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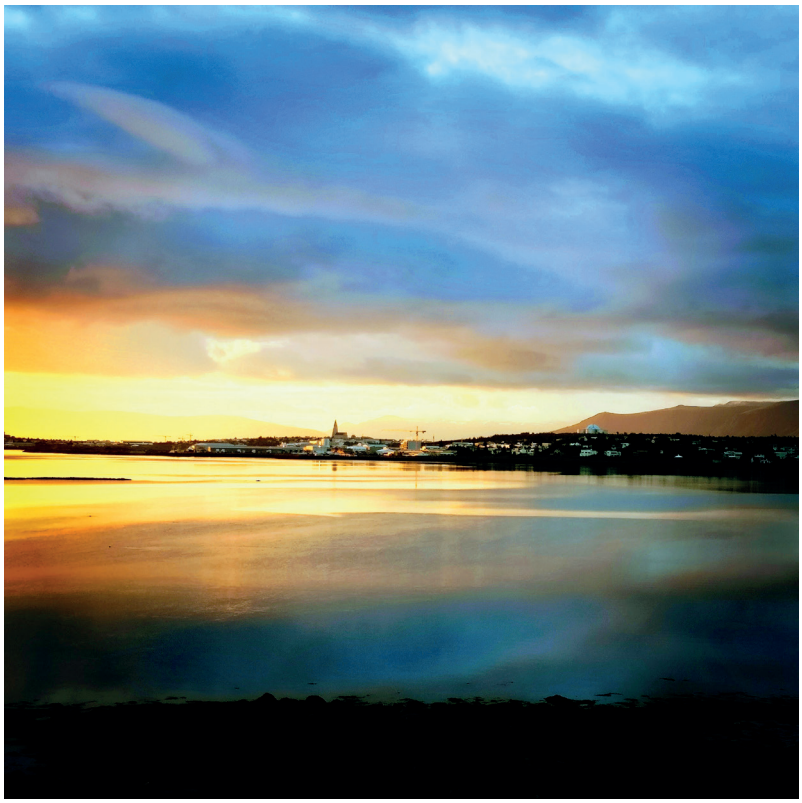

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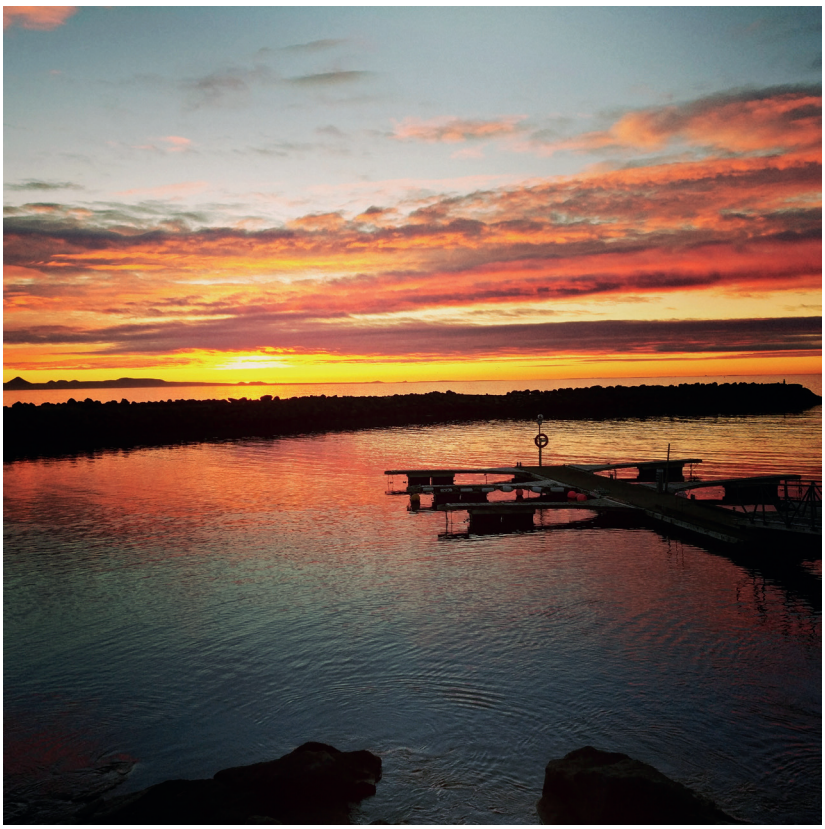

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ENDOTHELIAL DYSFUNCTION IN ATHEROSCLEROSIS AND TYPE 2 DIABETES: CLINICAL AND MOLECULAR STUDIES OF THE ROLE OF ENDOTHELIN AND ARGINASE

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ENDOTHELIAL DYSFUNCTION IN ATHEROSCLEROSIS AND TYPE 2 DIABETES: CLINICAL AND MOLECULAR STUDIES OF THE ROLE OF ENDOTHELIN AND ARGINASE

By

Arnar Rafnsson

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CONTENTS

ABSTRACT

Background
Atherosclerosis is a modern world scourge and the number of people worldwide diagnosed with type 2 diabetes (T2D) is on the rise, causing much morbidity and mortality. Endothelial dysfunction (ED) is an early sign of atherosclerosis and contributes to vascular complications in T2D. Endothelin-1 (ET-1) and arginase are two potent inhibitors of endothelium-derived nitric oxide (NO) and are known to cause ED. Short term studies have shown that blocking ET-1 can improve ED in T2D but no longer term studies have been performed. In addition, it remains unclear which ET receptor subtypes are beneficial to block.

Arginase is an enzyme that causes ED and oxidative stress by metabolizing the NO substrate L-arginine. The role of arginase in human atherosclerosis and its regulation by ET-1 is unknown. The aim of the project was to investigate the role of ET-1 for vascular dysfunction in atherosclerosis and T2D, to evaluate the efficacy of ET receptor blockade to improve endothelial function as well as investigating the possible interaction between ET-1 and arginase in atherosclerosis.

Materials and methods
In study I we investigated whether treatment with bosentan, an oral ET_A/ET_B receptor blocker could improve endothelial function in patients with T2D and microvascular complications. Endothelial function was tested with Endo-PAT (peripheral artery tonometry) and FMD (flow mediated vasodilatation).

In study II, the effect of selective ET_A receptor blockade vs. combined ET_A/ET_B receptor blockade on endothelial function was investigated in patients with coronary artery disease (CAD) and T2D.

In study III we investigated the extent of endothelial injury by measuring the number of endothelial progenitor cells (EPC) in the patients of Study I. The method used to isolate the EPC was flow cytometry by staining for cells positive for CD133, CD34 and KDR.

In study IV the biobank of carotid endarterectomies (BiKE) was used to determine the gene and protein expression of ET-1 and arginase in the atherosclerotic plaque, and to investigate the functional interaction between ET-1 and arginase activity in endothelial cells and macrophages.

Results
Study I demonstrated that 4 weeks oral treatment with the dual ET_A/ET_B receptor blocker bosentan improved peripheral microvascular endothelial function in patients with T2D and microalbuminuria.

In study II it was demonstrated that both selective ET_A and dual ET_A/ET_B receptor blockade markedly improved endothelial function in patients with CAD and T2D. However, the addition of ET_B receptor blockade did not improve endothelial function further.

Among patients with T2D and vascular disease, high plasma levels of ET-1 were associated with a higher number of EPC. The recruitment of EPC did not seem to be regulated via ET receptor activation since treatment with a dual ET_A/ET_B receptor blockade did not affect circulating EPC numbers.

Study IV demonstrated co-localization of ET-1 and arginase in human atherosclerotic plaques. ET-1 stimulated arginase expression and activity in endothelial cells as well as formation of reactive oxygen species in macrophages via an arginase-dependent mechanism.

Conclusion
Longer term treatment with a dual ET_A/ET_B receptor blocker improves endothelial function which may indicate a role in the treatment of microvascular complications of T2D. Both selective ET_A and dual ET_A/ET_B receptor blockade improved endothelial function in a first head to head comparison in this patient category.

Important interactions exist between the ET-1 pathway and arginase in human atherosclerotic plaques. Our data suggest that a part of the effect of ET-1 on NO and ROS production is through upregulation of arginase.

SAMMANFATTNING

Bakgrund

Atherosklerossjukdom och komplikationer till den utgör de vanligaste orsakerna till död och antalet människor med typ 2 diabetes (T2D) ökar ständigt och orsakar en enorm börda av mortalitet och lidande. Ett förstadium till ateroskleros är endoteldysfunktion (ED) som bidrar till de vaskulära komplikationer som orsakas av T2D. Endothelin-1 (ET-1) och arginas är två kraftfulla hämmare av produktionen av kväveoxid (NO) i endotelet och bidrar därigenom till ED. Korttidsstudier har påvisat att blockad av ET-1 förbättrar ED hos patienter med T2D men långtidsstudier saknas och det är fortfarande oklart vilka av ET-1 receptorerna som ska hämmas. Arginas är ett enzym som bidrar till ED och oxidativ stress genom att metabolisera L-arginin, substratet till NO-produktion. Betydelsen av arginas vid ateroskleros och dess reglering av ET-1 är okända. Syftet med projektet var att undersöka vilken betydelse ET-1 har i uppkomsten av vaskulär dysfunktion i ateroskleros och T2D samt att utvärdera vilken typ av ET-1 receptorblockad som har bäst effekt på endotelfunktionen. Dessutom avsågs att belysa sambandet mellan arginas och ET-1 vid arterioskleros.

Material och metoder

I delarbete I undersöktes om den oselektiva ET_A/ET_B-receptorblockeraren bosentan förbättrade endotelfunktionen hos patienter med T2D och mikrovaskulära komplikationer. Endotelfunktionen testades med hjälp av Endo-PAT (peripheral artery tonometry) och FMD (flow-mediated vasodilatation).

I delarbete II jämfördes effekten av selektiv ET_A-receptorblockad eller kombinerad ET_A/ET_B-receptorblockad på endotelfunktion hos patienter med koronarsjukdom (CAD) och T2D.

I delarbete III studerades antalet endoteliala progenitorceller (EPC) som en surrogatmarkör för kärlskada i studiepatienterna från delarbete I med flödescytometri.

I delarbete IV bestämdes gen- och proteinuttryck av ET-1 och arginas i aterosklerotiska plack från halspulsådern i Biobank of Karolinska Endarterectomies (BiKE). Dessutom undersöktes funktionella interaktionen mellan ET-1 och arginas i endotelceller och makrofager.

Resultat

Delarbete I demonstrerade att fyra veckors behandling med den kombinerade ET_A/ET_B-receptorblockeraren bosentan förbättrade den mikrovaskulära endotelfunktionen hos patienter med T2D och mikroalbuminuri. Däremot förändrades inte endotelfunktionen i ledningsärtär (FMD). I delarbete II visades att både selektiv ET_A- och kombinerad ET_A/ET_B-receptorblockad markant förbättrade endotelfunktionen hos patienter med koronarsjukdom och T2D. Tillägg av ET_B receptorblockad hade inte någon ytterligare effekt jämfört med selektiv ET_A-receptorblockad.

Delarbete III visade att hos patienter med T2D och manifest kärlsjukdom, korrelerade höga plasmahalter av ET-1 till ökat antal EPC. Rekryteringen av EPC verkar inte vara styrd av ET-1 receptorer eftersom behandling med kombinerad ET_A/ET_B-receptorblockad inte påverkade antalet cirkulerande EPC.

Delarbete IV demonstrerade att ET-1 och arginas är samlokaliserade i endotelceller och makrofager i humana aterosklerotiska plack. ET-1 stimulerade arginasexpression och aktivitet i endotelceller samt ökar produktionen av fria syreradikaler i makrofager genom en arginasberoende mekanism.

Konklusion

Fyra veckors behandling med kombinerad ET-1 receptorblockerare förbättrar endotelfunktionen och kan eventuellt ha en roll i långtidsbehandling av mikrovaskulära komplikationer vid T2D. Både selektiv ET_A- och kombinerad ET_A/ET_B-receptorblockad förbättrade endotelfunktionen markant i en första jämförande studie. Det finns en viktig funktionell interaktion mellan ET-1 och arginas i ateroskleros vilket kan tyda på ET-1 utövar sin effekt på NO och oxidativ stress via aktivering av arginas.

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II. **A Rafnsson**, A Shemyakin, J Pernow. Selective endothelin ET_A and dual ET_A/ET_B receptor blockade improve endothelium-dependent vasodilatation in patients with type 2 diabetes and coronary artery disease. Life Sci, 2014:**118**(2):435-9.

III. C Jung, **A Rafnsson**, K Brismar, J Pernow. Endothelial progenitor cells in relation to endothelin-1 and endothelin receptor blockade: A randomized, controlled trial. Int J Cardiol, 2013:**168**(2):1017-22.

IV. **A Rafnsson**, L Perisic Matic, J Yang, M Lengquist, A Mahdi, A Shemyakin, G Paulsson-Berne, G K Hansson, A Gabrielsen, U Hedin, J Pernow. Endothelin-1 is co-expressed with arginase in human atherosclerotic plaques and increases arginase activity in endothelial cells and macrophages. Manuscript.

LIST OF ABBREVIATIONS

ABH	(S)-amino-6-boronohexanoic acid
ADC	Arginine decarboxylase
AGEs	Advanced glycosylated end-products
AngII	Angiotensin II
ANOVA	Analysis of variance
ARB	Angiotensin receptor blockers
ARG	Arginase
ASL	Argininosuccinate lyase
ASS	Argininosuccinate synthetase
BH4	Tetrahydrobiopterin
BiKE	Biobank of Karolinska Endarterectomies
BMI	Body mass index
cDNA	Complementary deoxyribonucleic acid
CMH	1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine
CP•	3-carboxy-proxyl
CRP	C-reactive protein
ECE	Endothelin converting enzyme
ED	Endothelial dysfunction
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EPC	Endothelial progenitor cell
ESR	Electron spin resonance
ET-1	Endothelin-1
ETA	Endothelin receptor A
ETB	Endothelin receptor B
FITC	Fluoresceinisothiocyanate
FMD	Flow mediated dilatation
GCSF	Granulocyte colony-stimulating factor
GFR	Glomerular filtration rate
Hb1Ac	Glycosylated hemoglobin
ICAM-1	Intercellular Adhesion Molecule 1
IGF-1	Insulin-like growth factor-1
IGFBP-1	IGF-1 binding protein
KDR	Kinase insert domain receptor
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
mRNA	Messenger ribonucleic acid
MS	Minor stroke
NADPHox	Nicotinamide adenine dinucleotide phosphate oxidase
NO	Nitric oxide
nor-NOHA	Nω-hydroxy-nor-L-arginine
NOS	Nitric oxide synthase
OAT	Ornithine aminotransferase
ODC	Ornithine decarboxylase
ONOO−	Peroxynitrite
PAT	Peripheral arterial tonometry
PBMC	Peripheral blood monocytes
PCR	Polymerase chain reaction
PE	Phycoerythrin
PU	Perfusion units
RAGEs	Receptor for advanced glycosylated end-products
RHI	Reactive hyperemia index
RIA	Radioimmunoassay
ROS	Reactive oxygen species
RPLPO	Large ribosomal protein
SDF-1	Stromal-derived factor-1
SMA	Alpha-smooth muscle cell actin
SNP	Sodium nitroprusside
T2D	Type 2 diabetes
TcPO2	Transcutaneous oxygen tension
THP-1	Human monocytic cell line
TIA	Transient ischemic attack
VEGFR-2	Vascular endothelial growth factor receptor 2
VSMC	Vascular smooth muscle cell.
vWF	von Willebrand factor

INTRODUCTION

Development and clinical consequences of atherosclerosis

Cardiovascular disease due to atherosclerosis is a growing clinical problem throughout the entire world. It has an estimated death toll of about 18 million people per year [1]. The number of non-fatal cardiovascular events is at least 2-3 times as high. Atherosclerotic disease is caused by a constellation of risk factors including hyperlipidemia, hypertension, smoking, age and diabetes [2]. These risk factors result in the development of endothelial dysfunction which is thought to precede the formation of the atherosclerotic plaque.

The pathophysiology begins with the formation of fatty streaks in the subendothelial layer, a process dominated by an influx of macrophages attempting to clear out oxidized LDL particles in the intimal layer which results in formation of a lipid core [3]. Representatives of the innate immune system such as macrophages, dendritic cells and monocytes as well as the adaptive immune system such as T cells are present in the atherosclerotic core. The proliferation and migration of vascular smooth muscle cells leads to the formation of a fibrous cap over the core. In addition, both pro- and anti-inflammatory cell types are located within the plaque, leading to chronic inflammation which is the prerequisite condition for the plaque to develop (Figure 1).

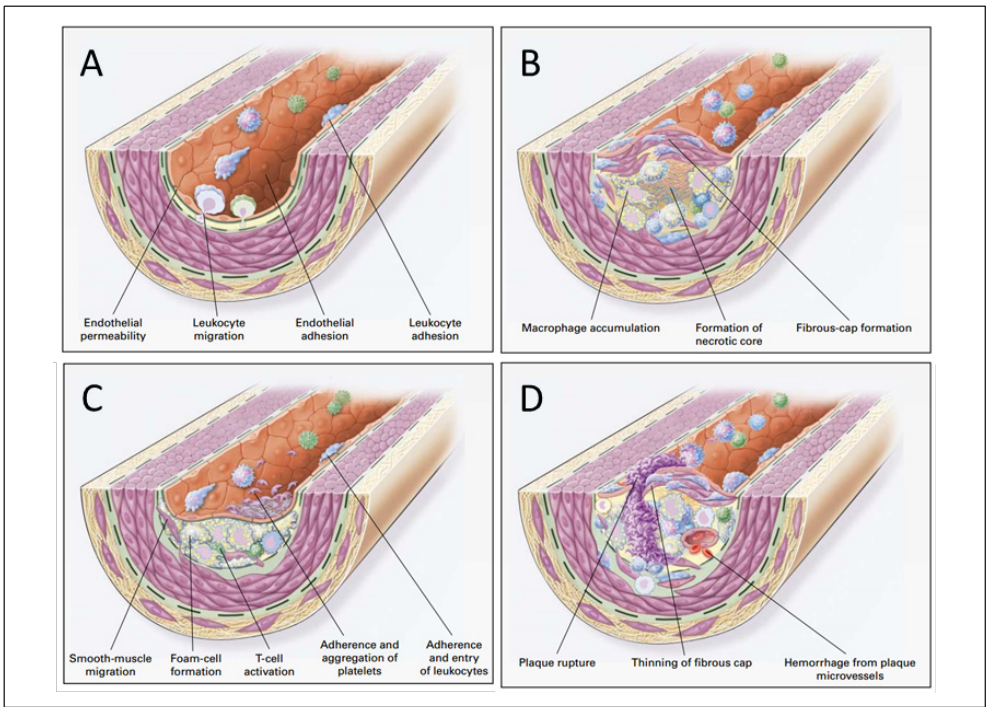

Figure 1. A) Endothelial dysfunction, B) Fatty streak formation, C) Development of an advanced complicated atherosclerotic lesion, D) Unstable fibrous plaque in atherosclerosis. Reproduced with permission from (scientific reference citation), Copyright Massachusetts Medical Society.

Later stages involve calcification of the fibrous cap and cell necrosis in the atherosclerotic core. This leads to a progressively decreasing lumen of the artery with the development of symptoms like angina pectoris and intermittent claudication of the extremities, depending on the anatomical location of the plaque. In some cases the plaque is "vulnerable" with a thin fibrous cap prone to rupture with increased risk for subsequent sudden thrombosis and acute occlusion of the artery leading to myocardial infarction or stroke [2]. The exact cause of the vulnerability is not fully clear, although inflammation is thought to be predominant, perhaps steering the cellular processes towards scar tissue breakdown and weakening of the fibrous cap [4].

The developmental process of atherosclerosis is thought to take years or decades with evidence of early plaque formation in young adults and even fatty streaks present in children. The main symptoms of an acute occlusion depend on the location of the afflicted artery. Occlusion of a coronary artery results in myocardial ischemia and myocyte necrosis referred to as myocardial infarction, the main symptoms of which are chest pain, difficulty breathing, nausea and sometimes vomiting. The clinical condition needs urgent treatment with anti-platelet therapy and percutaneous coronary intervention with balloon angioplasty to restore blood flow to the ischemic myocardium. A serious complication of coronary occlusion may well be ventricular fibrillation which results in sudden cardiac death.

If the carotid artery is afflicted with thrombosis and embolisation to the brain, the result is an ischemic stroke with almost instant neurologic deficits such as loss of movement/motor function, loss of sensation or loss of speech.

Owing to the vast world-wide burden there is still reason to delve into the finer details of the atherosclerotic disease process to work out the possible mechanisms and try to prevent or stabilize the vulnerable plaque that is the cause of acute cardiovascular events.

Type 2 diabetes

The prevalence of T2D is an enormous global burden with a doubling of cases since 1980. In year 2014, more than 9% of people over the age of 18 were estimated to have T2D [5]. Diabetes mellitus is an endocrine disorder characterised by chronically increased levels of blood glucose which initiates pathologic processes that have damaging effects on various organs [6], most commonly afflicted are the eyes, heart, kidneys as well as the peripheral vascular system. T2D is characterized by reduced sensitivity to insulin while type 1 diabetes is defined by the inability to produce insulin in the endocrine part of the pancreas. T2D is known to be a strong risk factor for atherosclerotic disease with a 2- to 3-fold increased risk of myocardial infarction compared to individuals without diabetes. This risk is equivalent to the risk of non-diabetic individuals with a prior coronary event [7]. In addition to an increased risk of macrovascular complications like myocardial infarction and stroke, the condition also causes microvascular complications including nephropathy, retinopathy and non-healing foot ulcers [8].

The molecular mechanism by which hyperglycemia causes endothelial dysfunction (ED) as an early event in the atherosclerotic process is incompletely understood. However, T2D is often accompanied by other cardiovascular risk factors such as hypertension, hyperlipidemia and obesity which all have a negative effect on endothelial function [9]. Recent work

indicates advanced glycosylated end-products (AGEs) as being central to the damage caused by hyperglycemia. AGEs are a heterogenous group of proteins, lipids and nucleic acids that have undergone an irreversible glycation and oxidation [10]. Elevated levels of AGEs are thought to support the formation of reactive oxygen and nitrogen species, further inducing formation of AGEs. In high enough numbers they induce oxidative stress in the mitochondria through the receptor for AGEs (RAGE). This then causes mitochondrial dysfunction resulting in cell death. These receptors are widely distributed throughout the body as well as being present on macrophages. In the macrophage they lead to internalisation of AGEs and subsequent disposal.

The treatment of T2D is multifactorial. Lifestyle management with physical exercise and weight loss together with glucose lowering medication, lipid lowering and antihypertensive therapy are needed. Glucose lowering drugs have historically shown little effect on cardiovascular complications, although recent additions have had more success [11]. Better results have been obtained with statin therapy and drugs lowering blood pressure but still diabetic complications cause a staggering amount of morbidity and mortality [12]. In light of this there is a need to develop therapies specifically targeting both the micro- and macrovascular complications caused by T2D.

The endothelium

The human artery consists of three distinct layers: Tunica intima, tunica media and tunica adventitia (Figure 2). Tunica adventitia is the outermost layer and is mostly made from resilient connective tissue that maintains structural integrity of the vessel as well as nerve endings and small vessels providing nutrients for the artery itself. The tunica media is the muscular layer with smooth muscle cells that maintain the vascular tone and modulate blood flow depending on the need of recipient organs. The tunica intima is composed of loose connective tissue and most importantly the endothelium, the layer of endothelial cells that line the inner wall of the artery and are the only cell type that is in contact with blood under normal circumstances [13].

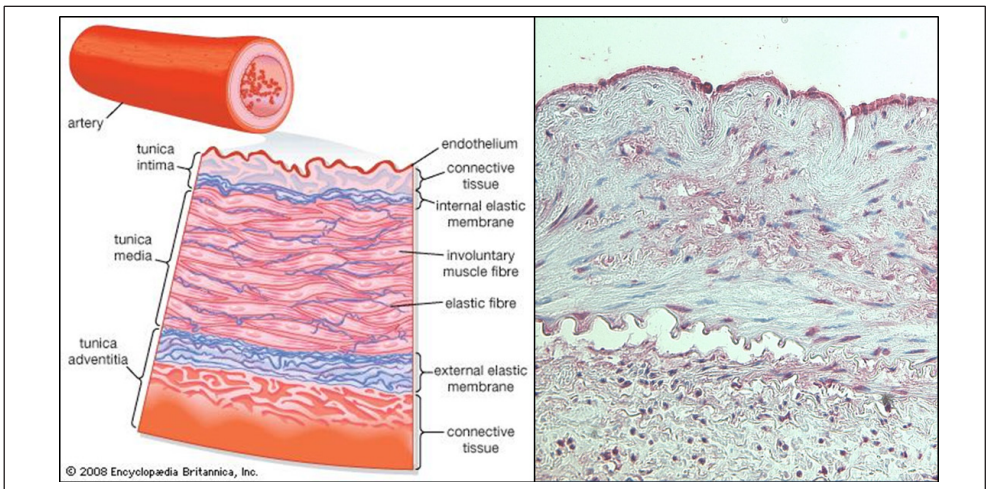

Figure 2. Anatomy of the artery on the left hand side, histological section on the right hand side, showing the endothelial layer stained in red towards the lumen. Copyright Encyclopaedia Britannica, Inc.

Nitric oxide in endothelial function and dysfunction

Endothelial dysfunction is characterized by reduced bioavailability of NO. Available data suggests that it is an important step in the pathogenesis of atherosclerosis and diabetic angiopathy due to increased vascular tone, vascular inflammation and oxidative stress [14-16]. Initially thought to be an inert cell layer, evidence shows that the endothelium acts as a transducer and integrator of both humoral and mechanical stimuli [17]. It responds to these stimuli by producing different factors that affect nearby cells as well as the endothelial cells themselves thereby maintaining the important vascular homeostasis (Figure 3). Nitric oxide (NO), discovered in 1980 by Furchgott, Zawadzki and Ignarro [18, 19], is the single most important molecule for the healthy homeostasis and reactivity of the vessel. NO is produced in the endothelial cells by the enzyme endothelial nitric oxide synthase (eNOS). Its production is dependent on the cofactor tetrahydrobiopterin (BH4) and the availability of L-arginine. NO activates guanylyl cyclase in the smooth muscle cells in the vessel wall, increasing the formation of cyclic guanosine monophosphate leading to vasodilatation.

NO is not only vasodilatory but also inhibits thrombocyte aggregation, smooth muscle proliferation and leucocyte chemotaxis [16]. It directly interferes with the rolling and adhesion of monocytes that would otherwise become macrophages. Its production and release is stimulated by a number of physiological stimuli, for example physiological flow forces (shear stress), acetylcholine, bradykinin, serotonin, adenosine diphosphate and histamine to name a few.

The delicate balance of a normally functioning endothelial layer can be disrupted by various factors. The classical risk factors of atherosclerosis like smoking, hypertension, diabetes mellitus and hyperlipidemia can disrupt the homeostasis, resulting in an activated/dysfunctional endothelium [20]. On a cellular level the risk factors decrease the production and bioavailability of NO. This decreased bioavailability of NO leads to vasoconstriction, increased extravasation of leukocytes and other cells of the immune system as well as oxidized LDL particles initiating the formation of an atherosclerotic plaque. Reducing these risk factors for endothelial function diminishes the risk of clinical events of atherosclerosis.

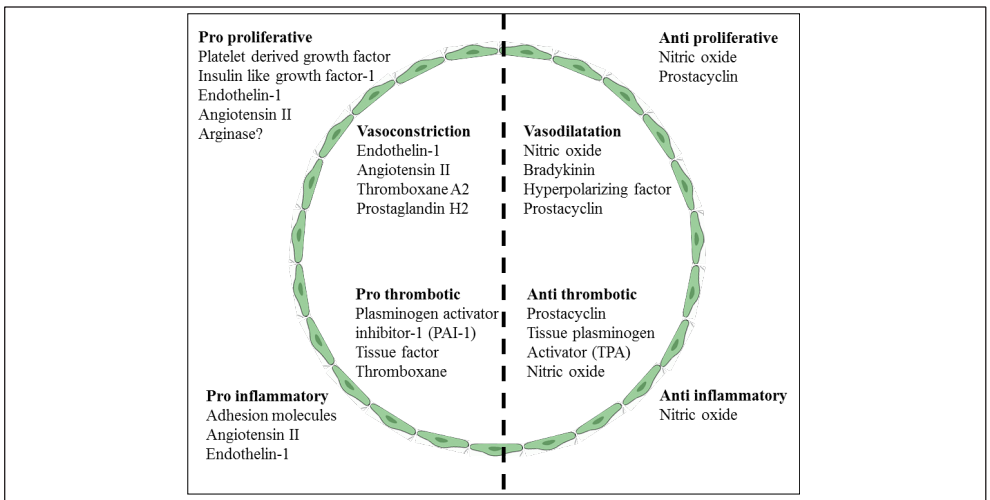

Figure 3. Endothelial layer functions.

Superoxide scavenges the NO molecule leading to decreased bioavailability. Endothelin-1 (ET-1) and angiotensin II, both potent vasoconstrictors and increased by several risk factors, increase the formation of superoxide by activating NADPH oxidase [21]. Another possible source of superoxide is eNOS itself, the enzyme that under normal circumstances produces NO. This reversal in the function of eNOS happens when levels of the cofactor BH4 is low by a mechanism referred to as uncoupling [22]. Also caused by the risk factors mentioned, is an increased activity of arginase, which causes a depletion of L-arginine, the substrate needed to produce NO, yet another cause of decreased bioavailability of NO.

The role of ET-1 in endothelial dysfunction and atherosclerosis

One of the factors, potentially contributing to the development of endothelial dysfunction in atherosclerosis is overabundance of endothelin, the 21 amino acid peptide. Endothelin is a potent vasoconstrictor initially described by Yanagisawa in 1988 [23]. It has three known isoforms, ET-1, ET-2 and ET-3, of which ET-1 is the most abundant and relevant in the context of atherosclerosis [24]. ET-1 is primarily produced by endothelial cells but also in several other cell types in the cardiovascular system. It exerts its effects by activating two pharmacologically distinct, G protein-coupled receptors, ET_A and ET_B. Both receptors are present on vascular smooth muscle cells mediating vasoconstriction, while the ET_B receptor is also present on endothelial cells mediating vasodilatation by releasing NO or prostacyclin (Figure 4). ET-1 exerts its effects mostly in a paracrine fashion being secreted from endothelial cells towards the smooth muscle cells of the arterial media [25].

Several studies have established that ET-1 has a role in atherosclerosis. ET-1 expression has been shown to be increased in animal models of atherosclerotic disease [26, 27].

In patients with atherosclerotic disease, administration of the precursor big ET-1 resulted in more pronounced forearm vasoconstriction than healthy controls indicating increased production of ET-1 [28].

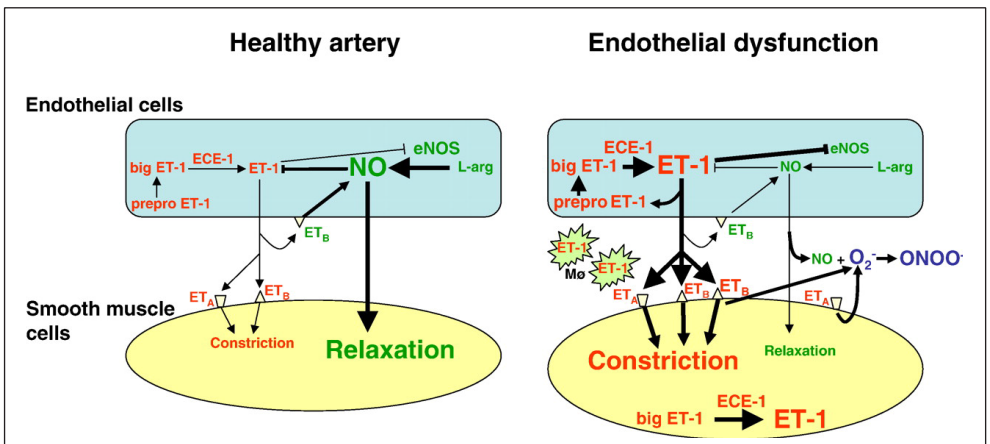

Figure 4. Balance of NO and ET-1 in endothelial and smooth muscle cells. ECE-1=endothelin converting enzyme-1, ET-1= endothelin 1, ET_A=endothelin receptor A, ET_B= endothelin receptor B, eNOS= endothelial nitric oxide synthase, NO= nitric oxide; $ONOO^-$=peroxynitrite, MØ=macrophage. Copyright © 2007, Oxford University Press.

It is not only that ET-1 is increased as a result of risk factors and early atherosclerosis but the relative expression of the receptors is also altered. According to an earlier study, expression of the ET_B receptor in macrophages and smooth muscle cells was increased in atherosclerotic lesions [29]. In an experimental study in patients with atherosclerosis, the vasoconstrictive response to ET-1 was not different from that of controls but an ET_B receptor agonist (sarafotoxin S6c) caused significantly more reduction in forearm blood flow in the patients with atherosclerosis, indicating an upregulation of the ET_B receptor [30]. However, in a mouse model of atherosclerosis there was a 30% reduction in atherosclerotic plaques in the group that received a selective ET_A receptor blocker [31] but comparison with a dual ET_A/ET_B receptor blocker was regrettably not performed. Collectively, the existing evidence points towards a central role of ET-1 in atherosclerotic disease. ET-1 is upregulated in patients with risk factors and functional studies show increased response in patients with atherosclerosis and its risk factors compared to healthy controls [32-34].

The role of ET-1 in type 2 diabetes

The exact pathophysiologic mechanisms behind vascular complications of T2D are not fully understood. Most likely a number of different factors contribute to the vascular damage. Evidence points towards an enhanced role of ET-1 in this respect.

Elevated plasma levels of ET-1 are found in patients with T2D [35]. Also, increased ET-1 correlates to impaired glucose uptake, HbA1c levels and the manifestation of microalbuminuria and retinopathy [36-38]. In addition, there seems to be an increase in the expression of ET-1 and both receptors in various animal models of diabetes [39, 40]. Experimental studies have shown acute improvement in endothelium-dependent vasodilatation with short-term infusions using ET receptor antagonists [41]. In another study, the improvement in endothelium-dependent vasodilatation in insulin-resistant subjects was achieved using dual ET_A/ET_B receptor blockade whereas no effect was observed following selective ET_A receptor blockade. This indicates that blocking the ET_B receptor may be important to achieve therapeutic vascular effects [42] again implying an enhanced role of the ET_B receptor in manifest endothelial dysfunction. To study the effect of ET-1 regulation on microvascular function in T2D patients, the nutritive capillaries in the nail folds were studied. An increase was seen in baseline and peak capillary blood velocity after infusion of the ET_A receptor blocker BQ123, whereas no change was seen in controls [43]. These observations indicate that short-term administration of ET receptor blockers acutely improves micro- and macrovascular function and may thus be of therapeutic value for patients with T2D. Data regarding the effect of long term oral administration of ET receptor blockers on endothelial function are limited. One study demonstrated that 6 months treatment with the oral ET_A receptor blocker atrasentan improved coronary endothelial function in patients with atherosclerosis [44]. However, only 2 out of 25 patients included in the active treatment group of that study had diabetes. Thus, the effects of oral administration of ET receptor antagonists on patients with T2D with manifest vascular complications has not been evaluated.

Arginase in atherosclerosis and type 2 diabetes

Arginase is an intracellular enzyme whose activity directly affects NO availability and therefore also endothelial function. It is present in two forms; Arginase 1 (Arg1) and Arginase

2 (Arg2). Arg1 is primarily found in the liver but is also expressed in other tissues like the myocardium, endothelial cells and smooth muscle cells (Figure 5). It is a cytosolic enzyme and an important part of the urea cycle in the liver removing excess nitrogen formed during amino acid and nucleotide metabolism. Arginase 2 (Arg2) is a mitochondrial enzyme and is mostly located in extrahepatic tissues, brain, kidney, small intestine, and immune cells as well as in the endothelial cells of the vasculature [45].

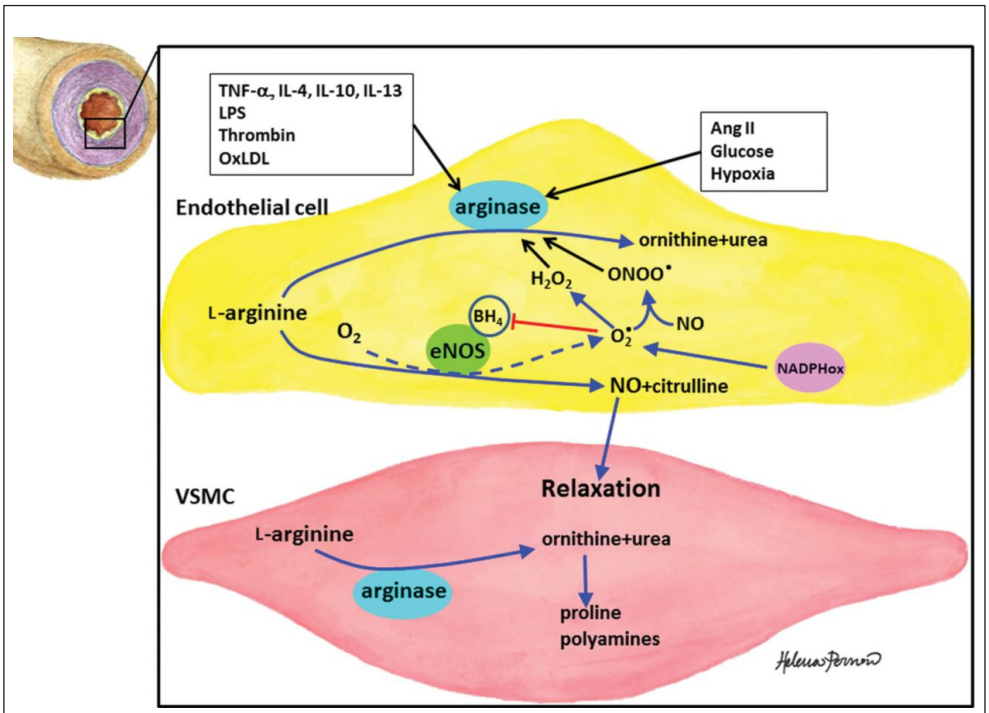

Figure 5. Schematic illustration of the action of arginase in the regulation of NO bioavailability and vascular function. Arginase is expressed in endothelial and vascular smooth muscle cells via regulation of cytokines, thrombin, hypoxia, reactive oxygen species, hyperglycaemia, and oxidized LDL. Increased activity of arginase will via hydrolysis of l-arginine to ornithine and urea reduce the availability of l-arginine for NO synthase (NOS), thereby reducing the production of NO. Lack of l-arginine will also result in 'uncoupling' of NOS whereby the enzyme produces superoxide instead of NO. Generation of superoxide by uncoupled eNOS and NADPH oxidase and peroxynitrite from superoxide and NO will further increase arginase activity and impair NO production via oxidation of tetrahydrobiopterin. Collectively, these changes will reduce the bioavailability of NO and contribute to endothelial dysfunction. In vascular smooth muscle cells, ornithine will increase formation of l-proline and polyamines which stimulate cell proliferation. Ang II= angiotensin II; BH_4=tetrahydrobiopterin; LDL=low-density lipoprotein; LPS=lipopolysaccharide; NADPHox=nicotinamide adenine dinucleotide phosphate oxidase; NO= nitric oxide; $ONOO^-$=peroxynitrite; VSMC=vascular smooth muscle cell. Copyright by Oxford University Press.

The role of arginase is thought to be an important key to L-arginine and NO homeostasis as well as the production of L-ornithine, a precursor to polyamines, necessary for cell proliferation. Arginase has also been shown to be a rate limiting step to this function in endothelial cells as well as being important in the production of proline, a building block for collagen [46, 47]. These studies imply not only the importance of arginase for endothelial cell proliferation but also its role in collagen synthesis, both vital for repairing the endothelial layer.

Increased arginase activity leads to decreased bioavailability of NO both through competing with eNOS for L-arginine, which is the substrate for NO production; and by uncoupling eNOS, leading to increased generation of reactive oxygen species (ROS) [48]. Arginase expression is stimulated by a number of proinflammatory factors like lipopolysaccharide, tumor necrosis factor-α and interferon-γ [47, 49] as well as oxidized LDL [50], glucose [51], reactive oxygen and nitrogen species [52] and hypoxia [53].

Arginase has been shown to be upregulated in atherosclerotic animal models [54, 55]. In a study of aged mice, blocking Arg2 led to increased NO production and decreased ROS production. It has also been shown that increased arginase activity is associated with endothelial dysfunction [56] and that overexpression of endothelial Arg2 induces endothelial dysfunction, hypertension and enhances atherosclerosis in mice [57]. Deleting Arg2 from endothelial cells in hypercholesterolemic mice reduced the formation of atherosclerotic lesions [58]. Endothelial arginase activity is elevated in conditions like hypertension and ischemia-reperfusion. This indicates a role in maintaining endothelial dysfunction in these states [59-61]. Furthermore, experiments with arginase inhibition have been shown to reduce aortic remodeling in chronically hypertensive rats probably inhibiting arginase-driven proliferation of smooth muscle cells and collagen synthesis [45]. This evidence implies an important role of arginase in the context of endothelial dysfunction and atherosclerosis.

Arginase expression is increased with elevated levels of glucose and its activity is increased in patients with T2D [62]. Arginase inhibition leads to improved endothelial function in patients with T2D and coronary artery disease (CAD) [63]. Pro-inflammatory cytokines increase arginase expression and activity in endothelial cells, whereas the anti-inflammatory cytokines IL-4, IL-10 and IL-13 increase arginase expression and activity in macrophages [64], mostly because of increased expression of Arg1 while Arg2 remains unchanged during stimulation [65]. This is in contrast to many animal models of atherosclerosis which indicate increased arginase activity with increased atherosclerotic burden [66].

Having established the role of arginase in both endothelial dysfunction and diabetes there is a need to investigate further its role in advanced human atherosclerosis. As a first step in this pursuit we decided to determine the expression of arginase in human atherosclerotic plaques and the functional interactions between the proinflammatory effect of ET-1 and arginase.

Determination of endothelial function

Endothelial function can be measured by observing the vascular response to shear stress, as the dilatation/constriction is dependent on the bioavailability of NO. Various methods can be employed for this purpose [67]. An early method involves infusing acetylcholine directly into the coronary arteries and measuring the dilation/constriction. Although measuring endothelial function directly in a clinically important vascular bed would be ideal, the invasiveness makes this a relatively impractical approach.

Peripheral measurements of endothelial function, such as flow mediated vasodilatation (FMD), have become more popular due to their non-invasive nature and established correlation to coronary endothelial function [68]. In addition studies indicate that FMD provides a meaningful addition to clinical risk factors in the evaluation of individual prognosis [69, 70]. The method involves the use of ultrasonography to visualize and measure the lumen of the brachial artery. Using a blood pressure cuff around the forearm, the blood flow is stopped by increasing the pressure above that of the systolic blood pressure and after five minutes the flow is reinstated by deflating the cuff. This leads to a post-ischemic hyperemia. The resulting increase in shear stress leads to the release of calmodulin from the inhibitor caveolin. Calmodulin then activates eNOS which produces NO from L-arginin. NO diffuses to smooth muscle cells activating guanylate cyclase, thereby increasing intracellular cyclic guanosine monophosphate which causes relaxation in the smooth muscle cells, resulting in dilation of the artery. The measured dilatation reflects the bioavailability of NO. FMD is technically challenging and requires extensive training and standardization.

Another method of measuring peripheral endothelial function is peripheral artery tonometry (PAT). PAT measures digital pulse amplitude in the fingertip through a device called Endo-PAT (Itamar Medical, Caesarea, Israel) [71]. The device covers the tip of the index finger and uses a pneumatic plethysmograph that applies a uniform pressure to the surface of the finger, allowing measurement of pulse volume changes. The PAT signal is recorded at baseline and following 5 min arterial occlusion using an inflatable cuff while the other arm serves as a control. Although relying on the same phenomenon, post-ischemic reactive hyperemia, this method studies endothelial function in the small arterioles of the fingertip whereas FMD studies the conduit brachial artery. Impairment in peripheral endothelial function measured with Endo-PAT correlates with coronary microvascular function in patients with atherosclerosis [72]. These measurements can predict cardiovascular events beyond traditional risk factors in specific subsets of patients [73].

Venous occlusion plethysmography of the forearm is yet another method of investigating peripheral endothelial function. Using blood pressure cuffs to repeatedly stop and return blood flow the sensitive mercury-in-silastic strain-gauge measures changes in forearm circumference. The setup allows for the administration of vasoactive drugs and the response measured as a change in forearm circumference owing to increased blood flow. The other arm can be used as control because the doses used do not have a systemic effect. Acetylcholine and serotonin are usually used to stimulate endothelium-dependent vasodilatation. They cause an increase in intracellular calcium levels that in turn release calmodulin from caveolin and a resulting increase in NO in the same way as shear stress. This method is excellent to study the pharmacological effects of different drugs in the individual but unsuitable to screen many for excess risk. It is invasive in nature owing to the arterial cannula used for infusion but less so than coronary catheterization [74].

Endothelial progenitor cells as a marker of endothelial injury

The endothelial progenitor cell (EPC) is a stem cell originating from the bone marrow. Previously thought to develop from precursor to mature endothelial cell only during embryonic development, it was shown by Asahara and colleagues in 1997 that these cells taken from

adults can differentiate into mature endothelial phenotype ex vivo [75]. These cells are identified by different surface markers using flow cytometry of cell suspensions. The markers CD34 and CD133 indicate that the cell is a hematopoietic stem cell and the VEGF receptor, also known as KDR, indicates that the cell is going to evolve to an endothelial cell [76]. EPCs home to sites of ischemia and vascular injury as an endogenous repair mechanism of damaged endothelium. They are thought to contribute to re-endothelialization, neovascularization and the restoration of blood flow at the site of injury [77]. Consequently, reduced numbers and diminished function of EPCs have been linked to endothelial dysfunction [78] as well as increased risk for atherosclerosis and cardiovascular morbidity and mortality (Figure 6) [79].

Cardiovascular risk factors have been linked to the number and function of circulating EPC. A reduced number of EPC is observed in patients with the metabolic syndrome [80], hypertension [81], family history of CAD [82], and hyperlipidemia [83]. Accumulating evidence indicates that diabetes mellitus is associated with impaired number and function of EPC. The number of EPC is reduced in patients with T2D and was found to negatively correlate with disease severity score [84], also increasing the risk of cardiovascular mortality and morbidity [85].

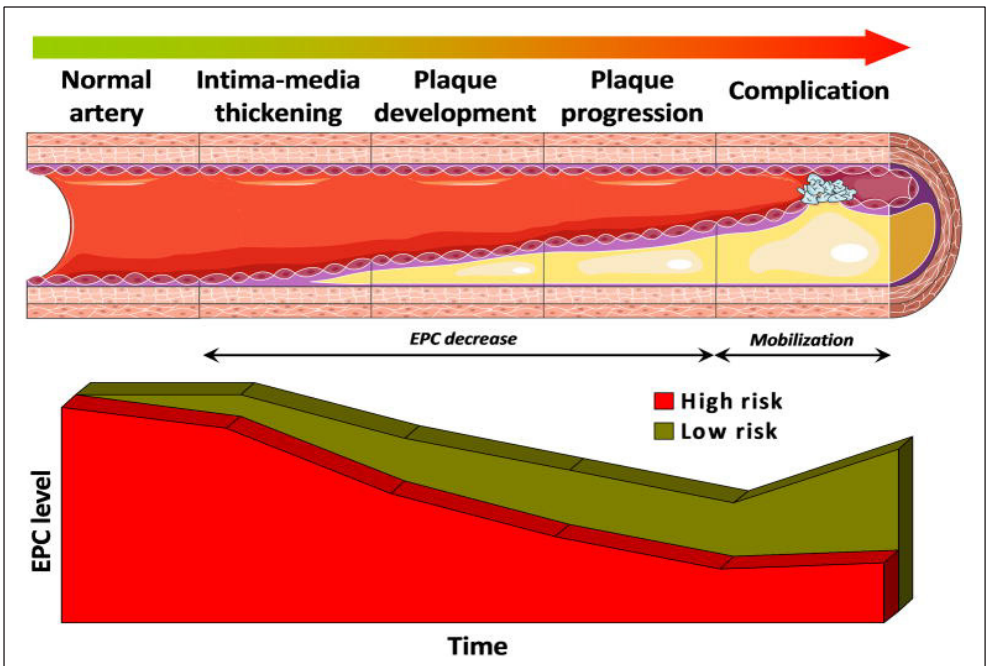

Figure 6. EPC mirror the natural history of atherosclerosis. The level of EPC (set at maximal in patients with normal arterial anatomy and function) starts to decline when cardiovascular risk factors appear in high risk patients. Further, EPC progressively decline with initial vascular remodeling (IMT), plaque development and progression. Lower EPCs are markers of high risk for future cardiovascular events. Finally, when complications occur (such as AMI or stroke) EPCs should be increased by bone marrow mobilization. When this mechanism is perturbed, a worse outcome can be predicted. EPC= endothelial progenitor cell. Circulation Research.**2012; 110: 624-637.**

Studies have indicated that there is a dynamic interplay between EPCs, reactive oxygen species and inflammation thus implicating EPCs in vascular repair [86]. In addition there seems to be an increase in the number of EPCs when individuals are treated with medication with anti-inflammatory properties like statins and angiotensin converting enzyme inhibitors in patients with cardiovascular disease [87]. The impact of ET receptor blockers on EPC numbers in patients with diabetes and cardiovascular disease remains unknown, however.

Summary

Based on the evidence presented above, there is a need to investigate further the effects of longer term ET receptor blockade on both macro- and microvascular function in patients with T2D and vascular complications. This is an ever increasing patient population with considerable mortality and morbidity and as of yet the therapeutic options remain insufficient. There is also a question whether to selectively block the ET_A receptor or to block both ET_A and ET_B for best therapeutic effect. ET_B causes vasodilatation when activated on endothelial cells under normal circumstances but as presented, with the development of atherosclerosis there seems to be a redistribution of the ET_B receptor to cells involved in the atherosclerotic plaque. Furthermore, it seems to be a contributing factor to endothelial dysfunction instead of vasodilatation. Both arginase and ET-1 play a role in endothelial dysfunction as well as in the development of vascular complications in T2D. The relationship between the two has never before been investigated in atherosclerotic disease.

PURPOSE

The overall aim of the project was to investigate the role of ET-1 for vascular dysfunction in atherosclerosis and type 2 diabetes and to evaluate the efficacy of ET receptor blockade to improve endothelial function. The specific aims were to:

I. Investigate the effect of oral administration of a combined ET_A/ET_B receptor blocker on endothelial function in patients with type 2 diabetes and microvascular dysfunction (I)

II. Compare the effects of selective ET_A receptor blockade and combined ET_A/ET_B receptor blockade on endothelial function in patients with type 2 diabetes and coronary artery disease (II)

III. Investigate the effect of oral administration of a combined ET_A/ET_B receptor blocker on the recruitment of endothelial progenitor cells in patients with type 2 diabetes and microvascular dysfunction (III)

IV. Determine the gene and protein expression of ET-1 and its receptors in the atherosclerotic plaque and its relation to arginase (IV)

MATERIALS AND METHODS

Study subjects

The studies were performed in accordance with the Declaration of Helsinki and were approved by the local ethics committee at Karolinska University Hospital. All patients gave their oral and written informed consent.

Studies I and III

A total of 46 patients with T2D of at least two years duration and microalbuminuria were recruited from the department of Endocrinology, Metabolism and Diabetes at Karolinska University Hospital. The patients were classified as having diabetes mellitus if fasting blood glucose exceeded 7.0 mmol/L (on at least on two occasions) or blood glucose concentration was >11.0 mmol/L two h after an oral glucose loading (75 g). Albuminuria was defined as urine albumin concentration >20 mg/L or >30 mg/L per 12 h and a ratio of albumin/creatinine >3.0 mg/mmol. Exclusion criteria were recent myocardial infarction or unstable angina (within the last three months), decompensated heart failure, changed dose of any vasodilator drug during the preceding six weeks, childbearing potential, impaired hepatic function (2 times the normal limit of serum aminotransferases), ongoing treatment with glibenclamide, cyclosporine or warfarin or any concomitant disease that may have interfered with the possibility for the patients to comply with or complete the study protocol. The baseline characteristics of the patients included in studies I and III are presented in Table 1.

Table 1. Baseline characteristics of study subjects in studies I and III. Values are presented as mean±SD or number of patients (n). ACE= angiotensin converting enzyme. ARB= angiotensin receptor blocker.

	Placebo (n=24)	Bosentan (n=22)
Age, years	63±9	62±8
Male/female, n	19/5	18/4
Body mass index, kg/m^2	31.5±4.0	28.9±7.4
Waist circumference, cm	111±11	109±10
Current smokers, n	1	2
Diabetes duration, years	17.7±9.6	14.9±6.3
Coronary artery disease, n	9	10
Peripheral artery disease, n	4	0
Retinopathy		
Non-proliferative, n	10	8
Proliferative, n	5	2
Neuropathy		
Moderate, n	5	7
Severe, n	6	2
Treatment		
Insulin, n	16	18
Oral glucose-lowering agents, n	16	13
Aspirin, n	15	13
Betablocker, n	8	12
ACE inhibitor/ARB, n	19	20
Calcium channel blocker, n	9	9
Statins, n	13	14

Study II

In study II we included 12 patients with T2D of at least two years duration and known CAD. They were recruited from the department of Cardiology, Karolinska University Hospital, during follow-up after coronary revascularization. Patients were classified as having diabetes mellitus if fasting blood glucose exceeded 7.0 mmol/L (on at least two occasions) or blood glucose concentration was >11.0 mmol/L after an oral glucose loading (75 g). CAD was defined as either previous coronary artery bypass surgery (CABG) or percutaneous coronary intervention (PCI). Exclusion criteria were myocardial infarction or unstable angina within the last six weeks, known Raynaud's phenomenon, peripheral vascular disease, arterial shunting or other vascular surgery of the study arm, change in dose of any vasodilator drug during the preceding six weeks, ongoing treatment with warfarin or any concomitant disease that may have interfered with the possibility for the patients to comply with or complete the study protocol. The baseline characteristics are presented in Table 2.

Table 2. Baseline characteristics of study subjects in study II. Values are presented as mean±SD. BMI=body mass index, HbA1c=glycosylated hemoglobin, eGFR=estimated glomerular filtration rate. ACEi=angiotensin converting enzyme inhibitor, ARB= angiotensin receptor blocker.

Characteristics	n=12
Age, years	64.8±9.0
Height, cm	175±5
Weight, kg	87.8±10.9
BMI, kg/m2	28.4±2.3
Hip-waist ratio	1.0±0.04
Glucose, mmol/L	8.4±1.9
HbA1c, mmol/mol	57.8±8.0
Creatinine, µmol/L	84±16
eGFR, mL/min	99±21
Hemoglobin, g/L	144±13
C-reactive protein, mg/L	2.1±2.0
Triglycerides, mmol/L	1.7±1.0
Cholesterol, mmol/L	4.2±1.0
HDL cholesterol, mmol/L	1.2±0.4
LDL cholesterol, mmol/L	2.2±0.9
Systolic BP, mmHg	138±13
Diastolic BP, mmHg	75±12
Treatment	
Aspirin, n	10
Oral antidiabetic agent, n	10
Insulin, n	6
Beta blocker, n	7
Nitrates, long acting, n	2
ACEi/ARB, n	9
Calcium channel blocker, n	9
Statin, n	10
Other antihypertensive treatment, n	2

Study IV

In study IV we had access to the Biobank of Karolinska Endarterectomies (BIKE) which is a database comprised of atherosclerotic plaques removed during carotid endarterectomies of either symptomatic or asymptomatic patients. Control tissue was obtained from iliac arteries of organ donors without atherosclerotic disease. Several clinical variables were registered, including gender, clinical chemistry, medication, and last recorded symptoms of plaque instability prior to the endarterectomy procedure. At the time of the study the database included 127 patients and 10 normal arteries. Patients undergoing surgery for symptomatic or asymptomatic high-grade carotid stenosis at the Department of Vascular Surgery, Karolinska University Hospital, were consecutively enrolled in the study and clinical data recorded on admission. Symptoms of plaque instability were defined as transient ischemic attack (TIA), minor stroke (MS) and amaurosis fugax (retinal TIA). Patients without qualifying symptoms within six months prior to surgery were categorized as asymptomatic. All samples were collected with informed consent from patients, organ donors or their guardians.

Methods

Flow Mediated Vasodilatation and Pulse Amplitude Tonometry

Study I uses two methods for determination of endothelial function: Flow mediated vasodilatation (FMD) and pulse amplitude tonometry (PAT). The patient is situated in a relaxed semi-recumbent position, a blood pressure cuff is placed on the forearm. An ultrasound probe is carefully positioned proximal to the cuff to visualize the brachial artery. On the index finger the Endo PAT probe is placed on index finger of both hands. The experiment begins with the blood pressure cuff being inflated to cause local ischemia of the forearm. After 5 minutes of ischemia, the pressure is released from the cuff reinstating blood flow. During the reperfusion the flow mediated vasodilatation is recorded on the ultrasound machine and the changes in the pulse amplitude in the index finger measured by the Endo-PAT device. Both methods make use of the physiological phenomenon called post-occlusive hyperemia resulting from the ischemia-reperfusion caused by the blood pressure cuff. The hyperemia leads to increased shear stress which stimulates NO release from the endothelium, resulting in an increase in the diameter of the brachial artery [41] (FMD) or the small artery pulse amplitude (PAT) in the fingertip (Figure 7) [88, 89]. Pulse amplitude was recorded electronically in both fingers and analyzed by a computerized, automated algorithm (Itamar Medical). The change from the baseline measurement is expressed as the reactive hyperemia index (RHI) which in part reflects vasodilator function of the digital microcirculation [90]. Previous evaluation of this method has demonstrated that RHI is to a large part dependent on NO bioavailability [88]. Endothelium-independent vasodilatation (EIDV) was determined following sublingual administration of nitroglycerine (0.4 mg).

This measurement has been shown to be an independent predictor of adverse cardiac events in the Framingham cohort [91]. FMD is calculated as per cent change from baseline in the diameter of the brachial artery measured with an ultrasound probe and the change in the diameter that is caused by the post-occlusive hyperemia. FMD has been shown to be impaired as a consequence of a range of classical risk factors [92].

Venous Occlusion Plethysmography

Study II uses venous occlusion plethysmography [93]. This is an invasive method capable of directly measuring the endothelium-dependent and endothelium-independent vasodilatation. Although invasive, this kind of study is valuable as it can measure immediate effects of infused agents on the endothelial function [94]. Forearm blood flow is measured simultaneously in both arms using the mercury-in-silastic strain-gauge technique. A venous occlusion cuff placed around the upper arm is inflated to 40 mmHg for 10 sec to obtain recordings of arterial inflow followed by deflation for 5 sec. During recordings of blood flow the circulation of the hands is occluded by a cuff inflated to 30 mmHg above the systolic blood pressure. A percutaneous catheter is inserted under local anesthesia in the brachial artery of the non-dominant arm for infusions and collection of blood samples. Another catheter is inserted in a deep cubital vein on the same arm for blood sampling. Endothelium-dependent vasodilatation is determined by intra-arterial infusion of serotonin (Ser; 21, 70 and 210 ng/min). Endothelium-independent vasodilatation is determined by infusion of sodium nitroprusside (SNP; 0.3, 1 and 3 µg/min). Each dose is given for 2 min at a rate of 2.5 ml/min. The NO-dependent property of the vasodilatation induced by serotonin has previously been validated in this model by our group [63].

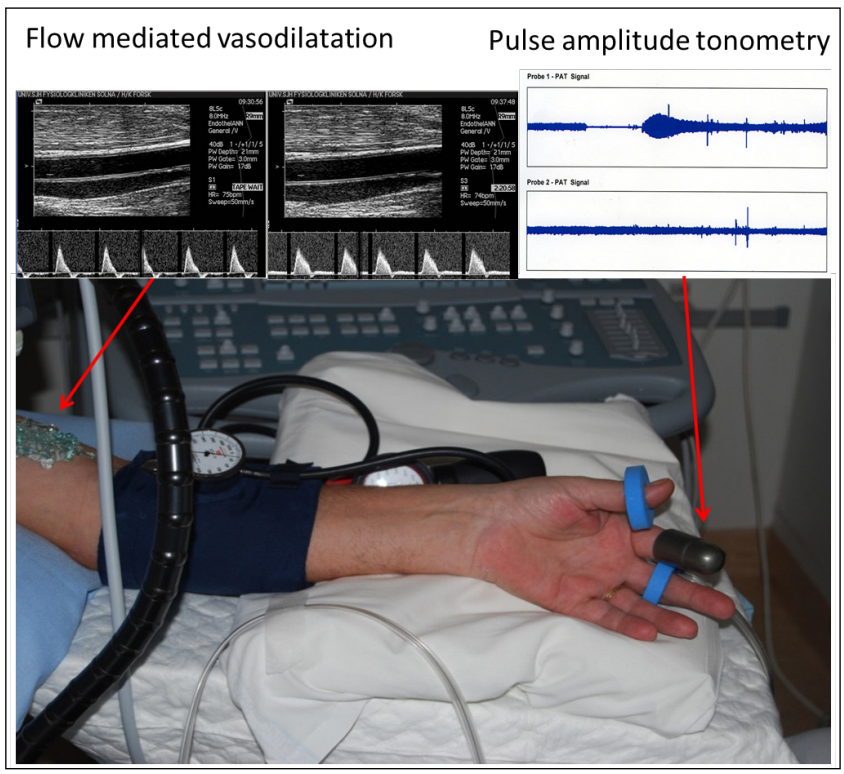

Figure 7. Pulse amplitude tonometry and flow-mediated vasodilatation examined simultaneously. The PAT signal represents endothelium-dependent dilatation in microvessels in the finger tip. FMD measures endothelium-dependent dilatation in a larger conduit artery (a. brachialis).

Transcutaneous Oxygen Tension ($TcPO_2$)

$TcPO_2$ is a noninvasive method to measure to quantify skin oxygenation. It is used in research as well as clinically to evaluate ulcer healing in diabetic ulcers and peripheral artery disease [95]. In study II it was used simultaneously with the venous occlusion plethysmography, a transcutaneous (tc) pO_2 probe (PF 5040, Perimed, Järfälla, Sweden) was attached to the forearm at the start of the study protocol for continuous recording. Readout was performed before and at the end of the infusions of antagonists in a standardized fashion and a photograph was taken to ensure the same placement on the different study occasions. The measured unit is mmHg.

Laser Doppler Flowmetry

Laser Doppler flowmetry is a technique based on the scattering of laser light by red blood cells. This corresponds to the blood flow in the skin underlying the probe and is a measurement of the skin microcirculation. When the beam of light encounters red blood cells, it scatters and the shift in light wavelength frequency depends on the blood cells average velocity (Figure 8) [96]. The sampling volume of current laser Doppler devices is between 0.5 and 1 mm^3, so the flow measured represents the average in at least 50 vessels, including arterioles, capillaries and venules of variable size and direction. The technique measures relative change in perfusion (called perfusion units, PU) which makes it possible to detect change in a given area after some type of challenge or treatment. The laser Doppler probe was placed on the forearm during setup of the experiment in study II and readout performed after conclusion of the venous occlusion plethysmography.

Flow Cytometry

Flow cytometry is a method employing laser light scattering properties to count cell types. When coupled to fluorescence labeling using different markers it is possible to label cells to count the different subtypes in a given sample. In study III, this method was used to isolate and count endothelial progenitor cells in a venous blood sample (10ml EDTA tubes). Different subpopulations can then be identified by using the hematopoietic progenitor cell marker CD34, the immature hematopoietic progenitor cell marker CD133, and the endothelial cell receptor VEGFR2 (vascular endothelial growth factor receptor-2, also known as kinase domain receptor, KDR), as described previously [97, 98]. Peripheral blood mononuclear cells (PBMC) are incubated with fluoresceinisothiocyanate (FITC)-conjugated anti-human CD34 monoclonal antibody (mAb) (BD Biosciences), phycoerythrin (PE)-conjugated anti-human

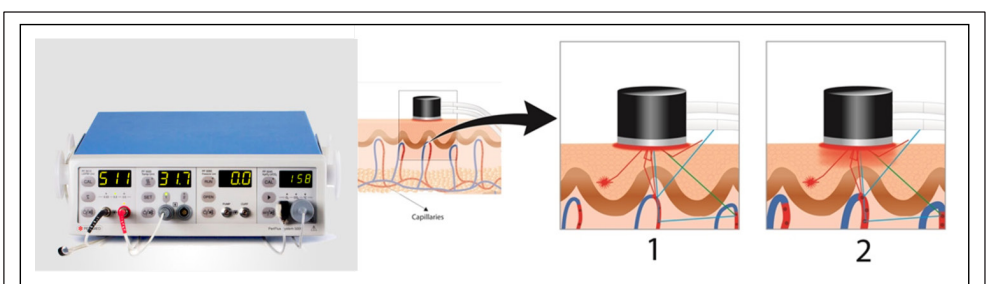

Figure 8. Laser Doppler showing close up of the probe during resting conditions (1) and during heating (2). Courtesy of Perimed.

CD133 mAb (Milteny Biotec) and allophycocyanin-conjugated monoclonal anti-VEGFR2 (R&D Systems) for 60 min at +4°C. The cytometer (CyAn, Dako, Denmark) then counts the frequency of peripheral blood cells positive for these reagents determined by a two-dimensional side-scatter fluorescence dot-plot analysis of the samples, after appropriate gating.

Study Protocols

Study I

The study was a placebo controlled, double blinded randomized trial including patients with T2D of at least 2 years of duration and documented albuminuria as a surrogate marker of vascular dysfunction. The study included a total of 46 patients of which 22 were randomized to receive the dual ET_A/ET_B receptor antagonist bosentan 250 mg bid and 24 patients were randomized to the placebo group (Figure 9). Treatment duration was 4 weeks and the patients underwent measurement of endothelial function at baseline and after the treatment duration. The primary endpoint for study I was change in microvascular function as measured by endo-PAT. Secondary endpoint was the change in endothelial function of the brachial artery measured by FMD.

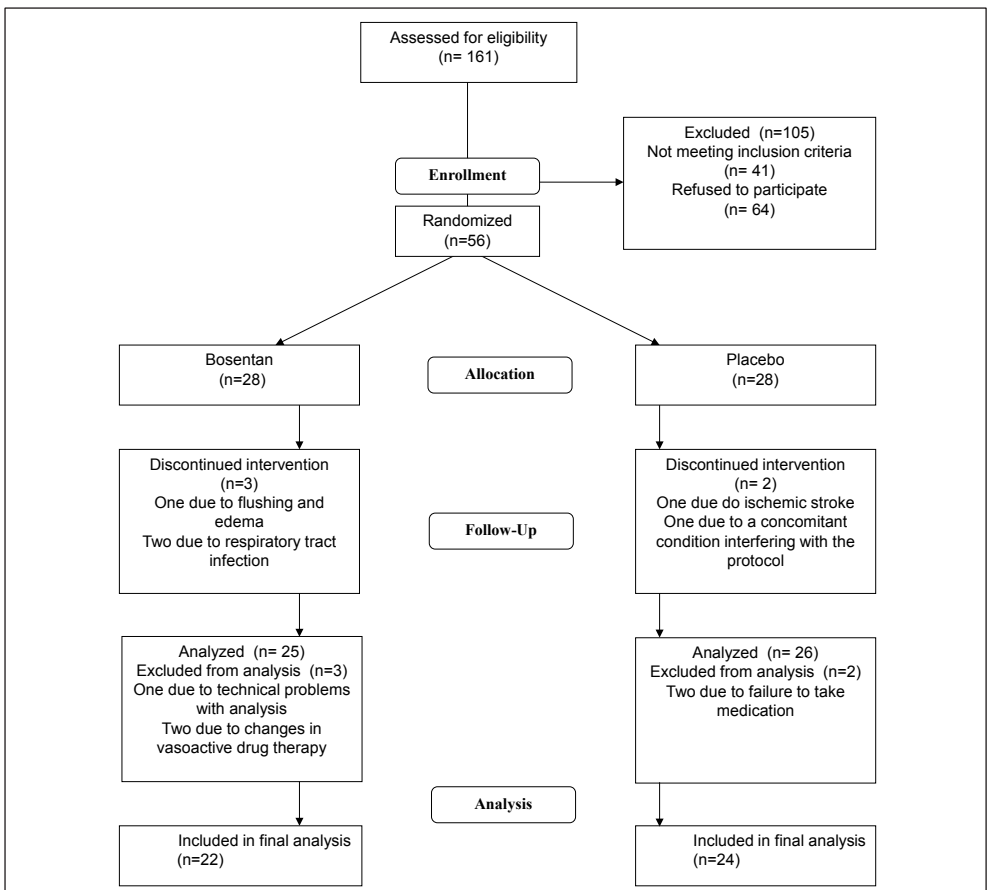

Figure 9. Flowchart for inclusion of patients in Studies I and II.

Study II

Twelve patients with T2D and CAD were included in this trial with cross-over design and blinded evaluation comparing the effects of selective ET_A receptor blockade and dual ET_A/ET_B receptor blockade. Endothelium-dependent and endothelium-independent vasodilatation was measured using venous occlusion plethysmography measuring forearm blood flow during intra-arterial infusions of serotonin and nitroprusside, respectively. The determination of endothelial function was performed before and after 60 minutes of intra-arterial infusion of either the selective ET_A antagonist BQ123 or the combination of BQ123 and the ET_B antagonist BQ788 (Figure 10).

A laser Doppler probe as well as a $TcPO_2$ probe were attached to the skin on the arm at the beginning of the experiment. The measured values are respectively, a mean value of flow during a period of time around 2 minutes, given in perfusion units (PU), and oxygen pressure in mmHg. At the end of the study the laser Doppler probe measured the thermal reactivity after 15 minutes of heating (44°C).

Study III

This study is based on the patient material from Study I. Blood samples were taken at baseline and at follow-up. Of the initial 46 patients in the study, blood samples for analysis of EPC subpopulations were successfully collected from 36 patients.

Different subpopulations were identified by using the hematopoietic progenitor cell markers CD34, the immature hematopoietic progenitor cell marker CD133, and the endothelial cell receptor VEGFR2. In addition to EPC subpopulations the number of apoptotic progenitor cells were measured using the Annexin-V Apoptosis detection kit. Circulating ET-1 was measured as a marker of activated endothelium and von Willebrand factor and ICAM-1 were used as markers of endothelial damage and were determined using enzyme linked immunoassay (ELISA).

Study IV

Two non-overlapping BiKE microarray datasets were used to determine the mRNA levels of genes of interest in human plaques. The larger 'discovery' dataset comprised n=127 carotid plaques and n=10 normal arteries. The smaller 'validation' dataset comprised n=50 plaques and n=5 normal arteries. Plaque material was analyzed with regard to mRNA expression of the genes *EDN1, EDNRA, EDNRB, ARG1* and *ARG2*. To investigate the protein expression

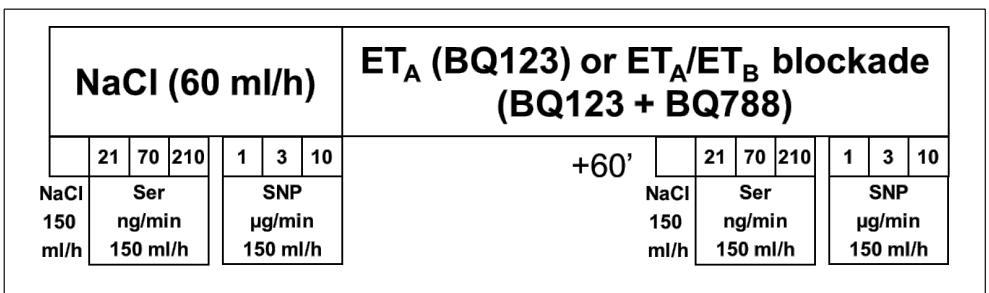

Figure 10. Protocol for study II.

of the genes of interest, the plaques were stained using immunohistochemistry, both single and double staining protocols to ascertain co-localization. Next the cells that showed co-localization of ET-1 and arginase, endothelial cells and macrophages were used in functional testing for a possible connection. The cells were stimulated with ET-1 and the mRNA expression of *ARG1 and ARG2* was determined by qPCR. To further explore the ET-1 stimulation, arginase activity was measured using spectrophotometry in both endothelial cells and macrophages. In the macrophages, reactive oxygen species were measured using electron spin resonance (ESR).

Biochemical analyses

Blood tests in studies I, II and III

In studies I and III fasting plasma glucose, Hb1Ac (Mono S), blood lipids, liver function tests were accessed with standard methods according to local laboratory routines. Insulin-like growth factor-1 (IGF-1) was analyzed by radioimmunoassay (RIA) and calculated using an age adjusted SD score: (10log(IGF-I level)-0.00625*age-2.555)/0.104 [99]. Insulin-like growth factor binding protein-1 (IGFBP-1) was determined by RIA [100]. ET-1 was measured using ELISA (R&D systems, Minneapolis, USA). High-sensitive C-reactive protein was analyzed using turbidimetry (Beckman Coulter, Fullerton CA, USA).

For study II, screening blood samples were taken on the morning of the first visit for analysis of blood glucose, HbA1C, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglycerides, C-reactive protein, creatinine, IGF-1 and IGFBP-1. Plasma ET-1 levels were measured by chemiluminescent ELISA (Quantiglo kit, R&D, Abingdon, UK) before and after completion of the treatment protocol. Glomerular filtration rate (eGFR) was estimated using the Cockroft-Gault formula.

Quantitative real time PCR for mRNA quantification

To estimate the translation activity of a specific gene it is possible to measure the amount of messenger RNA that is present. The method used in this work to quantify amounts of mRNA from a single gene is called polymerase chain reaction (PCR) and relies on the generation of cDNA, hybridization of complementary DNA primers and probes for activating DNA polymerase, and repeated heating/cooling cycles for the amplification of the probed gene. Real time PCR amplifies the target genes mRNA to measurable levels real time and makes use of gene specific probes produced commercially [101]. This method is utilized in study IV to measure mRNA levels of atherosclerotic plaques as well as in cell cultures. For qPCR, total RNA was reverse-transcribed using High Capacity RNA-to-cDNA kit (4387406, Applied Biosystems, Life Technologies, Carlsbad, CA). PCR amplification was done in 96-well plates in 7900 HT real-time PCR system (Applied Biosystems), using TaqMan® Universal PCR Master Mix (Applied Biosystems) and TaqMan® Gene Expression Assays (Arginase 1=ARG1: HS00968979_M1, Arginase 2=ARG2: HS00982833_M1, Applied Biosystems). All samples were measured in duplicates. Results were normalized to the equal mass of total RNA as well as the Ct values of RPLPO housekeeping control (HS99999902_M1). The relative amount of target gene mRNA was calculated by 2-ΔΔCt method and presented as fold change compared to the baseline expression.

Immunohistochemistry

Immunohistochemistry is a process to identify specific proteins in cells and tissue. The technique relies on the specific binding of antibodies to their antigens. In this way the protein of interest is localised in the tissue by the antibody which is coupled to an enzyme that can catalyse a colour reaction that can be visualised in a common light microscope or if the chromogen is fluorescent, a fluorescence microscope. This is a qualitative method that allows specific receptors and proteins to be located in tissues as well as co-localisation with other proteins by using double stainings. One of the weaknesses of the method is the specificity of the antibody used. If specificity is lacking the antibody can bind to other non-relevant structures. This error can be avoided by testing the antibody with antigen pre-adsorption [102]. In study VI the following antibodies were used for immunohistochemical staining: a polyclonal rabbit anti-human ET-1 (1:250; Abcam, Cambridge, UK), polyclonal rabbit anti-human ET_B (1:100; Alomone labs, Jerusalem, Israel), polyclonal rabbit anti-human ET_A (1:500; Alomone labs), Monoclonal mouse anti-human Arg1 and Arg2 (1:50, Atlas antibodies, Bromma, Sweden), monoclonal mouse anti-human CD163 (1:200; Dako, Glostrup, Denmark), monoclonal mouse anti-human von Willebrand Factor (VWF; 1:4000; Dako), and monoclonal mouse anti-human smooth muscle α-actin (1:600; Dako). Isotype rabbit and mouse IgG were used as negative controls. In brief, 5 µm sections were deparaffinized in Clear (Histolab, Gothenburg, Sweden) and rehydrated in ethanol. For antigen retrieval, slides were subjected to high-pressure boiling in DIVA buffer (pH 6.0). After blocking with Background Sniper, primary antibodies diluted in Da Vinci Green solution were applied and incubated at room temperature for 1 h. Detection was performed with Mach3 Probe and Mach3 Polymer Detection technology (BioCare Medical, Pacheco, CA, USA), using Warp Red and Vina Green, stained in a consecutive manner using MACH 2 double stain kit 2. Rabbit IgG1 and mouse IgG1 (BioCare Medical) were utilized as negative controls. The slides were then counterstained with Hematoxylin QS (Vector Laboratories, Burlingame, CA, USA), dehydrated and mounted in Pertex (Histolab). Images were taken using a Nikon OPTIPHOT-2 microscope equipped with digital camera and NIS-Elements software.

Arginase activity determined by spectrophotometry

Spectrophotometry is a method that measures the intensity of light passed through a sample solution of the chemical in question. Each compound or chemical absorbs, reflects or transmits light over a certain range of wavelength. This method is widely used for quantitative analysis and is particularly popular in enzyme-catalyzed reactions which we used in study IV to analyse arginase activity from different cell cultures. Endothelial cells and THP-1 macrophages were lysed by using fresh 1 mM EDTA, Triton X-100 (0.1%, MERCK, Darmstadt, Germany) and protease inhibitors (Roche) in PBS. Each sample was incubated at 37 °C for 1 h with either l-arginine (50 mM Tris·HCl at pH 9.7) + vehicle, l-arginine + ABH (0.1 mM; Enzo Clinical Labs, Farmingdale, NY, USA) or with l-arginine + nor-NOHA (1 mM). The concentration of the end product urea was determined by using spectrophotometry. The inhibition induced by arginase inhibitor was calculated as the difference between the urea production from vehicle-treated and inhibitor-treated samples.

Electron spin resonance for production of reactive oxygen species (ROS)

As reactive oxygen species have a half-life ranging from nanoseconds to a few seconds, the methods used to accurately quantify these short-lived chemical compounds have to have

fast reaction times and produce longer lived signal compounds [103]. One of those methods employs a hydroxylamine spin probe that binds to ROS and creates a stable compound and produces a measurable signal. Reactive oxygen species are molecules that have free radicals. Radicals have an unpaired electron and this causes them to have magnetic moment that can be detected by electron spin spectroscopy using a magnetic field [104]. Macrophages were treated with the arginase inhibitor (S)-amino-6-boronohexanoic acid (ABH; 1 mM), or vehicle for 30 min prior to incubation with ET-1 (1 nM). After 24 h incubation, the cells were incubated with 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH, 200 μM) for 30 min. The cell suspensions were frozen in liquid nitrogen. Reactive oxygen species (ROS) formation was detected by ESR using the following setting: center field 1.99 g, microwave power 1 mW, modulation amplitude 9 G, sweep time 10 s, number of scans 10, field sweep 60 G. The amount of CM• was determined from the calibration using 3-carboxy-proxyl (CP•, Noxygen Science Transfer & Diagnostics GmbH).

Statistical analyses

In study I data are presented as mean and standard deviation. A two-sided P-value <0.05 was considered significant. The data for analyses between the groups was calculated as change from baseline after completed therapy. Group comparisons were made using t-test. Data that were not normally distributed were log transformed. The number of patients per group needed to detect a difference in RHI=0.3 with a power of 80% with a two-tailed t-test at the 5% level was calculated to be 22 with SD=0.35 for difference in RHI. The improvement in by 0.3 was based on a previous study [89]. All statistical analyses were performed with GraphPad Prism 6.

In study II differences in the change in forearm blood flow induced by serotonin and SNP between the two different treatments were assessed by 2-way analysis of variance. Changes in baseline flow were assessed by 1-way repeated-measures analysis of variance followed by the Bonferroni multiple comparison test. Based on a previous study performed using a similar study protocol we have observed improvement in endothelium-dependent vasodilatation by 30% following dual receptor blockade [43]. Assuming similar effect in the present study the number of subjects was calculated to be 12 ($\alpha = 0.05$ and 80% power). A value of $P<0.05$ was considered significant. All statistical analyses were performed with GraphPad Prism 6.

In study III continuous variables were tested for normal distribution with the Kolmogorov-Smirnov test. Non-normally distributed continuous variables groups were compared by the Mann-Whitney *U* test, while others were analyzed by *t* test (2 sided, including Levene's test for equality of variances). Bivariate correlation (Spearman's rank correlation coefficient) was used to correlate circulating EPC counts with risk factors or drug treatment. Comparisons between before and after treatment were compared using a paired test. Statistical significance was assumed if a null hypothesis could be rejected at $p<0.05$. All statistical analyses were performed with IBM-SPSS, version 19.0 (IBM Inc.).

In study IV microarray dataset analyses were performed using a linear regression model adjusted for age and gender and a two-sided Student's t-test assuming non-equal deviation, with Bonferroni correction for multiple comparisons. Details of these analyses were previously described [105]. Spearman correlations were calculated to determine the association between

mRNA expression levels from microarrays (weak if Spearman $r<0.3$, moderate $0.3<r<0.6$ and strong $r>0.6$). Results from *in vitro* experiments were evaluated by the non-parametric Kruskal-Wallis test or ANOVA when appropriate. Data are presented as mean fold change with SEM. All calculations were performed using GraphPad Prism 6 and Bioconductor software. In all analyses p-value <0.05 was considered significant.

Results

Study I

Study subjects

Three patients in the active treatment arm (bosentan) stopped the study because of adverse events (one due to facial flushing and oedema, two due to respiratory tract infection) and three patients were excluded due to protocol violation (one patient due to only 4 min arterial occlusion, two patients due to change in vasodilatory medication during the study period). In the placebo group, four patients were excluded. One of these suffered an ischaemic stroke, two patients did not take the study medication as instructed and one patient was excluded due to a concomitant condition that interfered with his/her ability to comply with the study protocol. A total of 46 patients remained in the final analysis. The two groups were well matched at baseline with no significant differences in their baseline characteristics or medication (Table 1).

Bosentan increased microvascular endothelial function

The primary endpoint was change in digital RHI from baseline to follow-up measured by Endo-PAT. Baseline RHI was 1.82±0.50 for the entire study population. Baseline RHI was similar in the two groups. RHI did not change significantly from baseline (1.84±0.49) to follow-up (1.87±0.47) in the placebo group. In the bosentan-treated group, RHI increased from 1.73±0.43 at baseline to 2.08±0.59 at follow-up ($P<0.05$; Figure 11A). The change in RHI during the 4 weeks of treatment with bosentan was significantly greater than that of the placebo group (0.38, 95% CI 0.02, 0.74; Figure 11B). The change in RHI did not correlate to change in systolic blood pressure ($r=0.16$; $p=0.28$). Nitroglycerine-induced PAT signal did not change with treatment (1.20±0.44 at baseline and 1.24±0.30 at follow-up).

Baseline brachial artery FMD was comparable in the two groups. After 4 weeks treatment there was no significant change in FMD (0.016, 95% CI -1.38, 1.0; Table 3) or nitroglycerine-induced brachial artery dilatation in either group.

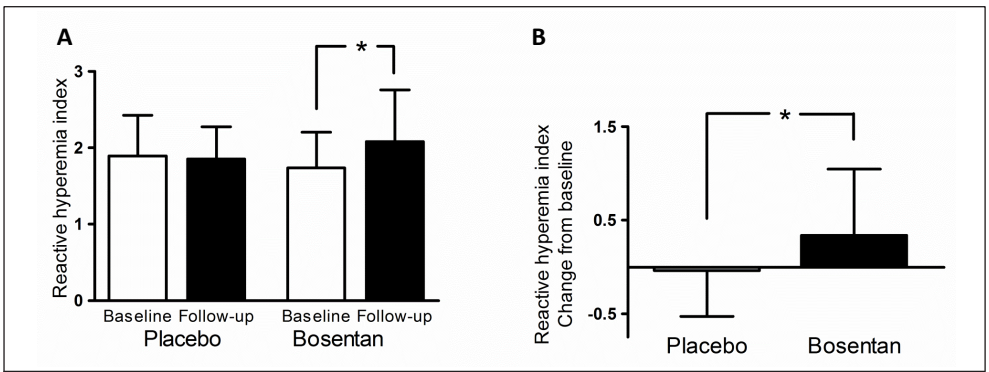

Figure 11. A: Reactive hyperaemia index (RHI) at baseline and follow-up for the placebo and bosentan groups. B: Change in RHI from baseline inthe two groups. Values are mean±SD; *$p<0.05$.

Increase in plasma ET-1 but no change in urine albumin/creatinine ratio

Treatment with bosentan resulted in a significant drop in hemoglobin from 134 g/L to 127 g/L (P<0.001; Table 3). There was no significant change in urine albumin/creatinine ratio in either group. During treatment there were no changes in lipid profile or blood glucose levels. Plasma levels of ET-1 increased from 1.5±0.6 pg/mL at baseline to 2.6±1.2 pg/mL (P<0.001) in the bosentan group which could be expected due to reduced ET_B receptor-mediated clearance of ET-1. ET-1 levels did not change in the placebo group (1.8±0.9 pg/mL at baseline and 1.7±0.7 pg/mL at follow-up). IGF-1 and IGFBP-1 did not differ at baseline between groups and were not affected by treatment. There was no change in hepatic transaminases during the treatment period.

Table 3. Treatment effects of 4 weeks treatment with bosentan. Values are presented as mean±SD. [a] P<0.001 vs. baseline. FMD; flow-mediated dilatation, SBP; systolic blood pressure, DBP; diastolic blood pressure, IGF-1 SD-score; age adjusted insulin-like growth factor-1, IGFBP-1; insulin-like growth factor binding protein-1, ASAT; aspartat aminotransferase, ALAT; alanine aminotransferase, VTI; velocity time integral.

	Placebo		**Bosentan**	
	Baseline	Follow up	Baseline	Follow up
FMD, change (%)	3.3±2.7	3.2±2.0	3.1±2.8	3.2±2.7
Brachial artery basal diameter (mm)	3.9±0.7	3.9±0.7	3.9±0.6	4.0±0.7
Brachial artery VTI (cm)	21±6.9	23±6.9	29±11	28±6.7
Systolic BP (mmHg)	151±25	150±21	149±24	143±18
Diastolic BP (mmHg)	78±9	79±8	81±10	77±11
Hemoglobin (g/l)	136.5±13.6	137.2±15.1	134.0±16.1	127.3±15.1[a]
Glucose (mmol/l)	9.5±3.9	9.9±3.6	8.6±2.5	8.4±2.7
Insulin (pmol/l)	107.5±143.1	95.8±97.6	129.2±121.3	118.3±93.1
IGF-1 (SD-score)	-0.47±1.4	-0.37±1.6	-0.39±1.2	-0.87±1.1
IGFBP-1 (µg/l)	50.9±33.8	51.3±33.8	50.7±35.6	54.1±30.7
ASAT (µcat/l)	0.51±0.45	0.48±0.25	0.39±0.14	0.48±0.31
ALAT (µcat/l)	0.61±0.71	0.56±0.53	0.47±0.24	0.58±0.42
uAlb/Crea (mg/mmol)	46.3±58.0	39.0±70.7	46.9±77.1	31.6±47.0
Triglycerides (mmol/l)	1.6±0.8	1.7±0.7	1.5±0.7	1.6±0.7
Total cholesterol (mmol/l)	4.0±0.9	4.1±1.0	3.7±0.7	3.7±0.5
HDL cholesterol (mmol/l)	1.0±0.3	1.0±0.3	0.9±0.1	0.8±0.2
LDL cholesterol (mmol/l)	2.4±0.9	2.4±0.9	2.1±0.5	2.1±0.5
HbA_{1c}, DCCT (%)	8.0±1.4	8.0±1.4	7.4±1.1	7.4±1.0
HbA_{1c}, IFCC (mmol/mol)	64.0±15.0	64.3±15.4	57.8±12.1	56.9±11.1
Creatinine (µmol/l)	101.9±51.3	107.3±56.6	125.2±96.0	115.7±85.0
hsCRP (mg/l)	3.0±3.3	3.4±2.9	3.2±3.2	4.8±7.7
Endothelin-1 (pg/ml)	1.8±0.9	1.7±0.8	1.5±0.6	2.6±1.1[a]

Study II

Study subjects

The patient characteristics are presented in Table 2. Blood pressure or heart rate did not change during the infusions of the ET receptor blockers. No adverse effects occurred during any of the experiments.

Both treatments improved endothelial function but only dual blockade increased basal flow

Both selective ET_A receptor blockade and dual ET_A/ET_B receptor blockade induced significant improvement in endothelium-dependent vasodilatation in patients with T2D and CAD (Figure 12). The magnitude of improvement did not differ between the two treatment regimes. Dual ET_A/ET_B blockade significantly increased basal blood flow (36.4±3 to 47.6±4 mL/min/1000mL, p=0.006) whereas ET_A blockade did not (37.4±3 to 42.1±3 mL/min/1000mL). Endothelium-independent vasodilatation was also increased significantly by both interventions (p<0.001).

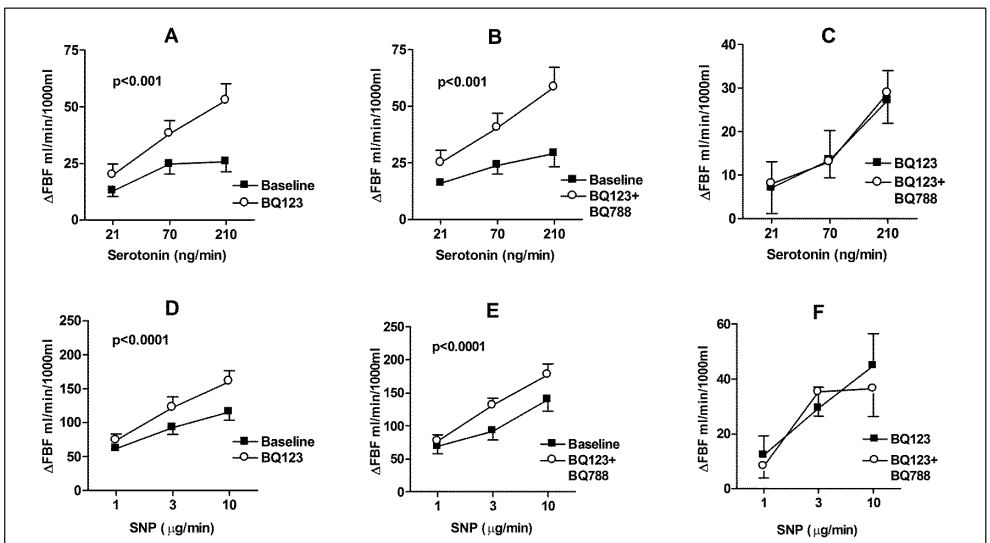

Figure 12. Endothelium-dependent vasodilatation (A–C) and endothelium-independent vasodilatation (D–F) determined as change in forearm blood flow (FBF) induced by serotonin and SNP, respectively. A and D: Before and during infusion of BQ123. B and E: Before and during infusion of BQ123 + BQ788. C and F: Change from baseline comparing the effect of the two treatments. Data are given as means and SEM.

Laser Doppler flow increased with ET_A blockade whereas TcPO2 increased with dual blockade

Laser Doppler flow was significantly higher after infusion of BQ123 than at baseline whereas there was a non-significant trend towards increased flow after infusion of BQ123+BQ788 (Figure 13A). There was no significant change after heating between the two treatments (increase of 1326±114% after BQ123 and 1459±215% after dual blockade).

$TcPO_2$ increased significantly during the administration of BQ123+BQ788 (Figure 13B) but administration of BQ123 had no effect.

Biochemical analyses

The baseline values of blood analyses are shown in Table 2. Arterial plasma ET-1 increased after administration of BQ123+BQ788 from 0.96±0.14 to 1.75±0.27 pg/mL ($P<0.01$) whereas it was not changed by administration of BQ123 alone, 0.96±0.18 to 1.1±0.18 pg/mL.

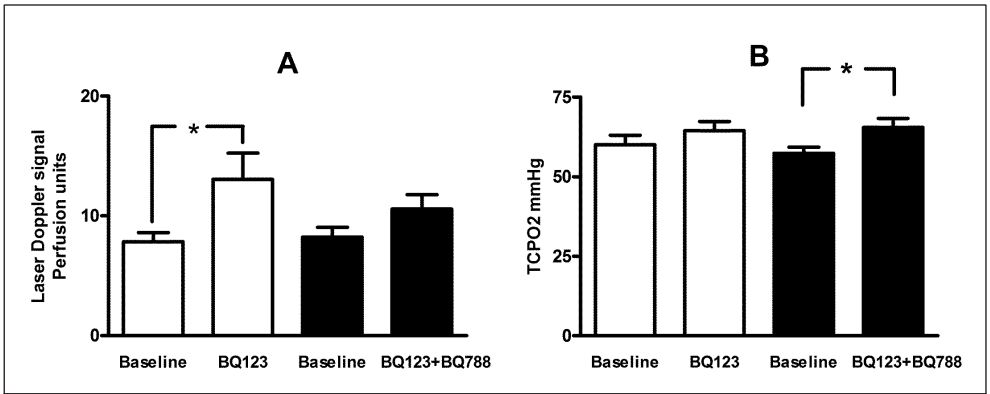

Figure 13. (A) Cutaneous microvascular flow and (B) Transcutaneous O2 tension at baseline and after infusion of BQ123 and BQ123 + BQ788, respectively. Data are given as means and SEM. *P<0.05.

Study III

Study subjects

The subjects of study III are the same as in study I.

Higher body mass index (BMI) and increased waist circumference associated with increased ET-1 and higher blood pressure

The baseline characteristics of the 36 subjects are summarized in Table 4. Baseline plasma ET-1 levels were 1.61 ±0.83 pg/mL. The cohort was divided into two groups based on the median (1.29 pg/mL) in order to compare the association between ET-1 levels on EPC and markers of endothelial damage. The groups with high and low ET-1 levels were well matched for age, gender and medication. Patients with high ET-1 levels had increased body mass index (BMI), larger waist circumference and had higher systolic blood pressure than those with low ET-1 levels. Other important risk factors for cardiovascular disease were similar in the two groups. Medication was similar in the two groups except for a higher proportion of patients treated with angiotensin receptor blockers (ARB) among those with high ET-1 levels.

Plasma ET-1 is related to EPC levels

Patients with higher levels of ET-1 had higher levels of CD34+CD133+ and CD34+KDR+ EPC (Figure 14). In contrast, there was no difference in CD34+ and CD34+CD133+KDR+ cells between patients with high and low ET-1 levels. There was no difference in the relative number of cells with markers of apoptosis between patients with high and low levels of ET-1

(22 ± 9% vs. 24 ± 15%). Furthermore, there was no difference in circulating levels of ICAM-1 (224.1 ± 48.0 ng/mL vs. 254.6 ± 77.1 ng/mL) or vWF (1.38 ± 0.62 kIE/L vs. 1.38 ± 0.76 kIE/L) between patients with high and low ET-1 levels.

Table 4. Baseline characteristics of the study population. Patients were divided according to their ET-1 level. The p-value indicates the comparison of the two latter groups. Ns: not significant.

	Total Study Population	Patients with ET-1 below median	Patients with ET-1 above median	p-value
N	36	18	18	
Demographics				
Age (years)	63.5 ± 7.7	62.4 ± 9.6	64.5 ± 5.3	ns
Gender (male / female)	29 / 7	15 / 3	14 / 4	ns
Height (cm)	175.8 ± 8.4	175.6 ± 6.4	176.2 ± 11.0	ns
Body weight (kg)	96.2 ± 17.1	91.2 ± 16.4	101.6 ± 16.6	ns
Body mass index (kg/m²)	31.2 ± 4.0	29.7 ± 3.4	33.5 ± 3.9	0.012
Waist circumference (cm)	110.5 ± 12.1	106.0 ± 12.4	114.8 ± 10.4	0.032
Blood pressure and heart rate				
Systolic blood pressure (mmHg)	149 ± 26	139 ± 25	159 ± 24	0.017
Diastolic blood pressure (mmHg)	79 ± 10	76 ± 11	80 ± 8	ns
Heart rate (1/min)	66 ± 8	66 ± 7	65 ± 8	ns
Risk factors and comorbidities n (%)				
Arterial Hypertension	32 (89%)	14 (78%)	18 (100%)	ns
Coronary artery disease	12 (33%)	7 (39%)	5 (28%)	ns
Smoking (yes and former)	18 (50)	9 (50%)	9 (50%)	ns
Hyperlipidemia	28 (78%)	13 (72%)	15 (83%)	ns
Medication n (%)				
Statin	22 (61%)	10 (56%)	12 (67%)	ns
ACE inhibitor	22 (61%)	12 (67%)	10 (56%)	ns
ARB	15 (42%)	4 (22%)	11 (61%)	0.041
Insulin	27 (75%)	12 (67%)	15 (83%)	ns
Metformin	19 (53%)	9 (50%)	10 (56%)	ns
Laboratory values				
LDL (mmol/l)	2.1 ± 0.7	2.0 ± 0.5	2.2 ± 0.8	ns
HbA1c (%)	6.8 ± 1.3	6.5 ± 1.0	7.1 ± 1.5	ns

Patients with high ET-1 levels had higher CRP levels (3.2 ±2.2 mg/L versus 1.4 ±1.2 mg/L; p<0.01). Furthermore, ET-1 and CRP correlated significantly to each other (R^2=0.562, p<0.001).

ET-1 receptor blockade did not alter EPC numbers

A secondary aim of the third study was to test the effect of dual ET_A/ET_B receptor blockade on EPC and markers of endothelial damage. As described in study I, patients were randomized to receive either bosentan or placebo for 4 weeks. Patient characteristics are shown in Table 1.

There was no effect of bosentan treatment on EPC counts either in comparison with baseline or with placebo after four weeks of treatment (Figure 15). In addition, there was no significant effect on the expression of markers of apoptosis in either treatment arm (25 ± 14% at baseline vs. 29 ± 17% at follow-up in the placebo group and 20 ± 10% at baseline vs. 20 ± 11% at follow-up in the bosentan group).

High ET-1 corresponded to higher CRP levels

Patients with high ET-1 levels had higher CRP levels (3.2 ±2.2 mg/l versus 1.4 ±1.2 mg/l; p=0.008). Furthermore, ET-1 and CRP correlated significantly to each other (R^2=0.562, P<0.001).

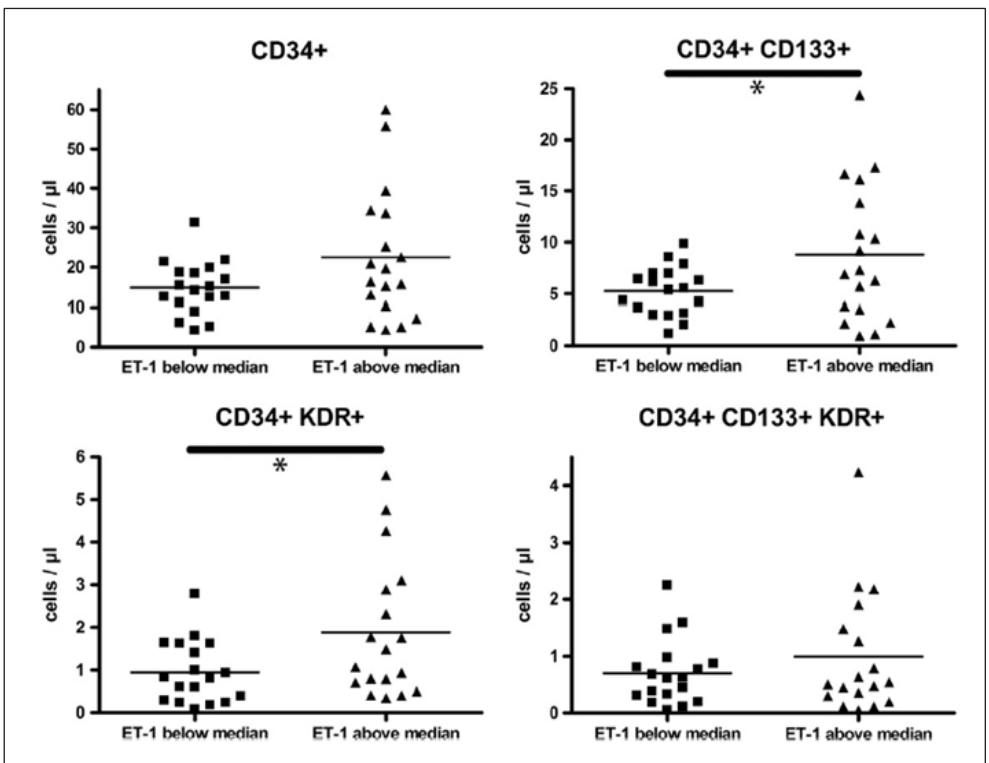

Figure 14. Different EPC subpopulations compared between patients with ET-1 levels below versus above the ET-1 median. *p < 0.05.

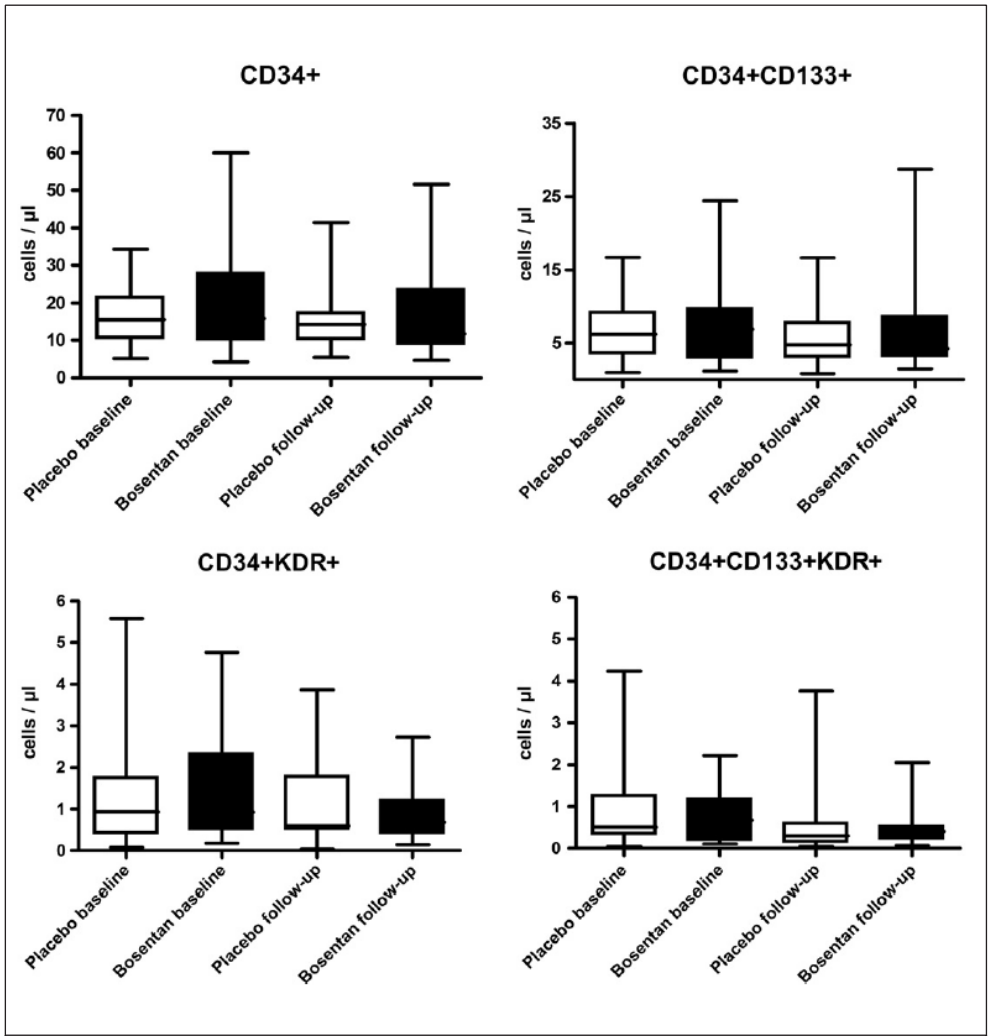

Figure 15. Different EPC subpopulations compared between patients receiving bosentan or placebo for four weeks before initiation of the treatment and after treatment. No significant differences or changes could be observed.

Biochemichal analyses

Treatment did not have any effect circulating levels of ICAM-1 and vWF. ICAM-1 was 263.5 ±80.5 ng/mL in the placebo group following treatment (-6.2 ng/ml change from baseline) and 230.6 ±65.4 ng/mL in the bosentan group following treatment (-3.4 ng/mL compared to baseline). The corresponding values for vWF were 1.50 ±0.80 kIE/L in the placebo group following treatment (-0.02 kIE/L compared to baseline) and 1.24 ±0.38 kIE/L in the bosentan group following treatment (-0.08 kIE/L compared to baseline). Similarly, there was no treatment effect on CRP levels (data not shown).

Study IV

Study subjects

The baseline characteristics for the BiKE database have been described in detail by Razuvaev et al [106].

ARG2 and EDNRA are downregulated in human carotid artery plaques

Using two non-overlapping BiKE microarray datasets we determined the mRNA levels of genes of interest in human plaques (Figure 16A and B). The larger 'discovery' dataset comprised 127 carotid plaques and 10 normal arteries. The smaller 'validation' dataset comprised 50 plaques and 5 normal arteries. Several genes of interest showed significant differences in expression levels comparing plaques vs. normal arteries in the discovery dataset (Figure 16A), and this could be further validated in the smaller dataset. In particular, the expression of *ARG2* and *EDRNA* was strongly down-regulated in plaques (Figure 16A and B) (*ARG2* log mean difference±SD=-0.40 ± 0.50, $p<0.05$, *EDNRA* log mean difference±SD=-2.30 ± 0.81, $p<0.01$).

Genes associated with the ET-1/Arg system show dysregulation depending on symptoms of atherosclerosis

Next, patients from the discovery dataset were stratified based on clinical symptoms of stroke, TIA or *amaurosis fugax* prior to surgery as symptomatic (n=87) or asymptomatic (n=40). Interrogation for expression of genes of interest revealed that *EDN1* and *EDNRB* were significantly upregulated in plaques from symptomatic patients (*EDN1* log mean difference±SD=0.12 ± 0.27, $p<0.05$, *EDNRB* log mean difference±SD=0.45 ± 0.81, $p<0.01$) (Figure 16C). Gene expression was also determined in PBMCs isolated from symptomatic and asymptomatic patients. In this analysis, *ARG1*, *ARG2*, *EDNRA* and *EDNRB* were all upregulated in PBMCs from symptomatic patients (*ARG1* log mean difference±SD=0.11±0.17, p=0.0005; *ARG2* log mean difference±SD=0.29±0.58, $p<0.05$; *EDNRA* log mean difference±SD=1.56±0.94, $p<0.001$; ET_B log mean difference±SD=1.53±1.06, $p<0.001$; respectively) (Figure 16D).

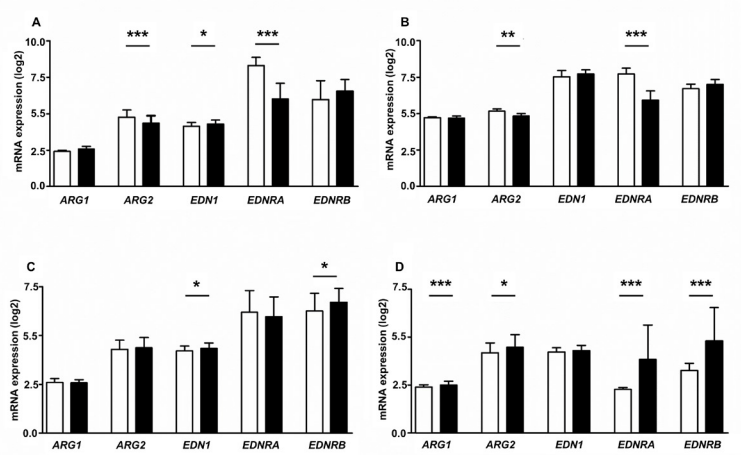

Figure 16. Upper row: Gene expression in normal arteries (white bars) and carotid atherosclerotic plaques (black bars) using (A) the discovery dataset and (B) the validation dataset. Lower row: Gene expression in (C) carotid atherosclerotic plaques and (D) peripheral blood monocytes obtained from asymptomatic (white bars) and symptomatic patients (black bars). Data are expressed as log2 with SD; *p<0.05, **p<0.01 and ***p<0.001. Abbreviations: *ARG*=arginase, *EDN1*=endothelin-1, *EDNRA*=endothelin receptor A, *EDNRB*=endothelin receptor B.

Of note, we also observed that *ARG1* mRNA displayed overall lower expression levels in both plaque tissue and PBMCs in comparison with *ARG2*.

Plaque localization of the ET-1/Arg system

To begin to delineate the localization of the ET-1/Arg system in plaques, mRNA expression levels for genes of interest from microarrays were first correlated to the levels of typical cell-specific markers and main processes in plaques. *ARG2* expression was significantly correlated with several markers of SMCs, macrophages and inflammation (Table 5). In addition, *ARG2* expression was significantly correlated with the expression of the *EDNRB* receptor.

Next protein levels and localization of the ET-1/arginase system were determined in individual endarterectomies by immunohistochemistry with antibodies against cell-specific markers (Figure 17). We observed widespread immunoreactivity for Arg1, Arg2, ET-1 as well as its receptors ET_A and ET_B in plaques. These proteins were all strongly expressed in the necrotic core, co-localizing with the macrophage markers CD163 and CD68. In addition, Arg2, ET-1 and ET_B receptors were also localized in vWF positive endothelial cells as well as in SMCs in the fibrous cap and remains of the media at the periphery of the plaque sections. Arg1 was detectable but mostly confined to the macrophage-rich areas and only weakly

Table 5. Correlations between expression of *ARG2* and markers of cells/processes in carotid atherosclerotic plaques from BiKE microarrays.
Abbreviations: PECAM-1=platelet endothelial cell adhesion molecule-1,RANK= Receptor activator of nuclear factor kappa-B, CD163=cluster of differentiation 163, IL1 **ß** =interleukin 1 **ß**, HIF1**α** =hypoxia-inducible factor1**α**, ET-1=endothelin-1, ET_A= endothelin receptor A, ET_B=endothelin receptor B, ECE1=endothelin converting enzyme, NOS=nitric oxide synthase, ARG=arginase.

Protein marker	*Gene Symbol*	Spearman r	P
Smooth muscle cells			
Smoothelin	*SMTN*	-0.3767	< 0.001
alpha smooth muscle actin	*ACTA2*	-0.3385	<0.001
Endothelial cells			
PECAM-1 (CD31)	*PECAM1*	0.3351	<0.001
von Willebrand factor	*VWF*	0.02935	0.74
Macrophages			
RANK	*TNFRSF11A*	0.4678	< 0.001
CD163	*CD163*	0.4072	< 0.001
Inflammation			
IL1ß	*IL1B*	0.4327	< 0.001
HIF 1α	*HIF1A*	0.6088	< 0.001
Endothelin/NO/Arginase pathway			
ET-1	*EDN1*	-0.1137	0.20
ET_A	*EDNRA*	0.1416	0.11
ET_B	*EDNRB*	0.3614	< 0.001
ECE1	*ECE1*	-0.01743	0.84
NOS1	*NOS1*	-0.16	0.07
NOS2	*NOS2*	-0.2309	<0.01
NOS3	*NOS3*	-0.0188	0.83
ARG1	*ARG1*	-0.02952	0.74

present in other cell types. ET-1 and the ET_A and ET_B receptors were co-localized with both isoforms of arginase in cells of the necrotic core, identified by the macrophage marker CD68 and sporadically also by alpha-smooth muscle cell actin (SMA).

ET-1 stimulates arginase activity and ROS production

Based on the co-localization of ET-1 and arginase in the atherosclerotic plaque, we investigated the functional interactions between the two *in vitro*. Human carotid endothelial cells (ECs) were stimulated with ET-1 and the expression of *ARG1* and *ARG2* was determined. ET-1 induced a rapid increase in *ARG2* mRNA expression already after 2 h of stimulation (Figure 18A) and this was accompanied by a 3-fold increase in arginase activity at 4 and 24 h (Figure 18B). In contrast, *ARG1* expression in ECs and THP-1 derived macrophages was consistently low and did not change in response to ET-1.

ET-1 also induced a significant increase in arginase activity in THP-1 derived macrophages although the mRNA expression of *ARG2* was not significantly increased (Figure 19A and B). The increase in arginase activity was blocked by macitentan and the selective ET_B receptor antagonist BQ788 (Figure 19B). The functional consequence of the increase in arginase activity by ET-1 stimulation was investigated by determination of ROS formation. Incubation of THP-1 derived macrophages with ET-1 increased ROS production by 30% (Figure 19C). The effect was completely blocked by the arginase inhibitor ABH.

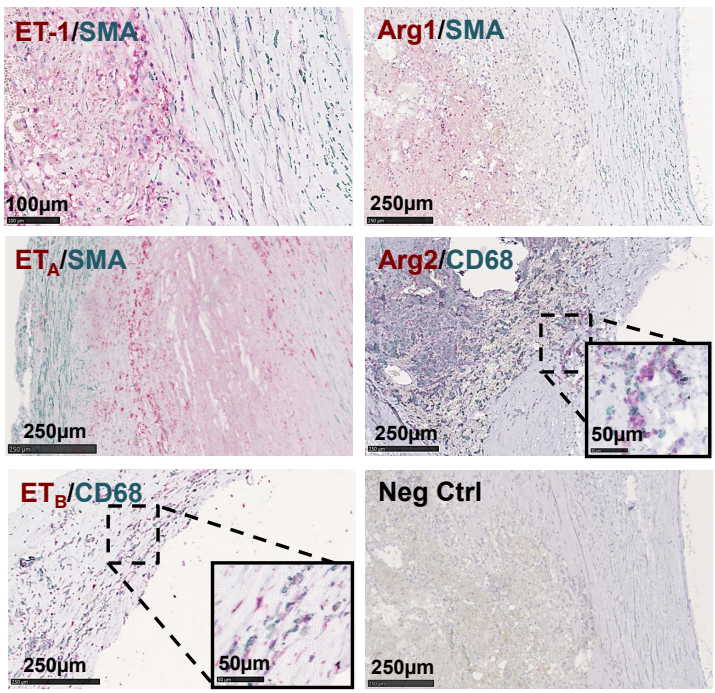

Figure 17. Double stainings of carotid atherosclerotic plaques by immunohistochemistry showing co-localisation of selected proteins. Abbreviations: Arg=arginase, ET-1=endothelin-1, ET_A=endothelin receptor A, ET_B=endothelin receptor B, vWF=von Willebrand factor, SMA=smooth muscle actin.

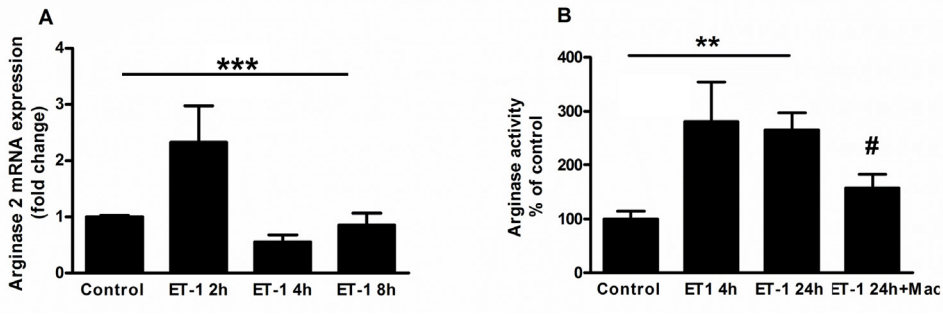

Figure 18. (A) Endothelial cell ARG2 mRNA expression and (B) arginase activity under control conditions and following stimulation with ET-1(1 nM, n=8) in the absence and presence of the dual ET receptor antagonist macitentan (10 μM, n=7). Data are given as mean and SEM and presented as relative change compared to control; **p<0.01, ***p<0.001 indicate significant changes over time, and #p<0.05 indicates significant difference vs. ET-1 24h.

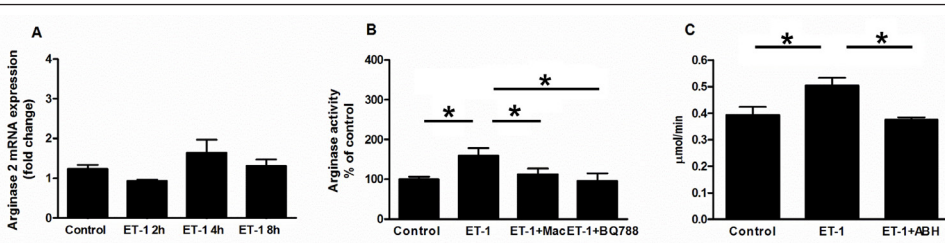

Figure 19. (A) ARG2 mRNA expression and (B) arginase activity in THP-1 macrophages under control conditions and following stimulation with ET-1 (1 nM, n=8) in the absence and presence of the dual ET receptor antagonist macitentan (10 μM, n=7) or the ETB receptor antagonist BQ788 (1 μM, n=7). (C) Production of reactive oxygen species by THP-1 cells under control conditions and following stimulation with ET-1 in the absence and presence of the arginase inhibitor ABH (1 mM, n=4). Data are given as mean and SEM and presented as relative change compared to control (A-B) or in absolute values (C); *p<0.05 indicate significant differences between groups.

DISCUSSION

Atherosclerosis is an enormous healthcare burden with high mortality and morbidity throughout the entire world. Endothelial dysfunction is thought to be the first step towards atherosclerosis and is also considered to be an integral part of the development of vascular complications in diabetes [14, 15]. Defined as decreased bioavailability of NO, endothelial dysfunction is probably the result of many different processes among which both ET-1 and arginase play important roles [59, 94]. This thesis explores these interactions utilizing several research methods; ranging from the cell, to the patient to a cohort of patients.

Effects of ET-1 blockade on endothelial function in patients with type 2 diabetes

Several studies have indicated that ET-1 plays a role in the development of vascular complications in diabetes. ET-1 levels are known to be higher in patients with diabetes mellitus [35] and endothelial function improves following acute intra-arterial administration of ET receptor blockers in patients with glucometabolic perturbations and manifest T2D [43, 107, 108] with or without manifestations of coronary artery disease. However, no previous study had addressed the question whether long-term oral administration of an ET receptor blocker improves endothelial function in patients with T2D and established vascular complications. The first study of this thesis therefore examined patients with T2D and microalbuminuria, who were randomly adminstered the oral endothelin receptor blocker bosentan or placebo. The results demonstrated that 4 weeks treatment with bosentan significantly improved digital microvascular function measured with the PAT signal compared to baseline and compared to placebo. These findings are relevant as previous studies have shown that a large part of the Endo-PAT signal during reactive hyperemia is dependent on NO availability [109, 110] and decreased RHI has been shown to be an independent risk factor for the development of cardiovascular events [91]. There was no change in brachial artery FMD which was measured simultaneously with the Endo-PAT. Previous studies have indicated that PAT and FMD provide distinct information regarding vascular function in smaller digital vessels vs. conduit arteries [111]. The results show that bosentan improves endothelial function in small vessels rather than the larger conduit arteries within the timeframe of the study. This is of particular interest in patients with T2D as they develop early disturbance in microvascular endothelial function [112]. Microvascular dysfunction in this patient category has been implicated in increased risk for cardiovascular outcomes [113].

These results indicate that ET receptor blockade may provide a beneficial therapeutic strategy to reverse microvascular dysfunction in T2D. A possible treatment effect associated with this outcome is a lower urine albumin/creatinine ratio. In this study we observed a non-significant trend towards a lowering of this ratio which is supported by a larger trial in which a reduction in urine albumin/creatinine ratio was observed in patients with diabetic nephropathy treated with the selective ET_A receptor blocker atrasentan [114].

The choice of ET receptor blocker is perhaps dependent on what condition is under consideration. Fluid retention is an issue with ET-1 receptor blocking. When the ET_A receptor is selectively blocked the renin angiotensin system is upregulated [115] with resulting fluid accumulation. When both receptors are blocked urine volume is reduced through an ET_B

receptor mediated mechanism in the kidneys [116]. Normally, the ET_A receptor is found in the smooth muscle cells of the vessel wall mediating vasoconstriction whereas the ET_B receptor is mostly found in the endothelial cells mediating vasodilatation and a smaller fraction present in smooth muscle cells mediating vasoconstriction. The net effect of ET-1 thus depends on the receptor localization and the balance of ET_A and ET_B receptors. Stimulation of the ET_A receptor causes vasoconstriction and increased oxidative stress, whereas stimulation of the ET_B receptor may cause vasodilatation in healthy subjects or vasoconstriction in patients with atherosclerosis due to upregulation of ET_B receptors on smooth muscle cells [24, 26]. Thus, the function of the ET_B receptor seems to change from stimulating vasodilatation via release of NO in healthy vessels to mediating vasoconstriction by activating receptors located on vascular smooth muscle cells in atherosclerosis.

The ET_B receptor is also upregulated on macrophages that are resident in the atherosclerotic plaque [29, 117] and in both macrophages and smooth muscle cells exposed to oxidized LDL [118]. An experimental study on mice demonstrated a marked reduction in atherosclerotic plaque formation when treated with a dual ET receptor blocker [119]. Unfortunately, the effect of selective ET_A inhibitor was not investigated in that study. Selective ET_A receptor blockade has been shown to increase coronary endothelial function in patients with atherosclerosis [44]. In another study selective ET_A receptor blockade improved microvascular nutritive capillary function in patients with T2D [43]. In a study including patients with insulin resistance and without known coronary heart disease only dual receptor inhibition showed improved endothelial function [107].

Collectively, available data suggest that both selective ET_A receptor blockade and dual ET_A/ET_B receptor blockade may improve endothelial function in the setting of T2D and CAD. However, a direct comparison has to date not been performed. This led to the design of study II in which the effect of selective ET_A receptor blockade vs. dual receptor blockade on ED was investigated in patients with T2D and manifest CAD. This is the first study where selective ET_A and dual ET_A/ET_B receptor blockers are compared in this setting in humans.

The main finding was that selective ET_A and dual ET_A/ET_B receptor blockade seemed to be equally effective. Both led to a marked increase in endothelium-dependent and endothelium-independent vasodilatation and there was no negative effect from blocking the ET_B receptor, perhaps owing to the changed role the receptor has in advanced vascular disease. The ET_B receptor is not only upregulated in the smooth muscle cells of the atherosclerotic plaque but also in the macrophages implying a possible role in generation of ROS. ROS in foamy macrophages have been linked to increased matrix metalloproteinase activity resulting in instability of the plaque [120]. Furthermore, this corrupted role of the ET_B receptor in individuals with vascular disease has been supported in a study of rats with pulmonary hypertension where the blood pressure drop was significantly larger in the group that received macitentan, a dual ET_A/ET_B receptor blocker, in addition to ambrisentan, an ET_A receptor blocker compared to ambrisentan alone. Conversely, when ambrisentan was added to macitentan vs macitentan alone there was no additional effect [121]. The increase in endothelium-independent vasodilatation has been shown in previous studies using ET receptor blockade and other interventions aiming at increasing NO bioavailability. In these studies endothelium-dependent and endothelium-independent vasodilatation increased in patients with T2D but not in patients without the disease [63, 107, 122].

Dual ET_A/ET_B blockade increased basal blood flow as well as the $TcPO_2$. Selective ET_A blockade on the other hand increased laser Doppler flow which might benefit wound healing, but available evidence is not convincing. The reason for the difference between the effects of the treatments are unclear. Laser Doppler measures total flow in the skin below the probe, both arterial and venous whereas $TcPO_2$ measures oxygen content in the skin. The response to ET-1 in the smaller vessels seems to be mainly dependent on ET_A and less so on the ET_B receptor [123]. In these smallest vessels, the vasodilatory response of ET_B receptor might still be significant and thus dual blockade not as effective. The increase in the $TcPO_2$ with dual blockade might indicate a potential target population of patients with diabetic foot ulcers. In a recent meta-analysis $TcPO_2$ was a reliable predictor of the healing of foot ulcers in diabetic patients as well as predicting risk of future amputations [124].

Effects of inflammation on EPC numbers in patients with T2D

Several different pathological conditions including atherosclerosis, diabetes, hyperlipidemia and hypertension lead to a reduced number of EPC [125]. Possible mechanisms could be exhaustion of the pool of progenitor cells in the bone marrow, impaired functional capacity within the bone marrow, reduced mobilization of EPC, or reduced survival and/or differentiation of mobilized EPC [126].

In contrast to the above there are a number of studies involving physiological and pathological stimuli that increase EPC levels. The factors that recruit EPC are insulin-like growth factor-1, growth hormone, GCSF, VEGF and stromal-derived factor-1 [127, 128]. Vascular inflammation has also been linked to increased EPC numbers. In a study from 2004, CRP levels correlated to EPC in patients with unstable angina [129]. Another study, involving exogenous vascular injury, linked increased EPC numbers with local vascular inflammation [130].

The main findings in study III were that in patients with T2D and vascular disease, high levels of ET-1 were associated with a higher number of EPC. Patients with higher ET-1 levels also had a higher CRP as well as higher systolic blood pressure, thus supporting the view that vascular inflammation triggers the recruitment of EPC. It has been shown in previous work that ET-1 is associated with hypertension and vascular inflammation [131].

Higher ET-1 levels were also associated with increased waist circumference in the present study, supporting previous work that indicated that being overweight, in the absence of acute vascular events, is associated with vascular inflammation as well as with increased EPC numbers [132].

In the present study there was no effect on the number or viability of EPC with oral ET_A/ET_B receptor blockade with bosentan. This observation suggests that recruitment of EPC does not seem to be regulated by ET-1 via a receptor-dependent mechanism. Other pharmacological compounds have been linked to beneficial effects on the number and function of EPC [133]. Few of these studies were randomized, but statins [134], angiotensin II receptor antagonists [135] and pioglitazone [136] have been shown to positively affect EPC in placebo-controlled randomized studies.

Together these observations support the theory that vascular inflammation is associated with increased levels of ET-1 and EPC. In this context it is important to note that only circulating EPC counts have been investigated. Earlier studies have shown the positive association between inflammation and the number of circulating EPC, but it has also been shown by different investigators that inflammation impairs EPC function [137]. Although the results were negative it might have been because the treatment time was only four weeks, a longer time period could be necessary to allow for the inflammation to decrease. Another possibility is that even though EPC numbers did not increase, their function might have improved.

ET-1 and arginase in human atherosclerotic plaques

Both ET-1 and arginase exert pro-inflammatory effects in the cardiovascular system and promote the development of atherosclerosis [31, 58]. Both reduce the bioavailability of NO and their expression is increased by risk factors for atherosclerosis. Although extensively studied by various groups, a possible connection between the two has never been established. Because NO bioavailability is similarily affected by ET-1 and arginase, it has been speculated that effects of ET-1 are dependent on arginase. Utilizing expression data from a unique biobank of human carotid atherosclerotic plaques, the approach encompassed an integrative analysis of members of the ET and arginase families. In our analysis we found that ET-1 along with its receptors, ET_A and ET_B and arginase, especially Arg2, were abundantly expressed in human atherosclerotic plaques. Both ET-1 and Arg2 were expressed in macrophages and endothelial cells as well as those smooth muscle cells associated with the fibrous cap. These observations support the notion that ET-1 and Arg2 may be of importance for plaque development and instability. The presence of ET-1 and arginase has previously been identified in atherosclerotic lesions [138, 139] but this is the first time they have been demonstrated to co-localize in human atherosclerosis.

The driving forces of atherosclerosis are the dysfunction of endothelial cells followed by an influx of monocytes that later turn into macrophages. The regulation of endothelial cells and macrophages is dependent on NO production and the counter-acting effects of arginase. Both have receptors for ET-1. Study IV demonstrated the presence of ET-1 and arginase in plaque areas dense with macrophages and in the endothelium. The functional interactions between ET-1 and arginase were investigated *in vitro*. Interestingly, ET-1 induced upregulation of *ARG2* expression and arginase activity in endothelial cells and increased arginase activity in THP-1-derived macrophages. To the best of our knowledge, this is the first demonstration of a regulatory effect of ET-1 on arginase expression and activity.

The stimulatory effect of ET-1 on arginase in endothelial cells is of interest considering the ability of ET-1 to reduce the bioavailability of endothelial NO and endothelium-dependent vasodilatation in patients with coronary atherosclerosis [140]. Arginase is known to be a critical regulator of NO production due to competition for the substrate L-arginine in endothelial cells [66]. Thus, the present findings indicate that increased arginase activity is an important mechanism by which ET-1 induces endothelial dysfunction. The importance of the interaction between ET-1 and arginase for endothelial cell and NO production is of interest to explore further in future studies.

In addition, THP-1-derived macrophages responded to ET-1 stimulation by increasing arginase activity as well as increased ROS production. No significant *ARG1* expression was detected after stimulation of THP-1 derived macrophages in this experiment, while *ARG2* expression was clearly present but not increased in a statistically meaningful way. Induction of Arg2 has been reported to reflect a polarization towards the pro-inflammatory subtype macrophage, M1 [141], while Arg1 has been associated with M2, the anti-inflammatory subtype [142]. However, the functional role of arginase in macrophages is complex and still elusive.

Arginase has previously been implicated in ROS production, which has been shown to take place in the mitochondria of macrophages [143]. Interestingly, it has been suggested that Arg2 promotes the pro-inflammatory responses of macrophages through mitochondrial ROS contributing to plaque formation [141]. Inhibiting Arg2 may thus represent a potential therapeutic strategy in atherosclerosis, especially as there is evidence that macrophages retain their plasticity after differentiation to one subtype [142].

Study IV provided clear evidence for an upregulation of ET-1 and ET_B receptor in carotid plaques from symptomatic patients. Furthermore, PBMCs of patients with recent symptoms had increased expression of the ET_B and ET_A receptors. Monocytes are paramount in atherogenesis and their influx into the artery wall and subsequent development into macrophages is vital for plaque formation [144]. The increase in ET_B receptor expression in plaques from symptomatic patients is in line with the previous observation of a change in the function of the ET_B receptor in atherosclerosis. Thus, the ET_B receptor contributes to vasoconstrictor tone and endothelial dysfunction in patients with atherosclerosis [26, 145, 146]. The connection to a more acute presentation of atherosclerosis implies an enhanced role of ET-1 and especially the ET_B receptor.

PERSPECTIVE

Taken together, the evidence points towards an enhanced role of ET-1 in the unstable plaque via the ET_B receptor in the proatherogenic environment. The clinical studies I and II indicate that blocking both the ET_A and ET_B receptors in patients with manifest vascular disease improves endothelial function. Compared to selective ET_A blockade, dual blockade increased skin oxygenation and is a possible treatment for chronic ulcers in patients with T2D as was suggested in a case report [147]. Study IV also implies that ET-1 has a role in plaque instability via increased production of ROS through Arg2. It also shows a possible link to the immune system as seen in the increased expression of both ET-1 and ET_B receptor in PBMC.

If we try to follow events in a possible scenario of atherosclerosis, risk factors increase the amount of ET-1 that causes an influx of primed monocytes into the vessel wall through the dysfunctional endothelium. On site the monocyte is influenced into the M1 phenotype through an Arg2 dependent pathway. The results indicate that both ET-1 and Arg2 are more involved in carotid plaques from symptomatic patients. As study IV showed, there is an increase in ET_B receptor in the plaque tissue of patients with more recent symptoms. It also showed that macrophages, when stimulated with ET-1, increased their ROS production. This increased ROS production has been shown to promote plaque instability [120]. Together with the results of the first and second studies this points toward an enhanced role of ET-1 and ET_B receptor in patients with diabetes and vascular disease.
The big question is whether there is a real therapeutic opportunity in either the ET-1 receptor blockers or an arginase inhibitor? Both systems are widespread throughout the body and thus a variety of different side-effects are possible. ET-1 receptor blockers are already established treatments for a limited number of disease states such as pulmonary vascular hypertension [148] and vascular complications of scleroderma [149]. In addition there is an ongoing trial aiming to determine wether the selective ET_A blocker atrasentan can reduce the risk of progression and delay the onset of end-stage renal disease in patients with T2D and nephropathy [150].

Studies with arginase inhibitors have shown promising results in human subjects. Intra-arterial infusion of nor-NOHA, an arginase inhibitor, improved endothelial function in patients with coronary artery disease with and without T2D [66]. Furthermore, arginase inhibition improved cutaneous microvascular function in patients with T2D and microvascular complications [151]. Other studies demonstrated improvement in endothelial function in patients with hyperlipidemia [152, 153]. Collectively, these observations indicate that arginase inhibition may be beneficial in human cardiovascular disease and T2D.

As a best case scenario the therapies would target the unstable atherosclerotic lesion. This treatment, in addition to traditional risk factor management and a healthier lifestyle, has the promise of reducing the incidence of the most severe manifestations of atherosclerotic disease.

Closer in the future is the possibility of shortening wound healing time in patients with diabetes foot ulcers using a dual ET_A/ET_B receptor blocker, a class of drugs already in use [149]. The ever increasing group of patients with T2D presents modern healthcare with a deluge of wounds needing costly long term management.

An important point to consider regarding drug therapies are side effects. ET-1 blockers are already in use with established side effects that include fluid retention, edema, increase in liver transaminases, headaches and facial flushing [154]. Arginase inhibitors are still in experimental phase. In these small trials the inhibitors seem to be well tolerated but larger studies are needed for a full profile of side effects [152]. A possible cause of concern is the disruption of the hepatic urea cycle which is vital for the individual. A number of different inhibitors have been developed but none are selective for either type of the enzyme [155].

LIMITATIONS

The studies of this thesis have several limitations, including a lack of female participants, study size, study duration and control factors.

In spite of efforts, the number of female participants in the studies is low. There seem to be gender differences in the contribution of the ET_B receptor in cutaneous vascular tone of healthy individuals [156]. Middle aged men seemed to have an increased ET_A mediated vasoconstrictor tone compared to women [157] although vasoconstrictor response to ET-1 was similar between the sexes. A possible cause is estrogen, as estrogen replacement therapy was shown to decrease the circulating levels of ET-1 in a study of postmenopausal women [158].

In studies I and III the same cohort was used and the sample size was small. Owing to technical issues the final number of samples available for analysis in study III was even smaller. Another possible weakness is the study duration. Four weeks is possibly too short to make a significant impact on a chronic disease like T2D and perhaps the reason that improvement was only detected in microvascular function and not macrovascular function.

Blood pressure decreased significantly in the treatment group compared to the control group. Tailoring a study where blood pressure reduction in the control group is the same, using a drug without vasoactive properties, is complicated.

In study II the number of patients is arguably low but there is no indication that a larger group would have changed the outcome as the effect was similar to the prediction in the power calculation.

In study IV the number of healthy arteries used as controls is low and from another anatomical location than the diseased carotid plaques. For *in vitro* studies, macrophages obtained by differentiating THP-1 cells were used, which is an immortalized monocyte-like cell line and not *ex vivo* obtained monocytes from individuals.

CONCLUSIONS

I. Four weeks oral treatment with the dual ET_A/ET_B receptor blocker bosentan improved peripheral microvascular endothelial function in patients with type 2 diabetes and microalbuminuria.

II. Both selective ET_A dual ET_A/ET_B receptor blockade markedly improved endothelial function in patients with coronary artery disease and type 2 diabetes.

III. ET-1 and Arg2 are co-expressed in human atherosclerotic plaques. ET-1 stimulated Arg2 expression and activity in endothelial cells as well as ROS formation in macrophages via an arginase-dependent mechanism. These results indicate an important interaction between the ET pathway and arginase in human atherosclerotic plaques.

IV. Among patients with type 2 diabetes and vascular disease, high levels of ET-1 are associated with higher numbers of EPC. The recruitment of EPC does not seem to be regulated by ET-1 receptor activation since treatment with a dual ET-1 receptor blocker did not affect circulating EPC numbers.

OVERALL SUMMARY AND CONCLUSION

Long term ET_A/ET_B receptor blockade improved the microvascular function in patients with T2D and CAD. Adding ET_B on top of selective ET_A blocker did not seem to improve endothelium-dependent vasodilatation although a small but significant effect was seen on tissue oxygenation compared to selective ET_A blockade. These effects might have therapeutic implications when it comes to treatment of diabetic microvascular complications. As illustrated in Figure 20, ET-1 has an integral part in the development of atherosclerosis and the progression of diabetic complications. Part of the effect might be through increased arginase expression and activity. This may indicate possible future targets in treating or hindering the progression of atherosclerotic disease.

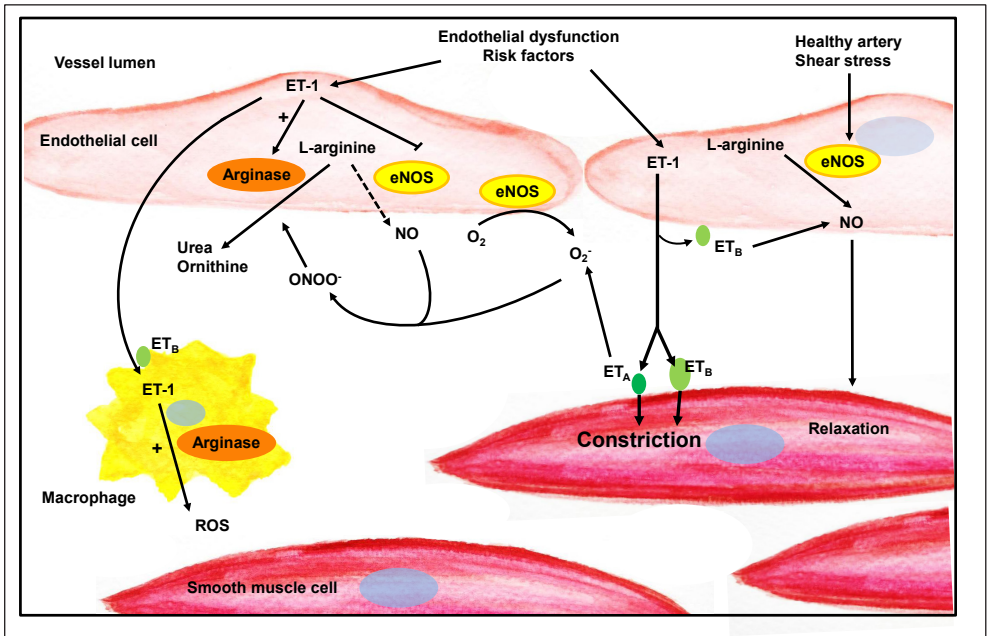

Figure 20. Schematic illustration of the roles of ET-1 and arginase and their interplay in endothelial cells and macrophages. ET-1 is upregulated by traditional risk factors. In addition, ET_B receptors are increased in vascular smooth muscle cells and on macrophages which contributes to increased vascular tone, impaired endothelial function and oxidative stress. ET-1 stimulates Arg2 expression and activity in endothelial cells as well as ROS formation in macrophages via an arginase-dependent mechanism. ET-1= endothelin 1, ET_A=endothelin receptor A, ET_B= endothelin receptor B, eNOS= endothelial nitric oxide synthase, NO= nitric oxide; $ONOO^-$=peroxynitrite, ROS= reactive oxygen species.

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