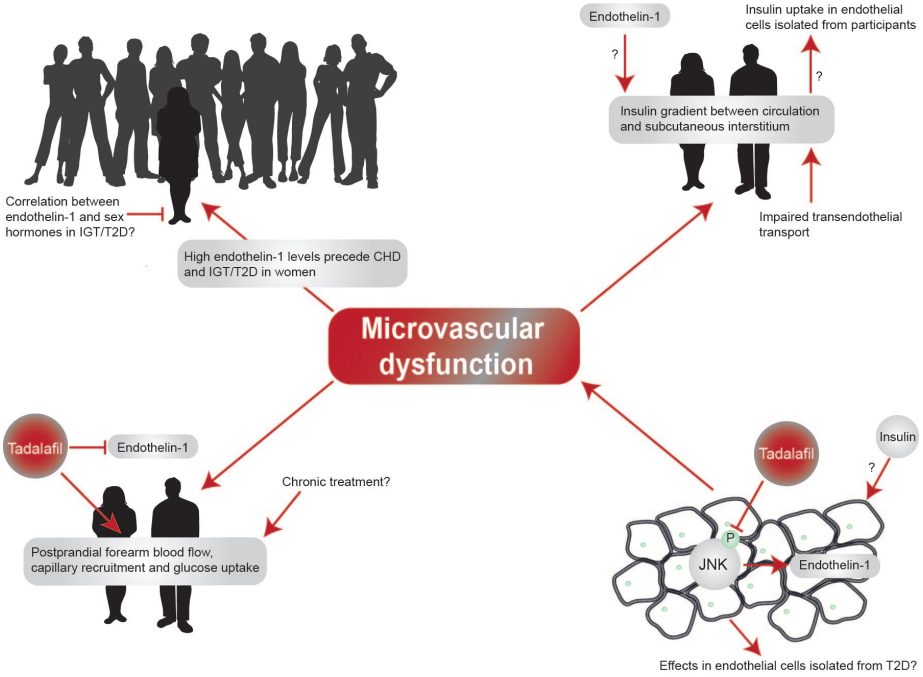

Figure 10. Overview of results and future perspectives of the studies included in this thesis. The mechanisms of microvascular function were approached in CHD and T2D by epidemiological, clinical and experimental studies. In paper I and II, endothelin-1 levels was investigated in the Vara-Skövde cohort, high endothelin-1 levels was shown to predict CHD and IGT/T2D in women but not in men. Thus, indicating a gender difference in the association between endothelial dysfunction and disease. Future studies in this cohort could further elucidate the association between sex hormones in IGT/T2D. In paper III the delivery of insulin to target tissues was investigated in T2D compared to lean controls in a clinical setting. This study showed a gradient of insulin between the subcutaneous interstitial fluid and the circulation in T2D, which possibly could be dependent on an impaired transendothelial transport of insulin. Further studies will explore the possible effects of endothelin-1 in these participants, as well as investigating insulin uptake in microvascular endothelial cells isolated from the subcutaneous adipose tissue. In paper IV and V the PDE-5 inhibitor tadalafil was investigated. Acute administration of tadalafil had positive effects on glucose uptake, blood flow and capillary recruitment in a clinical setting. A chronic study of tadalafils effects in T2D is in the first stages and will further elucidate these findings. In an experimental setting was tadalafil able to decrease phosphorylation of JNK, in addition to decreasing endothelin-1 secretion. In the future, it would be interesting to investigate the effects of tadalafil in the presence of insulin, and to study tadalafil effects in endothelial cells isolated from T2D.

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