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Abstract

Myocardial infarction and stroke are two of the most common causes of death in the world, mainly caused by a rupture of an atherosclerotic plaque. A vulnerable atherosclerotic plaque is a high-risk plaque that can form a thrombus by rupture. The phenotype of a plaque prone to rupture includes a thin fibrous cap, a large lipid core, accumulation of macrophages, a fissured fibrous cap and high-grade stenosis.

The aim of this thesis has been to investigate plaque vulnerability and vascular repair processes. We studied androgen-deprivation therapy for prostate cancer and its effects on plaque vulnerability in a mouse model of atherosclerosis where we alter shear stress to induce plaques with advanced or fibrous phenotypes. Androgen-deprivation therapy has been associated with increased risk for development of cardiovascular events and recent pooled analyses suggest that this primarily is the case for patients with pre-existing cardiovascular disease treated with gonadotropin-releasing hormone receptor agonists. In the present study, the gonadotropin-releasing hormone receptor agonists caused necrosis-like areas in fibrous plaques from ApoE deficient mice after 4 weeks of treatment. This suggests that there are direct drug related interactions with the atherosclerotic plaque. The effects of androgen-deprivation therapy on cardiovascular disease need to be further studied.

In the same mouse model just mentioned, we studied IL-22 deficiency in vascular tissue repair. IL-22 attenuates plaque development in advanced plaques, but not in fibrous lesions, and appears to ease the differentiation of SMC that promotes early plaque growth. The specific role that IL-22 plays in atherosclerosis is yet to be determined. We do know that IL-22 has been found in abundance in carotid plaques from symptomatic patients. Patients with acute myocardial infarction have also been found with a remarkable rise in IL-22 plasma levels, compared with patients with controls.

Diabetes is a riskfactor for cardiovascular disease. To investigate vascular repair mechanism in hyperglycemia per se, without the involvement of dyslipidemia and subsequent inflammation, we induced carotid neointimal hyperplasia in the Akita mouse. We found that hyperglycemia does not alter vascular repair in our mouse model of neointimal hyperplasia

After having to discard a study using diabetic OPN deficient mice, we have isolated and tested OPN deficient beta cells to gain a better understanding of the role of OPN in beta cell function. Our study showed that OPN deficiency in the mouse pancreatic beta cells results in negative alterations of beta-cell physiology that appear to be compensated for in a healthy physiological state. In the OPN-/- mouse we detected three important alterations in the beta cells: elevated Ca2+ levels, morphological modifications of the endoplasmic reticulum and altered insulin vesicle localization. These findings indicate that reduced levels of OPN could in the long run be a factor contributing to development of beta cell failure.

Key words: Atherosclerosis, ADT, shear stress, restenosis, plaque, diabetes

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For you

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Introduction

Stability is Key in Atherosclerosis

Atherosclerosis is the underlying disease behind cardiovascular events such as myocardial infarction and stroke. Atherosclerotic plaques begin to build up at an early age and continue to progress throughout our lives, but do not necessarily give any symptoms. Why is it that some atherosclerotic plaques cause clinical symptoms and others remain silent a lifetime?

The main focus of my thesis has been to study atherosclerotic plaque stability and vascular repair in an experimental mouse model. Atherosclerosis occurs at sites with disturbed blood flow and is a dysfunctional chronic inflammatory disease of the arterial wall. Arterial inflammation, repair mechanisms and their mediators play important roles for the stability of a plaque.

There are risk factors that can destabilize plaques making them more prone to rupture and subsequently cause clinical events. In this thesis I will lead you through androgen deprivation therapy and diabetes as cardiovascular risk factors. We will also explore the vulnerable plaque and factors that affect vascular repair.

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Original Papers

I. **Anki Knutsson**, Sabrina Hsiung, Selvi Celik, Sara Rattik, Ingrid Yao Mattisson, Maria Wigren, Howard I. Scher, Jan Nilsson, Anna Hultgårdh-Nilsson. *Treatment with a GnRH receptor agonist, but not the GnRH receptor antagonist degarelix, induces atherosclerotic plaque instability in ApoE-/- mice.* Scientific Reports, Article number: 26220 (2016) doi:10.1038/srep26220

II. **Anki Knutsson**, Sabrina Hsiung, Anna Roxå, Ellen Andersson, Sara Rattik, Uwe Rauch, Jakob Larsson, Jan Nilsson, Anna Hultgårdh-Nilsson. *IL-22 deficiency reduces progression of advanced atherosclerotic carotid plaques in ApoE deficient mice.* Manuscript.

III. **Anki Knutsson**, Sabrina Hsiung, Oscar van der Have, Jakob Larsson, Vignesh Murugesan, Uwe Rauch, Jan Nilsson and Anna Hultgårdh-Nilsson. *Decreased elastin content correlates with increased growth of carotid lesions in diabetic Akita mice.* Submitted manuscript.

IV. Anna Wendt, Inês G. Mollet, **Anki Knutsson**, Victor Strevens-Bolmgren, Anna Hultgårdh-Nilsson, Maria F. Gomez and Lena Eliasson. *Osteopontin affects insulin vesicle localization and Ca2+ homeostasis in mouse pancreatic beta cells.* Submitted manuscript.

Papers not included in this thesis

I. Bond AR, Hultgårdh-Nilsson A, **Knutsson A**, Jackson CL, Rauch U. *Cartilage oligomeric matrix protein (COMP) in murine brachiocephalic and carotid atherosclerotic lesions.* Atherosclerosis. 2014 Oct;236(2):366-72. doi: 10.1016/j.atherosclerosis.2014.07.029.

II. Engelbertsen D, Rattik S, **Knutsson A**, Björkbacka H, Bengtsson E, Nilsson J. *Induction of T helper 2 responses against human apolipoprotein B100 does not affect atherosclerosis in ApoE-/- mice.* Cardiovasc Res. 2014 Jul 15;103(2):304-12. doi: 10.1093/cvr/cvu131. Epub 2014 May 27.

III. Shami A, **Knutsson A**, Dunér P, Rauch U, Bengtsson E, Tengryd C, Murugesan V, Durbeej M, Gonçalves I, Nilsson J, Hultgårdh-Nilsson A. *Dystrophin deficiency reduces atherosclerotic plaque development in ApoE-null mice.* Sci Rep. 2015 Sep 8;5:13904. doi: 10.1038/srep13904.

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Abbreviations

α-SMA	smooth muscle α-actin
ADT	androgen deprivation therapy
AGE	advanced glycation end products
ApoB	apolipoprotein B
ApoE	apolipoprotein E
ATP	adenosine triphosphate
CD	cluster of differentiation
CVD	cardiovascular disease
DHT	dihydrotestosterone
EC	endothelial cell
ECM	extracellular matrix
eNOS	endothelial nitric oxide synthase
FoxP3	forkhead box protein 3
FSH	follicle-stimulating hormone
GnRH	gonadotropin-releasing hormone
HDL	high-density lipoprotein
HFD	high fat diet
HSPG	heparan sulfate proteoglycan
IDL	intermediate-density lipoprotein
IFN-γ	interferon gamma
IL	interleukin
IL-10RA2	IL-10 receptor alpha 2
IL-22BP	IL-22 binding protein
IL-22R	IL-22 receptor
IL-22RA1	IL-22 receptor alpha 1
IL-22RA2	IL-22 receptor alpha 2
ILC	innate lymphoid cell
iNOS	inducible nitric oxide synthase
L	ligand

LDL	low-density lipoprotein
LDLr	LDL receptor
LH	luteinizing hormone
LHRH	luteinizing hormone-releasing hormone
LOX	lysil oxidase
LRP1	LDLr related protein 1
MHC	major histocompatibility complex
MMP	matrix metalloproteinases
NO	nitrogen oxide
NOS	nitric oxide synthase
OPN	osteopontin
oxLDL	oxidized LDL
PDGF	platelet-derived growth factor
RORγt	retinoid-acid receptor-related orphan receptor gamma t
SMC(s)	smooth muscle cell(s)
SLRP	small leucine-rich repeat proteoglycan
STAT3	signal transducer and activator of transcription 3
STZ	streptozotocin
T1D	type-1 diabetes
T2D	type-2 diabetes
TGF-β	tumor growth factor-β
Th cell	T helper cell
TIMP	tissue inhibitor of MMPs
TNF-α	tumor necrosis factor alpha
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VLDL	very low-density lipoprotein

Background
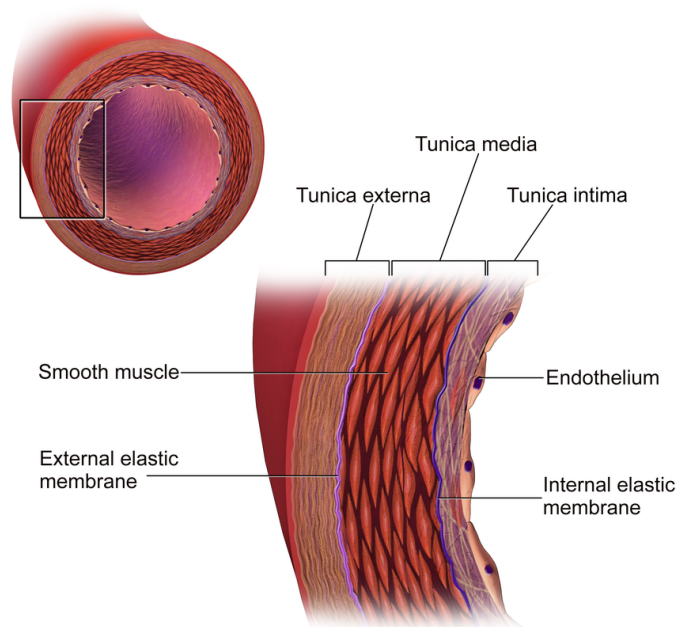
Figure 1. A healthy artery wall made up up three distinct layers. Image courtesy of Blausen 2014[1].

Atherosclerosis

The healthy artery

More than just pipes that carry fluid

Arteries transport blood from the heart supplying the body with oxygen and nutrients. The walls of the arteries are well adapted to their function; the vascular extracellular matrix (ECM) provides the mechanical properties needed such as elasticity, compressibility and tensile strength. As the heart pumps blood into the

arteries their elasticity allows them to stretch and accommodate much of the stroke volume. During diastole, the elastic fibers recoil and the arteries help push the blood onward. Except for giving the artery its mechanical properties, the ECM provides a ground for biological processes[2]. The vascular ECM is composed of collagens, proteoglycans, elastin and glycoproteins; each playing an important part in cell adhesion, migration and proliferation and is crucial in the repair process of an injured artery. The ECM exists in a balance that gives the arteries their unique properties. To maintain a healthy artery, a balance between synthesis and degradation of the ECM is necessary. If this balance is broken it can give rise to an altered ECM prone to vascular disease.

The structure of the artery wall consists of three distinct layers separated from each other by a dense layer of fenestrated elastin; the intima, media and adventitia. Each layer has a distinct function and arrangement of cells and ECM (figure 1).

The intima, the innermost layer facing the lumen, consists of an endothelium that sits upon a basement membrane. The basement membrane is a compartment of connective tissue enriched in laminins, fibronectin, hyaluronan and types IV and VIII collagen[3,4].

The media consists of vascular smooth muscle cells (SMCs) with an ECM consisting of collagen types I, III, V and XVIII, proteoglycans and fibronectin. In the healthy arterial wall, fibrillar collagen type I and III provide the tensile strength, while proteoglycans and hyaluronan give the arterial wall viscoelasticity, making the vessel sustainable to compression[5]. The contractile capacity of SMCs control the vascular tone and hence the blood pressure. The SMC can also exist in a synthetic phenotype to assist in rapid vascular tissue repair, as is discussed on page 20 under the section 'vascular repair'.

The adventitia, the outermost coat, is composed of fibroblasts, nerves and small blood vessels incorporated in an ECM of collagen types I and III, elastic fibers and vasa vasora[3,6].

Atherogenesis and vascular repair

Hypotheses on the initiation of the atherosclerotic plaque
There are hypotheses as to what happens at the very beginning of atherogenesis.

Ross and Glomset[7] proposed the 'response-to-injury' hypothesis where the initial step in atherogenesis is endothelial denudation that alters the adhesiveness for leukocytes and platelets. Shear stress then aids the desquamation of endothelium. The resulting injury causes adherence and aggregation of platelets at the site. Degranulating platelets release mitogenic factors that result in intimal proliferation of SMCs. This

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results in the synthesis of new ECM proteins by synthetic SMCs and often the deposition of intracellular and extracellular lipids.

The 'response-to-retention' hypothesis was presented by Tabas and Williams in 1995[8,9]. This hypothesis concludes that lipoprotein retention is the initiating step in the development of atherosclerosis. The retention of lipoproteins within the arterial wall is tightly linked to proteoglycans of the ECM. Apolipoprotein B-100, a protein associated with LDL, is retained within the arterial wall in areas with diffuse intimal thickening rich in proteoglycans; for example in areas of disturbed shear stress. Retained lipoproteins are susceptible to modifications such as oxidative or enzymatic[10]. Modified lipoproteins gain new epitopes, such as oxidized phospholipids, that are recognized by scavenger receptors as well as toll-like receptors. This activates and promotes the production of inflammatory cytokines and chemokines[11-13].

The concept of oxidative-modification hypothesis focuses on native lipoproteins not being atherogenic[14,15]. Modified lipoproteins, however, are readily internalized by scavenger receptors on macrophages that in turn initiate the cascade of events leading to atherosclerosis.

Atherosclerotic plaque development

Independent of what actually happens at the very start of atherosclerosis, the outcome is the same.

In the atherosclerosis prone artery there is an alteration of the composition of the ECM and a diffuse intimal thickening appears, consisting mainly of SMCs, elastin and proteoglycans[16,17]. Lipoproteins, such as LDL particles, invade the intima and adhere to proteoglycans[18]. Monocytes present in the vascular wall clear LDL through LDL receptors. Proteoglycans, having a high affinity for lipoproteins, retain lipoproteins in the intima[19]. Changes to the endothelium, such as altered shear stress, increase permeability and allows for a greater influx of LDL to the intima[20-22]. The retained LDL particles are susceptible to modification by oxidation or enzymes that in turn release phospholipids that can activate the endothelial cells of the intima. The expression of adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), E-selectin and P-selectin, are upregulated in activated endothelial cells and contribute to monocyte and platelet adherence[23-25]. Activation of the intimal endothelial cells can also be caused by patterns of hemodynamic flow, in particular low shear stress or oscillatory shear stress. These are found in the inner curvatures of arteries or near bifurcations respectively[26].

LDL receptors on monocytes and macrophages do not recognize modified LDL, but scavenger receptors do. The scavenger receptors do not have a negative feedback mechanism meaning that macrophages can continue to ingest modified LDL until they become foam cells[27,28]. The macrophages secrete high levels of proinflammatory

cytokines, growth factors and proteases that augment the ongoing inflammation and alter the environment and the composition of the ECM[29].

The SMCs of the media, that are kept differentiated in a healthy artery by the surrounding ECM, are all of a sudden surrounded by a cocktail of cytokines, growth factors, proteins and proteases[3,30]. This environment promotes a shift from a differentiated contractile state to modified SMC that gains the ability to migrate, proliferate and synthesize ECM proteins[3,30,31].

A cap rich in collagen, synthesized by SMCs, covers the atherosclerotic plaque, giving it stability and protection against rupture. Simultaneously as it is being built, the fibrous cap is exposed to matrix degrading enzymes produced by the cells of the atherosclerotic plaque[32,33]. The balance between cap synthesis and degradation is essential for keeping the plaque stable.

Vascular repair

The process of atherosclerotic plaque development as a response to injury has a close resemblance to that of wound healing that goes through the phases of inflammation, proliferation and remodeling[34]. In the initial steps of plaque development, as described above, activation of the endothelium recruits immune cells to the site of injury. SMCs are very plastic and can switch phenotypes[31]. In the media of an uninjured artery, the SMCs are contractile and express proteins involved in contraction, such as α-actin and myosin heavy chain. The contractile phenotype is a sign of a healthy artery, and thereof are contractile proteins widely used as markers for differentiated SMCs. Cytokines and growth factors, such as platelet derived growth factor (PDGF), released by activated endothelial cells and macrophages promote the modulation, or phenotypic switch, of the SMC[31]. During the switch to a synthetic phenotype, the SMCs lose much of their myofilaments, α-actin and have a higher number of Golgi apparatus and endoplasmic reticulum[5]. The modulation alters the protein expression of the SMCs that start to produce ECM components, growth factors and matrix-degrading enzymes. Synthetic SMCs are considered de-differentiated[35]. It is important to point out that modulated SMCs are a heterogeneous population. They can linger in an intermediate differentiated state, and be both contractile and synthetic at the same time[36]. The modulated synthetic SMCs can migrate from the media to the intima and produce various ECM proteins such as elastin and collagen that build the protective fibrous cap.

The vulnerable plaque

A vulnerable atherosclerotic plaque is a high-risk plaque that can form a thrombus, either by rupture or erosion. The phenotype of a plaque prone to rupture includes a thin fibrous cap, a large lipid core, the accumulation of macrophages, a fissured fibrous caps and high-grade stenosis[37]. Erosion causes endothelial denudation that exposes the subendothelial ECM. Damage to the atherosclerotic plaque exposes the highly thrombogenic plaque content to the blood stream resulting in thrombus formation[38]. Plaque ruptures account for about 60-80% of coronary artery events whereas endothelial erosion accounts for 20-40%.

Plaque rupture and erosion

Plaque rupture

The protective fibrous cap covering the atherosclerotic plaque is subjected to degradation (figure 2). It consists mainly of collagen type I, and SMCs that synthesize and maintain the matrix proteins of the fibrous cap. Matrix metalloproteinases (MMPs) produced by macrophages compromise the tensile strength of the fibrous cap[32,33,39]. It is crucial that there is a balance between synthesis and breakdown is necessary to maintain the integrity of the fibrous cap. Studies have shown that lesions of fatal thrombi in coronary arteries contain a reduced amount of mature cross-linked collagen and higher levels of collagen-degrading enzymes[39]. The accumulation of macrophages in a plaque naturally increases the grade of inflammation and as a result increased production of matrix degrading enzymes that thin the fibrous cap[37,40,41]. A vulnerable plaque is also characterized by having a lipid-filled necrotic core. The lipid core is created by 1) the extracellular trapping of LDL particles, like explained in the response-to-retention-hypothesis, and 2) from apoptotic/necrotic macrophages leaving behind a pool of extracellular lipids and cellular debris[42,43]. Defective efferocytosis, the clearance of the apoptotic cells, further triggers plaque necrosis[44]. A large lipid core is often accompanied by a thin fibrous cap[37].

Plaque erosion

Plaques with endothelial erosion differ phenotypically from those that are prone to rupture. They tend to lack the large lipid core and contain fewer inflammatory cells (figure 2). Proliferating SMCs are found along with a matrix rich in proteoglycans and hyaluronan, and neovascularization[39]. The two very different phenotypes of 'a vulnerable plaque' suggest that the pathologic mechanisms may differ between these two conditions. The inciting action that causes endothelial erosion has recently been pointed towards innate immunity. Endothelial cells express the Toll-like receptor-2 (TLR-2)[45] that upon ligand binding results in endothelial cell production of reactive-
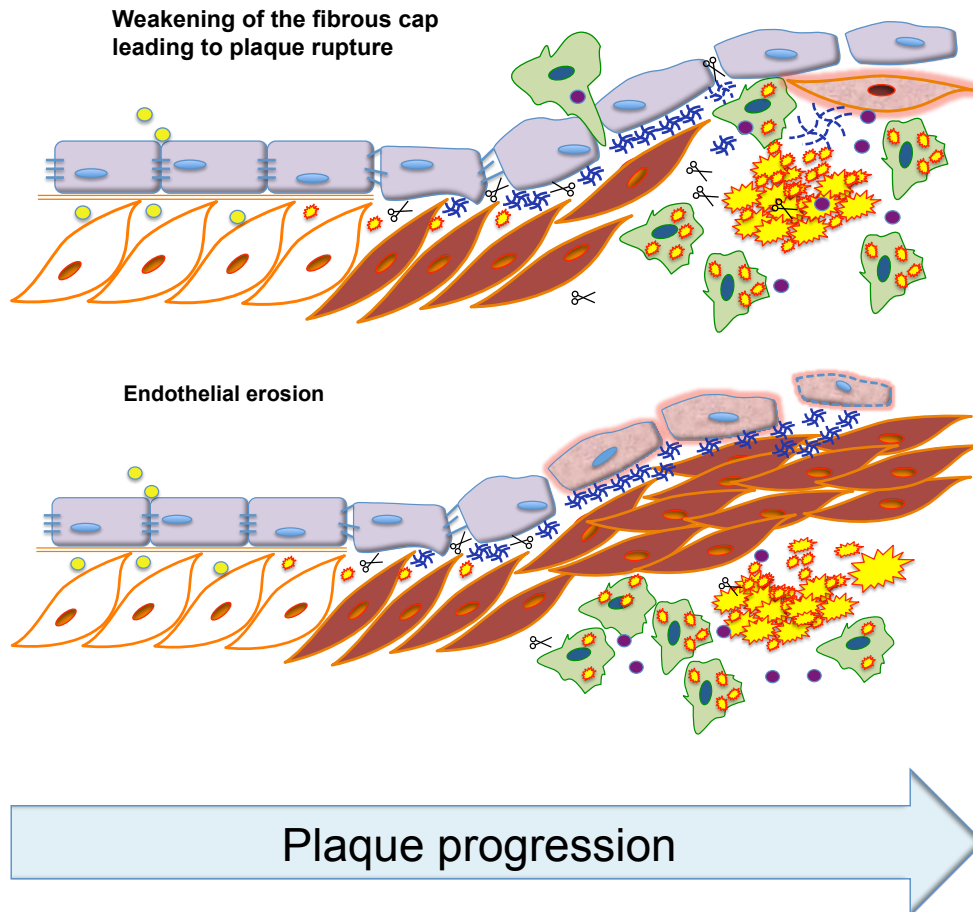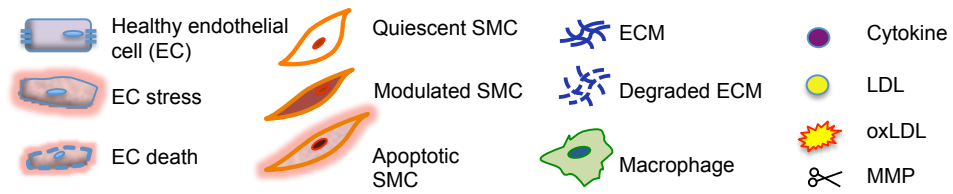
Figure 2. Atherosclerotic plaque development. The top figure shows the initiation of a plaque to the breakdown of the fibrous cap. The bottom figure shows the initiation of a plaque that ends with endothelial denudation.

oxygen species, ER stress and apoptosis[46]. Neutrophils are found at sites of endothelial erosion and studies have found them to augment the initial endothelial cell reaction to TLR-2 binding. Plaques with endothelial denudation have been found to be rich in hyaluronan that is one of the ligands to TLR-2. Quilard and coworkers have shown *in vitro* that TLR-2 agonism increased the activity of MMP-2 and -9 that are type IV collagenases that could degrade the basement membrane to which endothelial cells attach. As the endothelial cells die they release tissue factor and expose the subendothelial matrix to the blood stream, resulting in thrombi formation[46].

Lipids and cholesterol

Elevated plasma cholesterol is an independent risk factor for the development of atherosclerosis. Patients with familiar hypercholesterolemia have accelerated lesion formation even in the absence of other risk factors[47,48]. Cholesterol and triglycerides are essential in various biological processes and in order to be transported in the aqueous circulation they need to be packaged as lipoproteins due to their hydrophobic nature (table 1).

ApoB is the major protein component of all lipoproteins except HDL[49]. It occurs in plasma as two main isoforms, ApoB-48 and ApoB-100. Posttranscriptional RNA modification of ApoB-100 results in ApoB-48. The expression of ApoB-48 is limited to the availability of the mRNA editing enzyme. In humans, ApoB-48 and ApoB-100 are synthesized exclusively in the gut and liver, respectively. ApoB-48 is present on the intestine derived lipoproteins chylomicrons and the chylomicron remnants. ApoB-100 is synthesized in the liver and present on the liver derived lipoproteins VLDL and IDL[50]. In mice, however, the mRNA editing enzyme is active both in the liver and interstine[51]. The main ApoB produced in any location in mice is ApoB-48. Since mice express ApoB-48 in the liver ApoB-48 is expressed on VLDL, IDL and LDL[52].

ApoE is a glycoprotein component of all lipoproteins, with the exception of LDL. Both ApoB and ApoE are ligands to the LDL receptor on hepatocytes in the liver that clear chylomicrons and very low-density lipoprotein remnants (figure 3)[53]. ApoE can also bind the LDL receptor-related proteins (LRP1) and heparin sulfate proteoglycans (HSPG)[53-55]. The LDL receptor has a much higher affinity for ApoE compared with ApoB[56]. These receptors mediates lipid metabolism and cholesterol homeostasis[57]. Table 1 summarizes the five human lipoproteins in regard to their lipid components, apolipoproteins and origin.

In the methods section on page 39, ApoE will be further discussed.

Table 1. Summary of human lipoproteins and their content, apolipoprotein types and origin.

Lipoprotein	Lipid Component	Apolipoprotein	Origin
Chylomicron	triglycerides	ApoA-I,-II, -IV ApoB-48 ApoC-I,-II,-III ApoE	Intestine
VLDL	triglycerides	ApoB-100 ApoC-I,-II,-III ApoE	Liver
IDL	Cholesterol esters	ApoB-100 ApoC-I,-II,-III ApoE	VLDL
LDL	Cholesterol esters	ApoB-100	IDL
HDL	Cholesterol esters Phospholipids	ApoA-I,-II, -IV ApoC-I,-II,-III ApoE	Liver, interstine
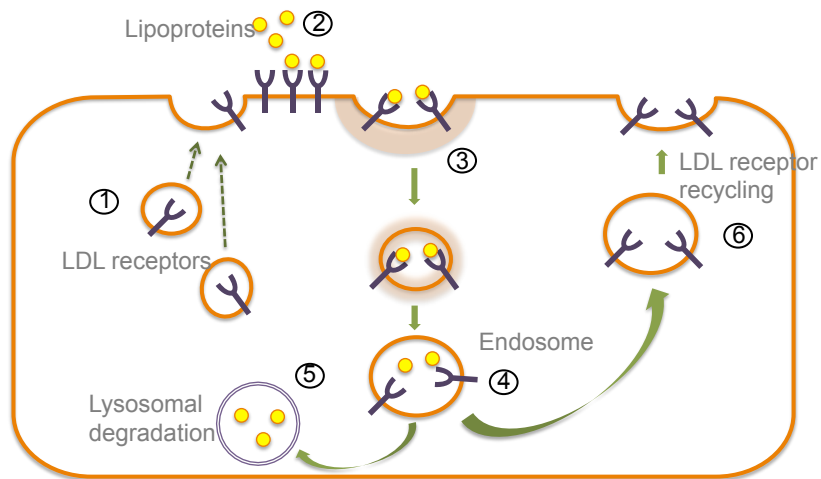
Figure 3. A schematic drawing of the LDL receptor and lipoprotein interaction. LDL receptors are synthesized and transported to the cellmembrane. Upon binding a lipoprotein containing ApoB or ApoE, the receptor is internalized in a clathrin-coated vesicle. The lipoprotein is subjected to lysosomal degradation and the receptor is transported back to the cellmembrane. ApoE has a higher affinity for the LDL receptor compared with ApoB. LRP1 and HSPG are two other lipoprotein receptors that are ainternalized in the same manners as the LDL receptor upon ligation with ApoE.

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Androgen Deprivation Therapy

In 1941 Huggins and Hodges discovered that prostate cancer is an androgen-sensitive malignancy[58]. Despite the years that have passed since the discovery, research is still being conducted to better understand and improve androgen deprivation therapy (ADT). The two major androgens in men are testosterone and dihydrotestosterone (DHT). Testosterone is mainly found in the circulation while DHT is the primary androgen in prostatic tissues. About 95% of the circulating testosterone is produced by the Leydig cells of the testis and the remaining 5% in the adrenal glands[59].

The growth and function of the prostate gland is regulated by androgens. Androgens are produced during fetal development by the testes to develop the prostate gland that continues to mature until the end of the puberty. The prostate continues to grow throughout life. Cell division is stimulated by androgens and can therefore assist in the development of cancer[60].

The most effective way of lowering testosterone levels is surgical castration that results in rapid and continuous testosterone suppression. However, most patients prefer chemical castration that has similar effects on the malignancy. There are various drug targets for suppressing testosterone in the treatment of androgen sensitive prostate cancer. To understand the targets, we have to understand how testosterone is produced (figure 4).

The male hypothalamic-pituitary-gonadal axis

Gonadotropin-releasing hormone (GnRH), also known as luteinizing hormone-releasing hormone (LHRH), is produced and released by the hypothalamus in the brain by GnRH-expressing neurons in a pulsatile fashion[61]. GnRH is a hormone that controls the production of androgens including testosterone in men, and estrogen in women. When the hypothalamus is stimulated by neurotransmitters such as dopamine and norepinephrine, GnRH is released and reaches the anterior pituitary gland through the hypothalamic-hypophyseal portal blood system. The anterior pituitary gland expresses GnRH receptors and responds to GnRH by producing luteinizing hormone (LH), follicle-stimulating hormone (FSH) and adrenocorticotropic hormone (ACTH). LH stimulates testosterone production in the testes and ACTH stimulates testosterone production in the adrenal glands. Testosterone regulates the hypothalamic- pituitary-gonadal axis through its negative

feedback on both the hypothalamus and the pituitary. This inhibits further stimulation of the pathway and the production of hormones.
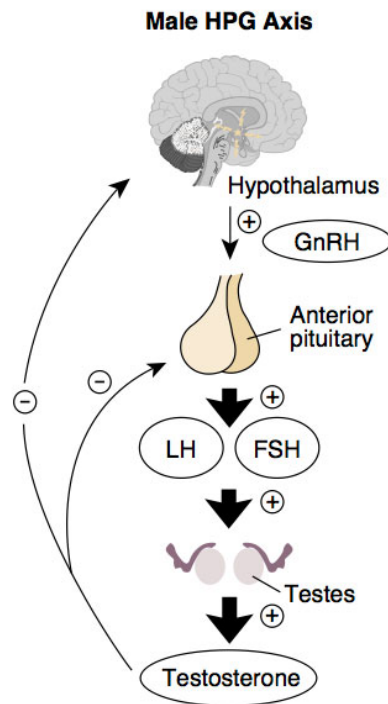
Male HPG Axis

Figure 4. The male HPG axis and the production of testosterone. GnRH is produced in the hypothalamus and stimulates the anterior pituitary to release LH and FSH. LH stimulates the tesosterone production in the testes. Testosterone, by a negative feedback mechanism, controls the release of GnRH. A GnRH receptor antagonist blocks the GnRH receptors in the anterior pituitary diminishes the production of testosterone. A GnRH receptor agonist binds the receptors in the anterior pituitary and by over-stimulation the GnRH receptors they are desensitized. Figure courtesy of Hiller-Sturmhofel, S.Bartke, A[62].

Targeting GnRH receptors

Agonists and antagonists both lower testosterone levels

The GnRH receptor is a class I G-protein coupled receptor. GnRH receptors are heterotrimeric G proteins of the $G_{q/11}$ family that activate phospholipase C with consequent Ca^{2+} mobilization and activation of protein kinase C isozymes[63].

The pulsatile fashion of release of GnRH from the hypothalamus is necessary for the secretion of gonadotropin[61]. Homologous receptor desensitization is achieved by

constant activation of the G protein coupled receptor family[64]. This is the target mechanism of GnRH receptor agonist. A constant release of GnRH suppresses gonadotropin and can thereby be utilized as a treatment for androgen sensitive prostate cancer. GnRH receptor agonists such as leuprolide are given as a subcutaneous injection that forms a depot. The depot continuously secrets the GnRH receptor agonist and desensitizes the receptor. This results in lowered testosterone levels. However, before the GnRH receptors are desensitized, there will be a surge of testosterone as a result of the GnRH receptor stimulation by the injected agonist. As the effect of leuprolide wears off, follow up injections result in small flares of testosterone levels.

The antagonist Degarelix occupies the GnRH receptor without activation. The effects of Degarelix are immediate. Testosterone levels reach that of castration within 1-3 days and are sustained with follow up injections[65].

ADT and cardiovascular disease

The influence of ADT on the cardiovascular system has been viewed as modifications of traditional cardiovascular risk factors. Current collected data, predominantly from usage of GnRH receptor agonists that have been used for decades, demonstrates an association between ADT and increased LDL and triglyceride levels, increased insulin resistance and decreased glucose tolerance, decreased lean body mass and increased fat mass; however, the fat accumulation seems to be subcutaneous rather than visceral[66-

[72]. These changes of cardiovascular risk factors during ADT can affect and accelerate atherosclerosis, possibly leading to a cardiovascular event. Besides affecting atherosclerotic risk factors, ADT has been associated with both arterial and venous thromboembolic events, including deep vein thrombosis, pulmonary emboli, arterial thrombosis and stroke[73,74]. The effect of ADT on the cardiovascular risk factor blood pressure is unclear. Interestingly, ADT enhances vascular endothelial function[75].

The GnRH receptor antagonist versus GnRH receptor agonists and cardiovascular risk

Albertsen and coworkers have suggested that ADT may be an independent risk factor for cardiovascular events. Emerging clinical data is pointing in the direction that there may be a difference in cardiovascular risk between the treatment with a GnRH receptor agonist and the GnRH receptor antagonist. GnRH receptor antagonist treatment halved the number of cardiac events within the first year of treatment in men with pre-existing cardiovascular disease (CVD), compared with GnRH receptor agonists according to an analysis of pooled data from randomized phase III/IIIb trials[76]. In the study, men with pre-existing CVD treated with the GnRH receptor antagonist had a 56% lower risk of suffering from a cardiovascular event than men treated with a GnRH receptor agonist. The absolute risk reduction is about 8.2%. Rosario et al[77] put this reduction of cardiovascular events nicely into perspective:

> 'The magnitude of this risk reduction can be put into context by considering treatments intended to reduce risk in populations at high risk of CV events. For example, the landmark 4S simvastatin study demonstrated a 34 % relative risk reduction versus placebo over 5.4 years[78]'

However, such a reduction was not seen in men without pre-existing CVD. The mechanisms of action that could contribute to increased cardiovascular risk with GnRH receptor agonist treatment, remains unclear. The observations with increased cardiovascular risk within months of ADT initiation and the main cardiovascular effects being observed in patients with pre-existing CVD[79,80], suggests that existing atherosclerotic plaques are directly affected by ADT, rather than altered metabolic factors. As previously described, myocardial infarction and stroke are caused by rupture of an atherosclerotic plaque leading to the formation of an occluding thrombus or emboli[81].

Interleukin-22 in atherosclerosis

The very specific role that IL-22 plays in atherosclerosis is yet to be determined. We do know that IL-22 has been found in abundance in carotid plaques from symptomatic patients[82]. Patients with acute myocardial infarction have also been found with a remarkable rise in IL-22 plasma levels, compared with patients with angina and controls[83]. IL-22 deficiency has previously shown to attenuate plaque formation in ApoE$^{-/-}$ mice[84].

In 1999 when Dumoutier and coworkers first discovered IL-22 in the mouse, it was named IL-10-related T cell-inducible factor, since it is part of the IL-10 related cytokine family[85]. IL-22 and IL-10 share a 22% amino acid homology, but they exert different effector functions. This is most probably due to the different expression pattern of their respective receptors[85-87]. IL-10 is a cytokine well studied in atherosclerosis. IL-10 inhibits the release of proinflammatory cytokines and matrix degrading enzymes, and is thought of as atheroprotective[88,89]. The role that IL-22 plays seems to be the link between immunity and tissue repair.

IL-22 is involved in tissue repair and protection. Anti-apoptotic proteins, such as, Bcl-xL, Mcl-1 and Bcl-2, are induced by IL-22 via the activation the STAT3 signaling pathway[90]. c-myc, cyclin D1, and Rb2, proteins involved in the cell cycle and cell proliferation, are thought to be downstream of the IL-22 signaling pathway[91,92]. The role of IL-22 in tissue repair has been studied in various tissues. IL-22 is considered as a survival factor for hepatocytes via activating the STAT3 pathway[93]. In a mouse model of inflammatory bowel disease, IL-22 has shown protective and even to exert therapeutic effects[94]. IL-22 is involved in a wider range of diseases such as malaria[95], psoriasis[96-99], rheumatoid arthritis[100-102] and is a mediator in would healing[88,103-105].

IL-22 production and the IL-22 receptor

IL-22 production and regulation

T cells, Th17 cells and ILC3

IL-22 is mainly produced by a subset of immune cells such as Th17[106], activated T cells[82], and innate lymphoid Type 3 cells (ILC3)[107].

The major producers of IL-22 in mice are Th17 cells[106]. Differentiation of naive T cells to the Th17 lineage requires stimulation with the cytokines IL-6, IL-1β and TGF-β. IL-23 is then crucial for the continued expansion of the Th17 cell[96,106,108]. While TGF-β is required for the differentiation of T cells to the Th17 lineage, 'optimal' concentrations are needed for maintaining IL-22 expression since TGF-β above a certain level suppresses IL-22 production[94,109]. IL-22 secretion by T cells and ILC3s is regulated by IL-1β, IL-23 and the agonists of the aryl hydrocarbon receptor (AhR).

IL-22 receptors

Membrane bound IL-22 receptor

The IL-22 receptor is a type-2 cytokine receptor composed of two subunits, IL-22RA1 and IL-10R2 (figure 5)[110,111]. IL-22 first binds to IL-22RA1, and then IL-10R2 binds the IL-22/IL-22RA1 complex[112]. IL-22RA1 is absent in immune cells but is highly expressed in the skin, pancreas, small intestine, lung, colon, kidney and liver[87,113]. IL-10R2 is ubiquitously expressed on both hematopoietic and non-hematopoietic cell lineages.

In a normal physiological state, the IL-22 receptor complex (IL-22RA1/IL-10R2) is not expressed on cells with a hematopoietic origin and therefore IL-22, unlike other cytokines, does not directly regulate the immune cells[87,114].

A growing body of evidence is suggesting that IL-22RA1 can be expressed on immune cells during pathological states. In a mouse model of arthritis CD4+ cells have been found to express IL-22RA1[115]. Circulating monocytes and tissue macrophages from patients with Sjögren's Syndrome have found to express functional IL-22RA1[116].

Soluble IL-22 receptor

Besides being regulated by growth factors and cytokines, IL-22 is regulated by a soluble receptor named IL-22 binding protein (IL-22BP) also called IL-22RA2 (figure 4)[85,111]. IL-22BP has a much higher affinity for IL-22 than the membrane bound IL-22RA1. IL-22BP is mainly produced by dendritic cells and is an important regulator of IL-22[117].
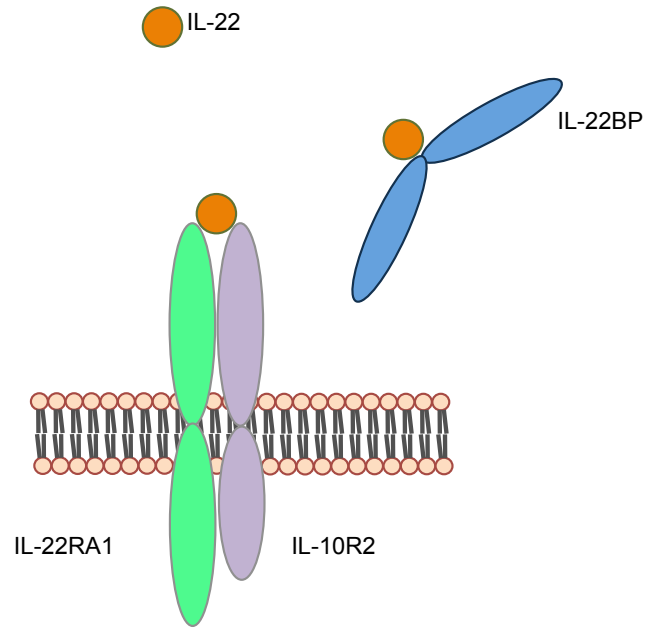
Figure 5. Schematic drawing of the IL-22 receptor complex, made up of two subunits, IL-22RA1 and IL-10R2. Here also showing IL-22BP that can regulate the bioavailable IL-22 levels by binding IL-22, leaving it unavailable to the IL-22 recptor.

Vascular complications in diabetes

According to the International Diabetes Federation, the prevalence of diabetes in persons between the ages of 20-79 in Sweden is 4.7%. Today, 1 in 11 people worldwide suffer from diabetes and that number is projected to rise to 1 in 10 by the year 2040.

In a broad overview, diabetes can be divided into two subtypes: diabetes type 1 (T1D) and diabetes type 2 (T2D). T1D is an autoimmune disease where the insulin producing β-cells of the pancreas are destroyed, resulting in insulin dependence. In T2D, glucose homeostasis is affected by genetic or lifestyle factors[118], such as high BMI[119]. A characteristic of T2D is insulin resistance. Initially there is an increase in insulin secretion to compensate the increased insulin demand that over time subsides resulting in hyperglycemia[118,120].

Diabetes is associated with a 2- to 3-fold increased risk of cardiovascular disease including acute myocardial infarction and stroke[121]. Independent of what type of diabetes; it is a risk factor for CVD. Diabetics have a higher incidence and severity of atherosclerosis, where both microvascular and macrovascular complications occur more frequently compared to the non-diabetic population[122-124]. The underlying mechanism behind the increased risk for CVD among diabetics, however, remains incompletely understood. Certain risk factors for CVD are present in diabetics including endothelial oxidative stress, formation of advanced glycation end products (AGE), dyslipidemia and disturbed platelet homeostasis.

Low-grade systemic inflammation and endothelial dysfunction have been seen as the culprits in diabetes that lead to vascular complications[125,126]. Much of the current research is focused on inflammation in diabetes. Atherosclerosis is an inflammatory disease and parallels are drawn between chronic low-grade inflammation and accelerated atherosclerosis in diabetics. A study performed almost two decades ago showed that specimen from diabetics taken from endarterectomies performed to treat symptomatic coronary artery disease, had larger lipid cores and a higher grade of macrophage infiltration compared with non-diabetics[127].

More recently, Edsfeldt and coworkers published a study that pointed to impaired fibrous repair responses rather than inflammation causing plaque vulnerability in diabetic patients[128]. Their study compared inflammatory markers and fibrous repair responses in symptomatic and non-symptomatic carotid plaques from diabetic and

non-diabetic patients. Edsfeldt et al have reported that impaired fibrous repair responses, rather than increased inflammation, drives atherosclerotic plaque vulnerability in diabetics. In their study, plaques from diabetics had a lower content of ECM proteins critical for maintaining plaque stability, like collagen and elastin, as well as tissue repair factors such as platelet-derived growth factor (PDGF) and MMP2. They did not find a difference in inflammatory markers in plaques from diabetics and non-diabetics. Interestingly, in the non-diabetics group, symptomatic plaques had an increase in inflammatory markers compared with asymptomatic. This was not seen in plaques from diabetics between the asymptomatic and symptomatic groups.

Pancreatic islets and osteopontin

One might wonder how a paper on islet cell exocytosis ends up in a thesis concerning atherosclerosis. It is a long, but interesting, story.

I had a project regarding the effects of osteopontin (OPN) deficiency on atherosclerotic plaque development in an ApoE^{-/-} mouse model where we induced diabetes using streptozotocin (STZ). Our question was:

> 'Does osteopontin-deficiency affect the atherosclerotic plaque progression in an STZ-induced diabetic mouse model?'

Type 1 diabetes is an autoimmune disease that leads to β-cell destruction[129]. In pathological states, such as T1D and atherosclerosis, OPN is upregulated in blood serum[130]. By using ApoE^{-/-} and ApoE^{-/-}OPN^{-/-} mice we wanted to investigate the effect of OPN-deficiency on atherosclerotic plaque development in diabetic and non-diabetic mice using a shear stress modifier. Previous studies using OPN knockout mice have shown that OPN deficiency attenuates plaque development[131-133].

Streptozotocin (STZ)

STZ is an antibiotic that was originally found in soil bacteria. It has a structural resemblance to glucose and can enter the pancreatic islets. STZ is highly toxic to -cells and is used to treat metastatic cancer of the islet cells. In experimental situations, it is used to induce hyperglycemia and T1D[134].

We encountered an unexpected problem with ApoE^{-/-}OPN^{-/-} mice suffering from severe hyperglycemia and dehydration after STZ treatment. The mice had to be euthanized and the study was put on hold. The experiment was repeated with similar

results. Interestingly, given the same dose of STZ, only 60% of the control-ApoE^{-/-} mice developed diabetes. Taken together, ApoE^{-/-}OPN^{-/-} mice are much more susceptible to STZ-induced diabetes.

At the initial stages of diabetes there is an infiltration of macrophages and T cells in the islets causing inflammation knows as insulitis. The cytokines produced by the immune cells contribute to β-cell dysfunction and destruction[135,136]. OPN is a glycoprotein produced by various cell types such as epithelial cells, T cells, macrophages and SMCs and has been shown to mediate various tumors survival through PI3-K/Akt signaling pathway[137-139]. In addition, OPN expression is induced by a multiple factors such as cytokines associated with acute phase inflammation like IL-1β and TNF-α and hyperglycemia, hypoxia and angiotensin-II[140]. It also activated in the islets and lymph nodes in non-obese diabetic mice[141]. Previously it has been shown that OPN is essential for the immune response in the islets to pivot toward the protective Th2 profile[142].

An important mechanism of STZ-induced diabetes, which could have importance in human Type 1 diabetes, is overproduction of NO by iNOS that is induced by cytokines[134,143]. Katakam et al suggested that OPN has a protective role by mediating an endogenous negative feedback loop against NO in the islets. NO is hypothesized to affect β-cell function by inducing apoptosis and suppressing glucose-stimulated insulin release[144,145].

We could speculate on the importance of OPN for islet survival, or we could do an actual experiment. We teamed up with the Islet Cell Exocytosis group at Lund University in Malmö and ran a pure islet study using our OPN deficient mice.
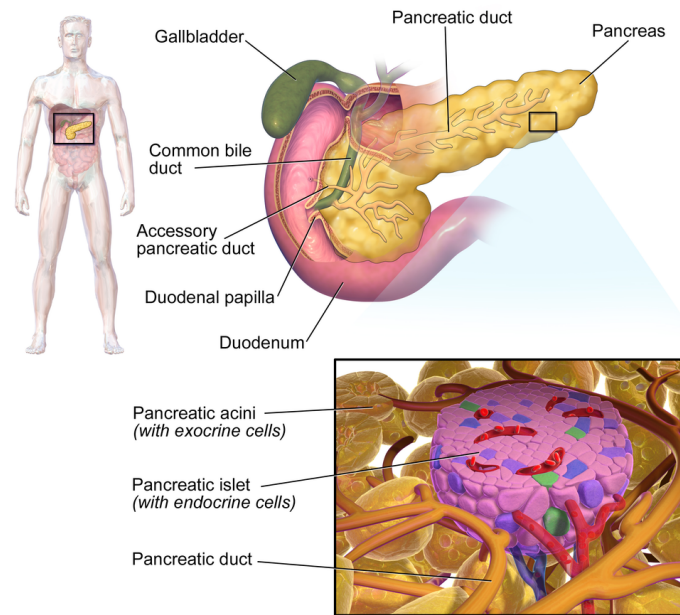
Figure 6. This image shows the location and structure of the pancreas. Beta cells are found in the pancreatic islets. Image courtesy of Blausen 2014[1].

Islets of Langerhans

The islets of Langerhans are groups of specialized cells in the pancreas that make and secrete hormones (figure 6). Paul Langerhans who discovered them in 1869 thought that they looked like small islets, hence the name islets of Langerhans. There are five cell types in an islet: alpha cells that make glucagon that raises the blood glucose level, beta cells that make insulin to lower glucose levels, delta cells that make somatostatin, PP cells that make pancreatic polypeptide, and epsilon cells that produce ghrelin[146]

Insulin is the primary glucose-lowering hormone in the body and the release is triggered by glucose. A mechanism referred to as the 'stimulus-secretion coupling pathway' of the beta cells, is a Ca^{2+} dependent release of insulin[147]. As described in figure 7, glucose crosses the beta cell membrane via glucose transporters. Inside the beta cell, glucose is metabolized resulting in an ATP increase that triggers the closure of the ATP-sensitive potassium channels in the cell membrane. This results in a depolarization of the cell membrane, leading to the opening of voltage-gated Ca^{2+} channels. The increase in Ca^{2+} signals for insulin-containing vesicles to fuse with the cell membrane and release their insulin content[148]. However, prior to fusing with the

cell membrane, the vesicles have to be transported close to the cell membrane. This is exerted through a cascade of events collectively called docking and priming[149,150].
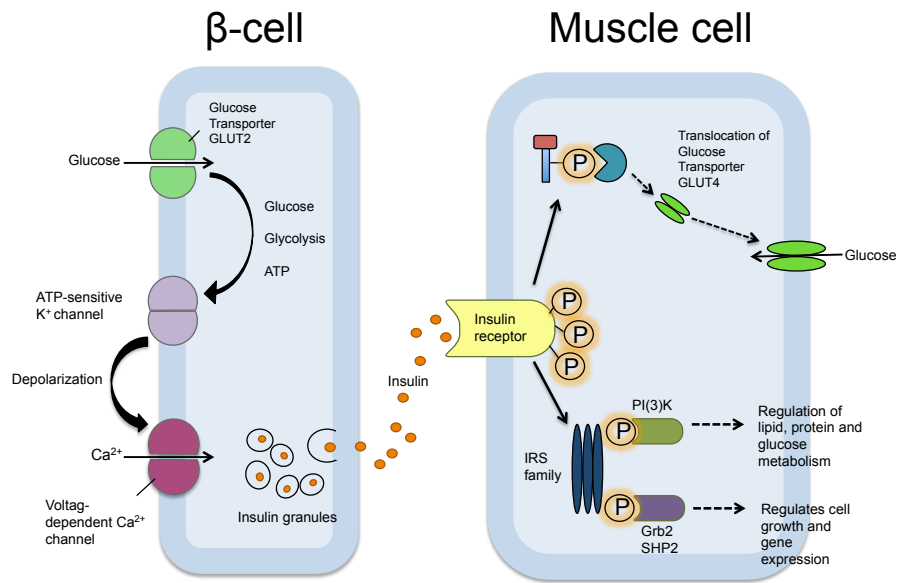
Figure 7. A schematic drawing of the beta cell and the release of insulin. Upon stimulation with glucose, the beta cell produces ATP. ATP-sensitive potassium channels close, leading to the depolarization of the cell membrane and the opening of the voltage dependent calcium channels. The increase of intracellular Ca^{2+} signals the insulin containing vesicles to fuse with the cell membrane and release insulin. Insulin can then act on insulin receptors, for example on a muscle cells, as drawn above. Stimulation of the insulin receptor allows glucose to enter the cell. Insulin regulates lipid, protein and glucose metabolism as well as the growth and gene expression of a cell.

Osteopontin and islets

OPN has been described as a protective protein for islets in T1D and has been found upregulated in both pancreatic islets and ducts during beta cell destruction using STZ in rats. In the same experiments, OPN weakened the STZ-induced cytotoxicity in part via an NO regulatory mechanism[151]. OPN, known as an anti-apoptotic factor, has shown to prevent apoptosis as well as stimulate cell proliferation in human beta cells[152,153]. In contrast, genome wide association studies of single nucleotide polymorphisms in humans has suggested that the gene encoding OPN to be associated with an increased vulnerability to the development of type 1 diabetes[154,155]. In type 2 diabetes OPN is suggested in the involvement in adipose tissue inflammation and insulin resistance[152]. These contrary findings need further evaluation. OPN is indeed a pleiotropic and versatile protein.

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Main Methods

Mouse models of atherosclerosis

Diet

The 'western diet' also called a high fat diet (HFD), is a standard experimental rodent food that mimics the components of a typical North American diet. It contains maize starch, cocoa butter, casein, glucose, sucrose, cellulose flour, minerals, and vitamins; 17.2% protein, 21% fat [62.9% saturated, 33.9 unsaturated and 3.4% polyunsaturated], 0.15% cholesterol, 43% carbohydrates, 10% H2O, and 3.9% cellulose fibers). It smells like a chocolate dessert you want to eat! Perhaps because the chocolate company Cloetta delivers the cocoa butter.

The ApoE deficient mouse

Mice do not develop atherosclerosis naturally; they are in fact highly resistant to plaque development due to their lipid profile. Mice, unlike humans that carry most of their plasma cholesterol on LDL, carry cholesterol mainly on HDL[156]. Mice do not express the cholesteryl ester transfer protein that transfers cholesterol ester from HDL to VLDL and LDL; which is considered an anti-atherogenic factor[157]. In the ApoE deficient mouse model, despite the great increase in total plasma cholesterol levels, the HDL levels are halved compared with wild type control mice. The triglyceride levels are approximately 68% higher than those of normal animals. Taken together, ApoE deficient mice have a shift in plasma lipoproteins from HDL to remnants of chylomicrons and VLDL; a highly atherogenic lipid profile.

ApoE is a glycoprotein component of all lipoproteins, with the exception of LDL, and a ligand to the LDL receptor, LRP1 and HSPG that are present on various cell types such as hepatocytes in the liver that clear chylomicron remnants and IDL (figure 3)[53]. In the ApoE deficient mouse lipoprotein particles recirculate and accumulate in the blood stream, leading to hyperlipidemia[156,158]. Plasma cholesterol increases 5-fold in the ApoE deficient mouse on a normal chow diet compared with wild type mice, and a striking 15-18-fold on a western diet[156,159].

The atherosclerotic lesion progression in the ApoE deficient mouse is similar to that of other animals and humans; involving the same cell types[159]. Plaques spontaneously develop in areas of disturbed stress like the aorta, aortic arch and in the subvalvular regions[159,160]. Atherosclerotic plaque progression is greatly accelerated when the ApoE deficient mouse is fed a western diet[159].

Shear stress

Shear stress is defined as the frictional drag force that liquid flow exerts on the vessel wall and has the unit dynes/cm^2, Newton/m^2 or Pascal. Shear stress can be laminar, pulsatile or disturbed, and is determined by flow rate, vessel diameter and fluid viscosity. The endothelium lining the vasculature is directly subjected to the force and patterns of shear stress. It is important to keep in mind that shear stress is involved in both normal physiologic and pathophysiologic vascular biology. Shear stress modulates many factors involved in the protection or disease progression in the arteries, such as vasoactive compounds, growth factors, and factors of the coagulation cascade, ECM and inflammation. The pathogenesis of the atherosclerotic plaque is affected by lowered disturbed shear stress or oscillatory shear stress. Low shear stress reduces endothelial repair processes and increases the permeability of lipoproteins, heightens leukocyte adhesion and reactive oxygen species. In straight sections of an artery, unidirectional laminar flow resides that creates an environment that promotes a healthy artery with anti-inflammatory, antithrombotic, anticoagulative, profibrinolytic and antihypertrophic properties[161].

Modifying shear stress experimentally

The shear stress modifier, better known as 'the cast'

The experimental and controlled development of atherosclerosis
Based on observational studies showing that plaques usually develop in regions near bifurcations or along the inner curvature of the arteries, Cheng et al developed a 'shear stress modifier'; a perivascular plastic cast, that alters shear stress leading to development of atherosclerotic plaques (figure 8)[26]. When the blood reaches the proximal end of the cast, the shape of the cast creates an area of low shear stress, leading to the formation of plaques with an advanced phenotype. Inside the cast shear stress is high and no plaque development is seen due to atrophy of the SMCs in the media. Distally to the cast oscillatory shear stress is created that promotes a plaque

with a more fibrous phenotype. In the discussion, under the section 'the advanced and fibrous plaques' on page 47, shear stress is further discussed.

This model enables us to retrieve two plaques with different phenotypes on the same artery, in the same mouse. We place the cast on the right common carotid artery and use the left as a control. The carotid is a nice straight artery and allows for great cross sections that make quantifications reliable.
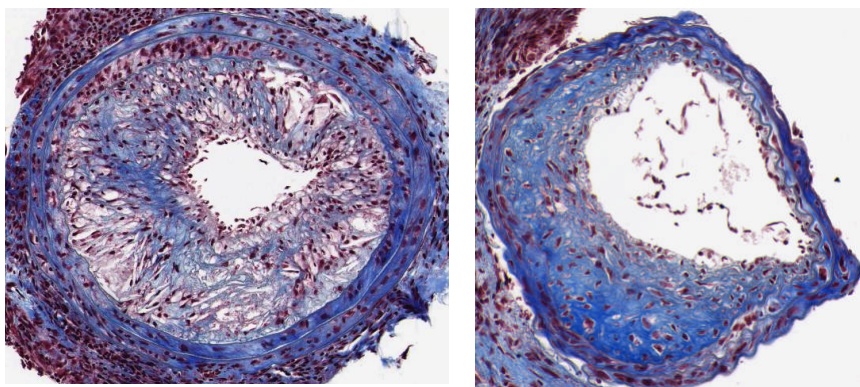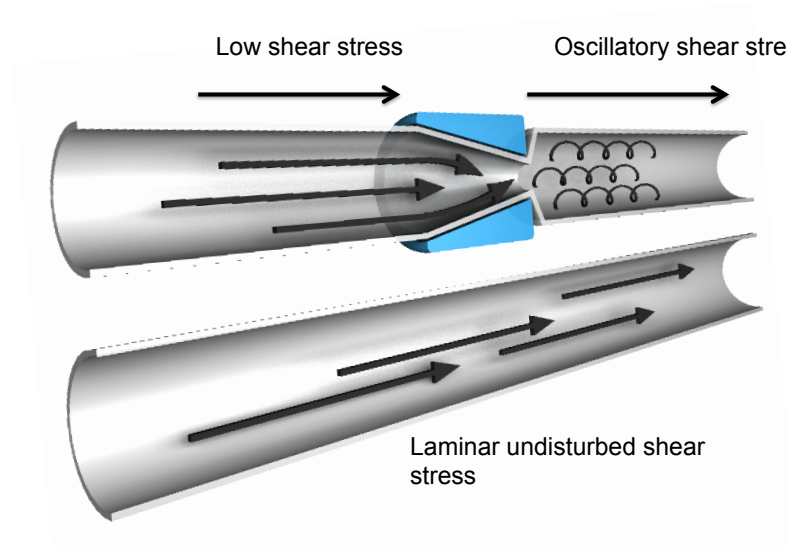
Figure 8. The shear stress modifying cast seen in blue in the top picture. The cast creates an area with lowered shear stress proximally to the cast and one area with oscillatory shear stress distally to the cast. The resulting plaques are phenotypically different. Lowered shear stress induces plaques with a more advanced inflammatory phenotype. Oscillatory shear stress promotes plaques with a fibrous, more stable plaque phenotype.

Neointimal hyperplasia

The experimental neointimal thickening

Neointimal hyperplasia can be induced by placing a non-occlusive plastic tubing (internal diameter, 0.51mm; length, 3mm) around the mouse carotid artery (figure 9)[162]. This method serves to imitate the balloon angioplasty method that has been used to denude endothelial cells to provoke neointima hyperplasia and vascular remodeling in rats[163]; a method that cannot be used in mice due to the size of the mouse carotid. The resulting neointima consists mainly of SMCs and ECM. This type of lesion does not contain lipids and the degree of inflammation varies with time after cast placement[162,164,165].

We used this cast on 25-week old Akita and wild type mice for the duration of 7 weeks to study the effects of hyperglycemia on vascular repair mechanisms in the absence of lipids.
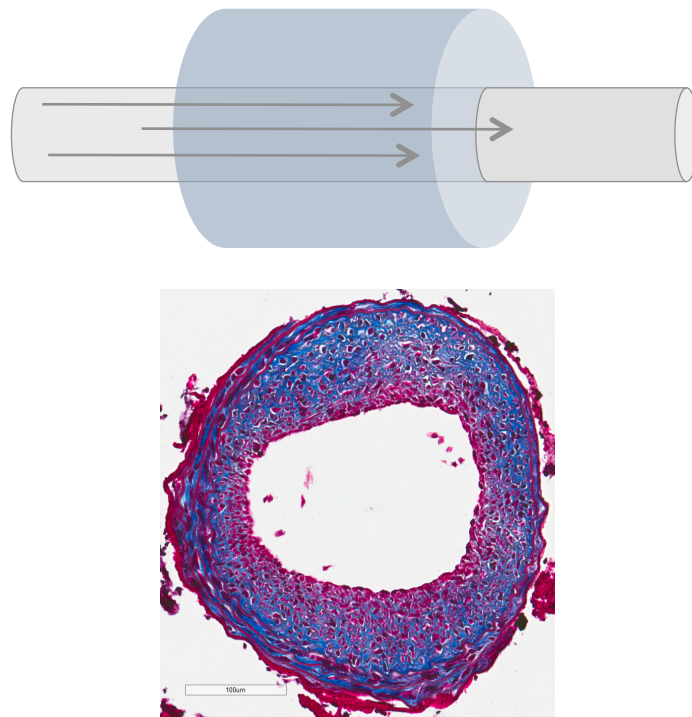
Figure 9. The non-occlusive injury inducing cast and an example of the resulting lesion visualized by at Masson's trichrome stain.

Aims and Key Findings

Paper I

Aim

To study the effects of a GnRH receptor antagonist and a GnRH receptor agonists on existing atherosclerotic plaques in ApoE$^{-/-}$ mice using a shear stress modifier.

Key findings

Four weeks after drug administration we detected that mice treated with the GnRH receptor agonist leuprolide had plaques with increased necrosis-like areas and macrophage infiltration compared with those untreated or treated with the GnRH receptor antagonist degarelix (figure 10). This effect was only seen in fibrous plaques formed in the oscillatory shear stress region. We also located the GnRH receptor in the atherosclerotic plaque.
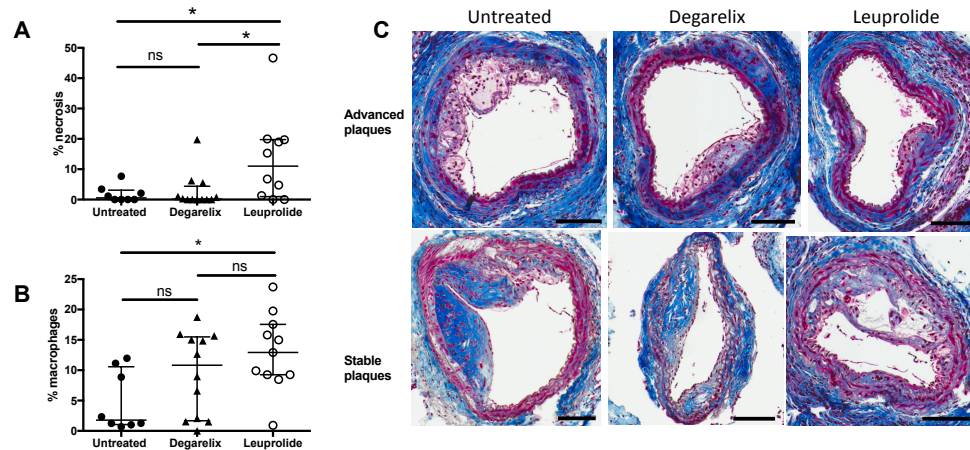
Figure 10. Increased necrosis-like areas (A) and macrophage (B) areas in leuprolide treated mice. (c) representative images of plaques with advanced and stable phenotype, bottom right image showing a stable plaque from a leuprolide treated mouse with necrosis.

Paper II

Aim

To study how IL-22 deficiency affects advanced and fibrous atherosclerotic plaque development in ApoE$^{-/-}$ mice using a shear stress modifier.

Key findings

IL-22 deficient mice developed smaller and less advances plaques in the low shear stress region of the cast where advanced inflammatory plaques arise. These plaques contained more contractile SMCs per plaque area and lacked necrotic-like areas (figure 11).
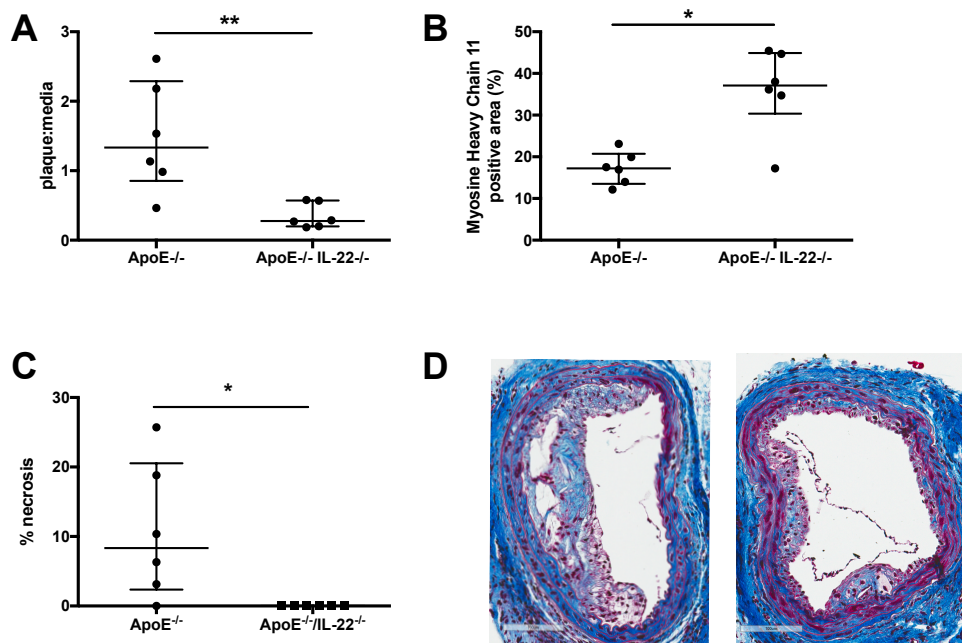
Figure 11. IL-22 deficiency attenuates advanced plaque formation (A). IL-22 deficient mice had more contractile SMCs per are (B) and lacked necrosis (C). Representative images of plaques from ApoE$^{-/-}$ and ApoE$^{-/-}$IL-22$^{-/-}$.

Paper III

Aim

Atherosclerotic plaques from subjects with diabetes have been reported to contain reduced amounts of stabilizing fibrous components. The aim of this study was to investigate if diabetes affects vascular repair responses.

Key findings

Hyperglycemia due to impaired β-cell function does not affect vascular repair responses (figure 12).
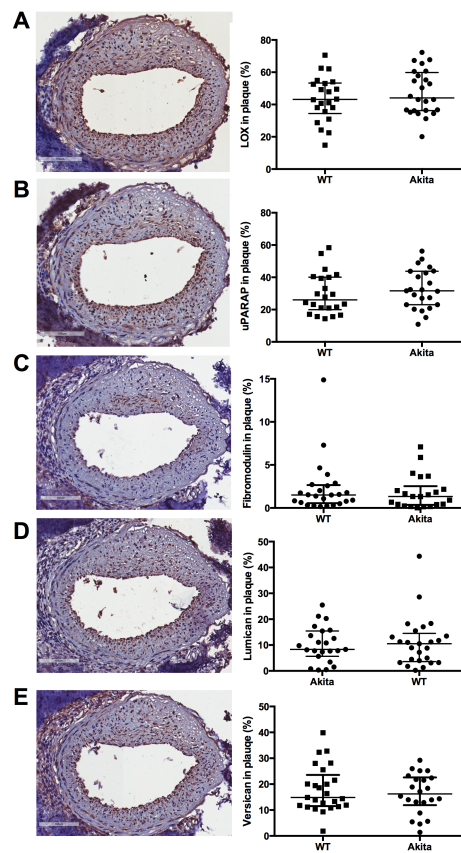
Figure 12. Expression of proteins involved in tissue turnover. Representative images and analyses of LOX (A), uPARAP (B), fibromodulin (C), lumican (D), and versican (E).

Paper IV

Aim

To investigate the role of OPN in insulin secretion we characterize islets of Langerhans from OPN deficient mice with respect to morphology, Ca^{2+} handling and capacity to secrete insulin. OPN has previously been implicated in the development of insulin resistance.

Key findings

Lack of OPN resulted in a reduced number of docked insulin granules. Ca^{2+} clearance was also altered in islets from OPN$^{-/-}$ mice, but insulin secretion was not affected in islets from OPN$^{-/-}$ mice compared with islets from WT mice (figure 13).
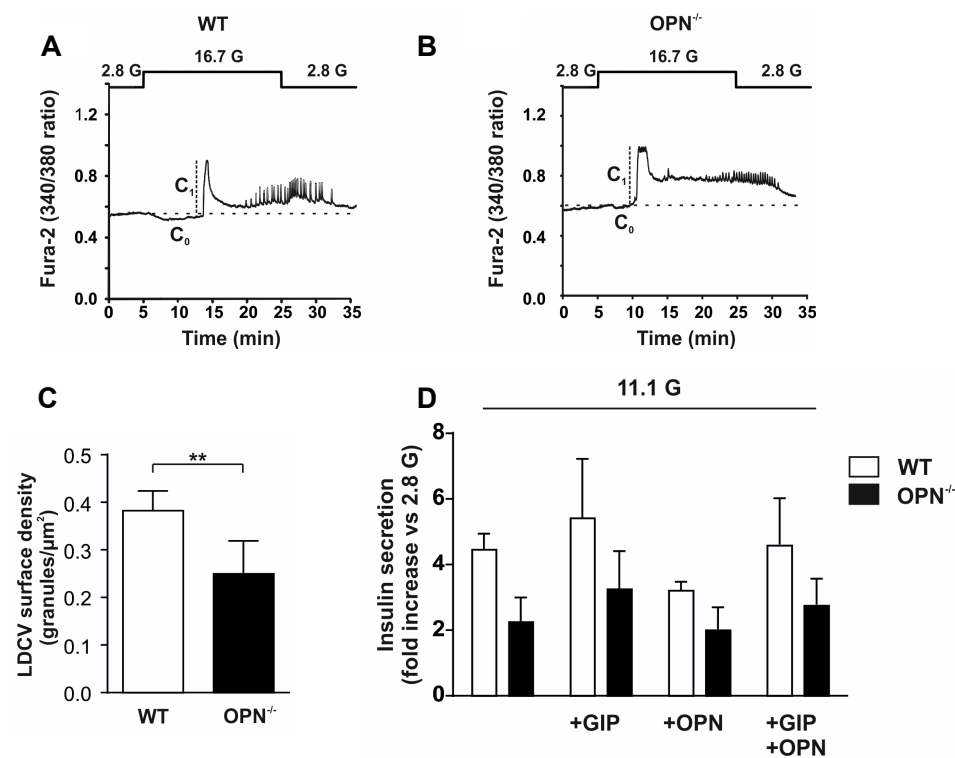
Figure 13. Ca^{2+} clearance in islets from (A) WT and (B) OPN$^{-/-}$ mice, showing an altered clearance in OPN$^{-/-}$. (C) OPN$^{-/-}$ mice have fewer docked insulin granules. (D) Insulin secretion is not altered in OPN$^{-/-}$ mice.

Discussion and Future Perspectives

The aim of this thesis has been to investigate plaque vulnerability and vascular repair processes. We studied ADT and its effects on plaque vulnerability, and IL-22 in tissue repair, both in a mouse model of atherosclerosis where we alter shear stress to induce plaques with advanced or fibrous phenotypes. To investigate vascular repair mechanism in hyperglycemia per se, without the involvement of dyslipidemia and subsequent inflammation, we have induced carotid lesions in the Akita mouse. After having to discard a study using diabetic OPN deficient mice, we have isolated and tested OPN deficient beta cells to gain a better understanding of the role of OPN in beta cell function.

Atherosclerotic plaque vulnerability and vascular repair

ADT is a cardiovascular risk factor for men with CVD where drug interactions destabilize the existing atherosclerotic plaque. IL-22 is a cytokine unlike other cytokines with its limited receptor expression on non-immune cells, linking immunity and tissue repair processes.

The advanced and fibrous plaques

The shear stress modifier, described on page 41, gives rise to two separate plaques with different phenotypes. The plaque formed proximally to the cast, in low shear stress, has high macrophage content, limited ECM, a thin fibrous cap and most often lipid cores. It is generally larger than the plaques formed distally to the cast. In our lab we have referred to this plaque as a 'proximal plaque' or an 'inflammatory plaque' or a 'low shear stress plaque'. It has been difficult to decide on a term that describes the plaque and does not confuse the reader. The plaque phenotype is that of a vulnerable plaque, but we do not know for certain that it actually is vulnerable. We have chosen to refer to this plaque as an advanced plaque.

The plaque formed distally to the cast, in a region with oscillatory shear stress, has a dense ECM and higher SMC content than the advanced plaques. Large lipid cores are

uncommon. This plaque has a phenotype of a stable plaque, but we do not know that it is stable. We have chosen to refer to this plaque as a fibrous plaque.

It is important to keep in mind that these plaques are very different from one another. Hemodynamic forces, particularly variations of shear stress regulate physiological and pathologic aspects of vascular endothelial function. The characteristics of shear stress can be a determinant of protection as well as susceptibility and development of atherosclerosis. Endothelial cells are sensitive to variations in the characteristics of flow that generate shear stresses; our cast model with low and oscillating shear stress give pathologic changes in the artery wall that lead to atherosclerosis. Shear stress is a force upon the endothelial cells and a mechanical stimulation that communicates via the cytoskeleton to the ECM, intracellular junctions and the nuclear membrane[166]. Depending on the type of shear stress, the endothelial cells will react subsequently.

The inventors of the cast model[26,167] have studied the effects of low shear stress and oscillatory stress on plaque formation. Cheng and coworkers found a 3-fold upregulation of VCAM-1 and intercellular adhesion molecule-1 in the lowered shear stress region compared with controls. In the oscillatory shear stress region VCAM-1 was only upregulated by 50%. C-reactive protein expression was increased by 3-fold and 2-fold in the lowered shear stress region and oscillatory shear stress region respectively compared with controls. The vascular endothelial growth factor was increased by 5-fold in the lowered shear stress lesions exclusively. Interleukin 6 (IL-6) was increased by 14-fold in the lowered shear stress and by 2-fold in the oscillatory shear stress region compared with controls. IL-6 inhibits endothelial nitric oxide synthase (eNOS) activation[168]. NO is an endothelium-dependent vasodilator of the underlying SMCs that promotes the inhibition of platelet adhesion and platelet aggregation. Low shear stress also has the specific expression of KC (CXCL1), IFN-γ–inducible protein–10 (IP-10) and fractalkine.

Taken together, the manners in which low shear stress and oscillatory shear stress stimulate and activate the vascular wall give rise to plaques with very different phenotypes.

The GnRH receptor antagonist is associated with lower cardiovascular risk

Fibrous plaques and ADT

It is terrible enough to be given a prostate cancer diagnosis without having to deal with the potential risk of a cardiovascular disease as a side effect to the treatment. Men with preexisting CVD are more susceptible for cardiovascular events during ADT. However, treatment with a GnRH receptor antagonist more than halves the

number of cardiovascular events in treated patients with preexisting CVD compared with GnRH receptor agonists.

Testosterone is found to be involved in vascular health; men with CVD generally have lower testosterone levels. Low testosterone levels affect several cardiovascular risk factors including cholesterol levels, endothelial dysfunction and inflammation[169,170]. ADT, by lowering testosterone levels, heightens traditional cardiovascular risk factors such as insulin resistance; dyslipidemia, hypertension and increases body fat mass[74].

GnRH receptor agonist treatment results in testosterone flares before the GnRH receptors are desensitized. These flares have been the postulated as a potential cardiovascular risk factor as high testosterone levels increase angiogenesis in the atherosclerotic plaque[171], increases platelet aggregation[172] and neutrophil migration[173].

The effects that testosterone depletion has on body composition accumulate over time and with them, so do the traditional risk factors. Interestingly, a study has shown that men who undergo orchiectomy have an increased risk for developing diabetes, but not myocardial infarction, coronary heart disease or sudden cardiac death[174]; making ADT the culprit and not testosterone. As a support of this, cardiovascular events can appear in close proximity to the start of ADT treatment. It has been suggested that ADT perhaps directly affects the atherosclerotic plaque in a manner that destabilizes existing atherosclerotic lesions, accounting for the short-term adverse effect of ADT.

In our study, paper I, we investigated the short-term effects of ADT. We treated male ApoE$^{-/-}$ mice with preexisting atherosclerosis with GnRH receptor antagonists and agonists for the duration of four weeks. The drugs we used are the same as in the clinic, but we gave a ten-fold higher dose based on weight. Flat preparations of the aortas show that ADT did not affect the over all plaque development; all mice had the same plaque burden. Mice given the GnRH receptor agonist gained weight compared with the untreated mice. In contrast, mice given the GnRH receptor antagonist, lost weight compared with the untreated mice. This phenomenon is seen in the clinic as well. Triglycerides and cholesterol levels were similar between the treatment groups.

We did not see any effects of ADT in the advanced plaques, only in the fibrous plaques. In the fibrous plaques we saw an increase in necrosis in mice treated with a GnRH receptor agonist, as well as an increase in macrophages compared with untreated or GnRH receptor antagonist treated mice. Histologically it appears that the cells 'expressing the macrophages cell marker' have expanded and undergone apoptosis/necrosis in GnRH receptor agonist treated mice. It is now accepted that SMCs in atherosclerotic plaques can take on a macrophage like behavior and express macrophage markers such as CD68 and F4/80[175]. If the macrophages were the cells most affected by GnRH receptor agonist treatment, we would expect to see an effect of ADT on the advanced plaques that are macrophage rich. It is a possibility that the affected cells in the fibrous plaque are SMCs expressing macrophage markers that we

cannot detect due to the lack of markers that detect modulated SMCs. Indeed, we analyzed human coronary SMCs *in vitro* together with GnRH receptor agonist and antagonist and found their viability or rate of apoptosis not to be affected by the drugs. The *in vivo* situation in the plaque with on-going inflammation, various cell types and perhaps an altered matrix, is a much more complex situation than the petri dish hosting only one cell type. *In vivo* the SMCs may be affected by the drugs directly or indirectly. Another possible reason that we only see an effect in the fibrous plaques could be that the ECM-rich fibrous plaques hosts a different subtype of macrophage compared with the advanced plaque, a subtype that may be less responsive to ADT, compared with the advanced plaque. Moreover, we cannot exclude that the low and oscillatory shear stresses affect the uptake of the GnRH receptor drugs differently. A very important detail is our staining for the GnRH receptor in the plaques where we found them to colocalize mainly with T cells. Only occasionally we found macrophages or SMCs that expressed the receptor. We tested the plasma for cytokines involved in inflammation and T helper responses, but found them to be similar between the groups. It appears that the GnRH receptor agonists and antagonist act directly on the GnRH receptors in the plaque. Perhaps the T cells, in response to ADT, locally regulate the cells of the plaque.

There are quite a few questions left to answer that we could not pursue in the present study. For this study, we used male ApoE^{-/-} mice that are known to develop less atherosclerosis in comparison to female mice that we normally use. On top of this, the males in this experiment developed much less atherosclerosis compared with the mice that we normally work with in our shear stress modifying model. This left us with very limited amounts of material to work with. Cryosections of the plaque tissue could give us answers regarding the influx and efflux of lipids that may be influenced with testosterone levels[176]. However, the fibrous plaques from mice treated with the GnRH receptor agonist were difficult to section. The plaques collapsed after sectioning, leaving a smear along the media. This problem was not experienced with the advanced plaque, or any of the plaques of the other groups. We speculate that this artifact is related to the increased amount of necrosis in the plaques and hence, making them difficult to cryosection due to the lack of tissue structure.

We are curious as to what happens in the plaques of GnRH receptor agonist treated mice that leaves such necrotic cores. The only way to find out is to repeat the experiment and adding an additional time point, and prolong the cast experiment to generate more plaque tissue. In our new study we treat the mice for two, or four weeks. We are hoping that the two-week time point will give us a clue as to what is happening before the onset of necrosis. We have also taken precaution and pretreated our frozen samples in order to give more rigidity to the samples that lack a sturdy structure.

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We have found the GnRH receptor to be expressed mainly on T cells. How do GnRH receptor agonists and antagonist affect the T cell? An in vitro experiment is planned to elucidate this question. And in turn, how do GnRH receptor stimulated T cells affect macrophages and SMCs?

Advanced plaques and IL-22

In paper II we studied advanced and fibrous plaques from ApoE^{-/-} and ApoE^{-/-} IL-22^{-/-} mice. We found that IL-22 deficient mice had smaller advanced plaques that were' less advanced', meaning that they had no necrotic cores and a higher percentage of contractile SMCs. We did not detect any differences in the fibrous plaques between the two genotypes.

Part of what makes IL-22 intriguing is the limited expression of the receptor subunit IL-22RA1. In a normal physiological state, the IL-22 receptor complex (IL-22RA1/IL-10R2) is not expressed on cells with a hematopoietic origin and therefore, IL-22, unlike other cytokines, does not directly regulate the immune cells. IL-22RA1 is expressed in tissues such as liver, skin, intestine, colon, kidney, pancreas and lungs[87]. This gives the immune system a target that is not itself, linking tissue repair and immunity. In our study we have seen limited expression of IL-22RA1 in the media of uninjured carotid arteries. After cast placement, IL-22RA1 is abundantly expressed throughout the media. IL-22RA1 is also expressed throughout the plaque. To find out what cells express also the receptor subunit in the plaque, we double stained IL-22RA1 with myosin heavy chain for SMCs, CD31 for endothelial cells, CD68 and F4/80 for macrophages. We found the IL-22 receptor subunit on SMCs, both in the media and plaque. A few occasional endothelial cells expressed the receptor. Surprisingly, we also found CD68 and F4/80 cells to express IL-22RA1 since cells of the hematopoietic origin are thought not to express the receptor. However, studies are emerging where IL-22RA1 is found on immune cells under pathological conditions, such as Sjögren's syndrome[116]. As mention previously, SMCs in atherosclerotic plaques can take on a macrophage like behavior and express macrophage markers such as CD68 and F4/80[175]. However, we have not yet distinguished between macrophages expressing IL-22RA1 and macrophage-like SMCs in the plaque material.

The advanced plaques from IL-22 deficient mice were significantly smaller and contained more contractile SMCs compared with the controls. Our hypothesis is that IL-22 facilitates the phenotypic modulation of SMCs and that quiescent SMCs are atheroprotective [177]

As discussed earlier, low and oscillatory shear stress activate the endothelial cells, and the subsequent response with cytokine release and upregulation of receptors differ depending on the type of shear stress. The developing plaques emerge from different

cascades of events and differ from one another in respect to cell content, degree of inflammation and ECM content, as previously described. A potential mechanism explaining why the effect of IL-22 deficiency is restricted to advanced plaques could be that collagen, found in abundance in the fibrous plaques, modulate the SMCs phenotype in the fibrous plaques. A study has shown that collagen IV and collagen I differentially affect smooth muscle phenotypic modulation through various pathways[178]. The type of collagen surrounding the SMCs in the fibrous plaque may be a stronger determining factor for the SMCs fate than IL-22 deficiency. Fibrous plaques contain less inflammatory mediators and immune cells than the advanced plaques; activated immune cells produce IL-22. Thus, the amount of bioavailable IL-22 in the fibrous plaques may be lower than in the advanced lesions and this could explain the lack of effect in the fibrous plaques between the genotypes.

The SMCs are often overlooked as solely do-gooders because of their contribution to plaque stability with their ECM production. A SMC can be proinflammatory[179]. Activated SMCs aid in the recruitment and maturation of inflammatory cells and release chemokines such as CCL2, CSF-1, IL-6 and CX3CL1 that enhance the ongoing inflammation. The release of proinflammatory cytokines and the effect of cytokine signaling on SMC growth and migration have been well described by Raines and coworkers, if you would like to further indulge in the subject[180].

A study by Ackers-Johnson proposed myocardin to protect the contractile, noninflammatory SMC phenotype; and loss of myocardin permitted SMC modulation and inflammatory activation. Myocardin-null mice develop larger atherosclerotic plaques. Taken together, there are indications that keeping SMCs quiescent attenuates plaque formation. Our IL-22 deficient mice developed significantly smaller advanced plaques compared with the controls and had a larger content of contractile SMCs. IL-22 has previously been reported to induce migration and proliferation of pulmonary SMCs[181,182] and Rattik et al have found contraction-associated proteins such as α-actin, caldesmon and vinculin mRNA in the arteries from ApoE$^{-/-}$IL-22$^{-/-}$ mice. *In vitro*, SMCs stimulated with IL-22 had reduced mRNA expression of α-actin and caldesmon[84].

To further investigate our hypothesis that IL-22 deficiency promotes a contractile SMC phenotype, we are planning on running *in vitro* experiments. We would like to study if IL-22 affects the migration and proliferation of SMCs in culture. We will run western blots to analyze the protein expression of markers of the synthetic and contractile SMC in our cultured cells, as well as investigate the mRNA expression.

Hyperglycemia and vascular repair

In paper III we have studied the effects of hyperglycemia on vascular repair. The basis for our study Edsfeldt and coworkers study that pointed to impaired fibrous repair responses rather than inflammation causing plaque vulnerability in diabetic patients[128]. They compared inflammatory markers and fibrous repair responses in symptomatic and non-symptomatic carotid plaques from diabetic and non-diabetic patients. Edsfeldt et al have reported that impaired fibrous repair responses, rather than increased inflammation, drives atherosclerotic plaque vulnerability in diabetics. In their study, plaques from diabetics had a lower content of ECM proteins critical for maintaining plaque stability, like collagen and elastin, as well as tissue repair factors such as platelet-derived growth factor (PDGF) and MMP2. They did not find a difference in inflammatory markers in plaques from diabetics and non-diabetics. Interestingly, in the non-diabetics group, symptomatic plaques had an increase in inflammatory markers compared with asymptomatic. This was not seen in plaques from diabetics between the asymptomatic and symptomatic groups.

Using the cast described on page 42, we induced intimal hyperplasia in the carotid artery of female diabetic Akita mice and WT controls. The mice were fed a regular chow diet. We wanted to eliminate dyslipidemia to study vascular repair in a setting with only hyperglycemia.

In Edsfeldts study, diabetics had less of the stabilizing matrix proteins collagen and elastin. In our study, we did not find a difference between the hyperglycemic and normoglycemic mice. The neointima formation was the same between the groups. We quantified various ECM proteins, receptors and enzymes involved in vascular repair and remodeling such as collagen, elastin, versican, fibromodulin, lumican, AGE, uPARAP and lysyl oxidase (LOX). We also looked at cleaved collagen using an antibody from Ibex that detects collagenase cleaved collagen I and II. It appears that without dyslipidemia, hyperglycemia does not affect vascular repair processes.

The Akita mouse and our cast model of neointima formation are well established and documented. Due to unexpected events, we do not have glucose measurements from our mice. We have measured the plasma insulin levels and they are significantly lower in the Akita mice. This should reflect their hyperglycemia.

As a continuation to this project, we have recently completed, but not analyzed, a study using mice heterozygous for glucokinase on an ApoE^{-/-} background. We would like to study if hyperglycemia, together with dyslipidemia, affects tissue repair in advanced and fibrous plaque phenotypes.

Osteopontin and beta-cell function

Our study showed that OPN deficiency in the mouse pancreatic beta cells results in negative alterations of beta-cell physiology that appear to be compensated for in a healthy physiological state. In the OPN^{-/-} mouse we detected three important alterations in the beta cells: elevated Ca^{2+} levels, morphological modifications of the endoplasmic reticulum and altered insulin vesicle localization. These findings indicate that reduced levels of OPN could in the long run be a factor contributing to development of beta cell failure.

The OPN deficiency has been reported to protect from insulin resistance that is associated with changes in adipocyte biology and adipose tissue inflammatory status. In the development of HFD-induced insulin resistance, OPN is emerging as a key mediator [152,183]. OPN has shown to impair differentiation and insulin sensitivity of primary adipocytes and to induce inflammatory signaling[184]. OPN mRNA and protein expression is upregulated in adipose tissue from obese mice and humans[185,186]. Despite the collection of data supporting the potential of OPN as a therapeutic target for obesity-induced complications, the potential risk of beta cell defects should be considered due to our findings. In addition, looking outside the field of insulin-resistance, OPN has been described as a protective protein for islets in T1D. OPN is an anti-apoptotic factor that has shown to prevent apoptosis as well as to stimulate cell proliferation in human beta cells[152,153]. One study has suggested that soluble OPN can in a variety of situations help beta cells survive an otherwise lethal insult. Furthermore, it has been shown that OPN is drastically increased in rat islets after STZ administration, a phenomena that might represent an endogenous mechanism to protect the islets against cytotoxicity perhaps in part in due to NO regulatory mechanism[142,151]. In one of our studies mentioned earlier, mice deficient of OPN did not survive STZ treatment, a treatment that was well tolerated by WT mice, suggesting a protective role of OPN against insults to the beta cell.

We have a few question left to answer. Can pancreatic islets from older OPN^{-/-} mice compensate for the alterations in their beta cells? Do glucose levels change with age in OPN^{-/-} mice?

It appears that OPN has a protective role in the survival of pancreatic islets and that OPN is the culprit in the decrease of insulin sensitivity. These contrary findings need further evaluation. OPN is indeed a pleiotropic and versatile protein.

Summary in Swedish

Populärvetenskaplig Sammanfattning
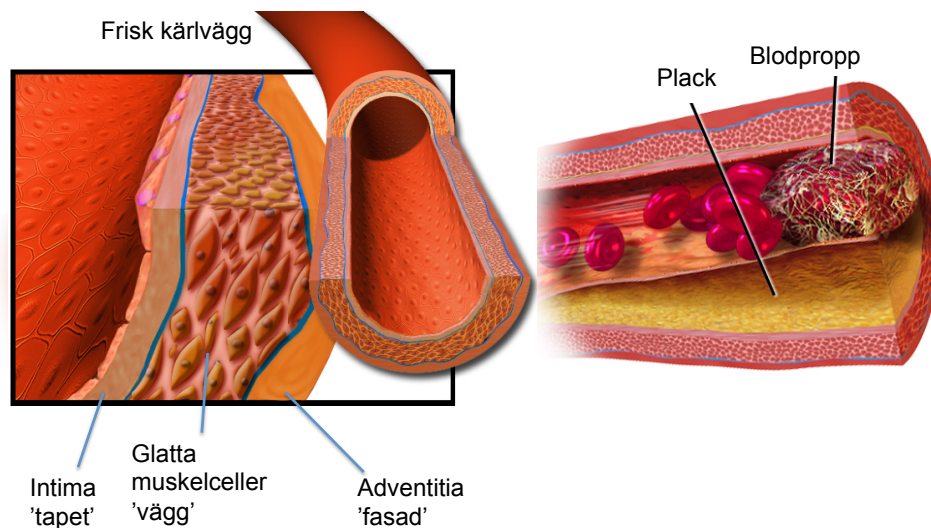
Figur 1.
Bilden till vänster visar hur en artär är uppbyggd. En artär har tre lager: intima, media och adventitia. En kärlvägg kan likanas vid en husvägg där man har tapet, vägg och fasad. Bilden till höger visar hur ett åderförkalkat kärl kan orsaka en blodpropp som täpper till blodkärlet. Bild: Blausen 2014[1].

Alla drabbas av hjärtkärlsjukdom på något vis. Antingen har man själv haft en hjärtinfarkt eller stroke, eller så är man anhörig till någon har drabbats. Den bakomliggande sjukdomen kallas för ateroskleros, eller åderförkalkning som man i folkmun säger. Hjärtkärlsjukdom är den vanligaste dödsorsaken i världen.

Kärlväggen består av tre olika lager: intima, media och adventitia (figur 1). Den kan liknas vid en husvägg som är uppbyggt av tre lager: tapet, vägg, och fasad. När blodet pumpas ut från hjärtat ska det leverera syre både upp till huvudet och ner till fötterna, vilket innebär att kärlet måste böja och förgrena sig. Precis vid förgreningar och krökar uppstår det turbulens och virvlar i blodet. Dessa typer av blodflöden kan irritera och leda till inflammation i kärlen. Inflammationen sker inte i själva

blodbanan, utan i kärlväggen, i intiman (figur 1). I de irriterade områdena i kärlen har kolesterol från blodet lättare att ta sig in i kärlväggen där det ansamlas och härsknar med tiden. Kroppen som har en utmärkt förmåga att självläka försöker reparera skadan i kärlväggen och bryta ner det ansamlade fettet. De glatta muskelcellerna som finns i median i kärlväggen (figur 1) aktiveras av skadan och producerar bindväv som en del av läkningsprocessen. Likt ett sår på huden som lämnat kvar ett ärr, bildas ärr inuti kärlväggen. Ärret i kärlväggen kapslar in fett och den pågående inflammationen i ett försök att dölja skadan. Detta gör att en bula bildas, ett så kallat plack, som minskar kärlets hålrum där blodet transporteras. Det är sällan ett plack växer sig så stort att det täpper till hela kärlets hålrum. Faran med plack är att de kan brista, och då kommer innehållet i kontakt med blodet som blixtsnabbt bildar en blodpropp som stoppar blodflödet (figur 1, höger bild). Detta händer i till exempel hjärtats kranskärl när man får en hjärtinfarkt (figur 2). Vi kallar svaga plack som har hög risk för att brista för 'sköra plack'. Plack som har mindre risk för att brista kallar vi för 'stabila plack'.
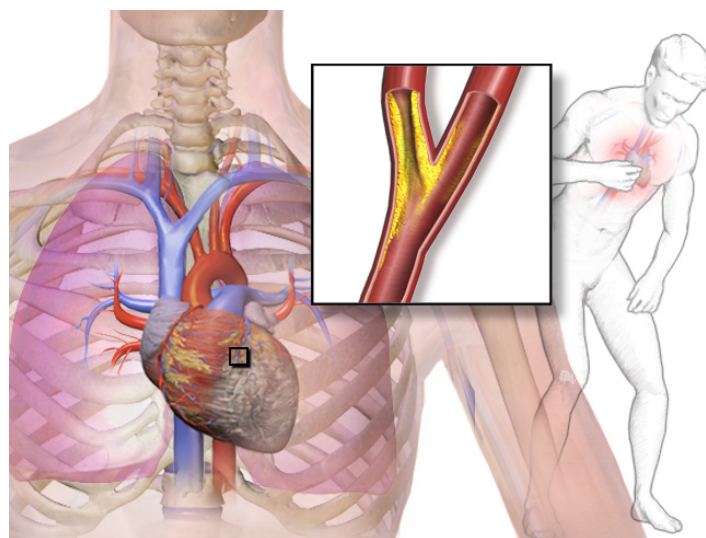
Figur 2
Bilden visar hur åderförkalkning bildats vid en förgrening i ett kranskärl i hjärtat. Denna typen av plack kan vara tyst och inte ge symptom, eller orsaka hjärtinfarkt eller kärlkramp. Bild: Blausen 2014[1].

Ateroskleros börjar redan i unga år och pågår livet ut hos alla människor. Varför drabbas bara vissa av hjärtkärlsjukdomar så som hjärtinfarkt och stroke? Varför brister vissa plack, men andra inte? Det finns riskfaktorer som kan bidra till att plack lättare brister. I min avhandling har vi undersökt diabetes och testosteronsänkande läkemedel som riskfaktorer för att drabbas av hjärtkärlsjukdom, samt hur proteinet IL-22 påverkar de glatta muskelcellernas förmåga att delta i läkningsprocessen.

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Studie I

Prostatacancer är den vanligaste cancerformen hos män i Sverige. Prostatacancer är testosteronkänsligt, och en del av behandlingen består av att sänka testosteronhalten i kroppen för att hindra cancerns framfart. På marknaden finns två typer av läkemedel. Ett som stimulerar testosteronproduktionen så mycket att systemet blir utmattat och testosteronhalten sjunker. Ett annat relativt nytt läkemedel blockerar signalerna för testosteronproduktion. Det är tidigare känt att testosteronsänkande läkemedel är en riskfaktor för hjärtkärlsjukdom, speciellt för män med redan dokumenterad hjärtkärlsjukdom. När man sänker testosteronhalten hos en människa minskar insulinkänsligheten, fettmassan i kroppen ökar och muskelmassan minskar. Dessa är traditionella riskfaktorer för hjärtkärlsjukdom och man har spekulerat i om effekten av läkemedlet på kroppssammansättningen och en ökad plackbildning kan ha varit orsaken bakom den ökade risken för hjärtkärlsjukdom. Det som talar emot detta är att många patienter insjuknar redan inom ett år efter påbörjad behandling och plackutveckling är en långsam process som kan sträcka sig över decennium. Medelåldern för diagnos av prostatacancer är 68 år, en ålder då man redan har utvecklade plack i kärlen. Detta skulle kunna tyda på att testosteronsänkande läkemedel påverkar befintliga plack i kärlen och gör dem sköra. Studier har visat att den typ av läkemedel som först stimulerar testosteronproduktionen innan den sänker den, orsakar 56% fler incidenter av hjärtkärlsjukdom än det läkemedel som omedelbart blockerar testosteronproduktionen.
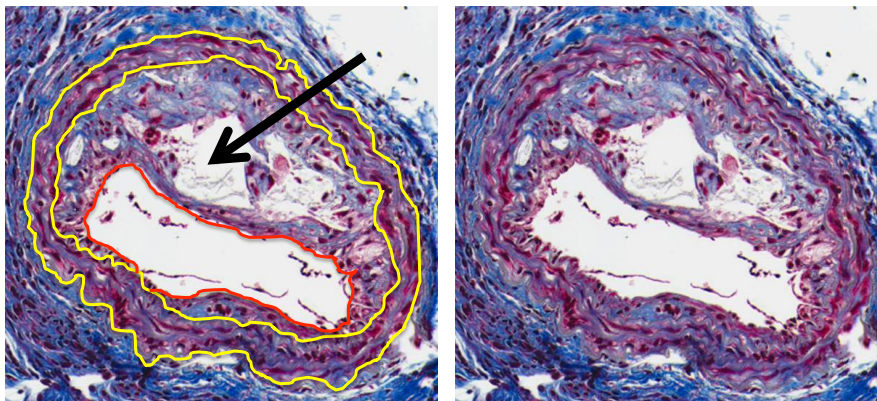
Figur 3.
På bilden till vänster är kärlväggen utritad med gula linjer. Den röda linjen visar blodbanan. All vävnad mellan den röda och den innersta gula linjen är plack. Här är ett exempel på ett plack som innehåller strukturer (de vita, tomma områdena markerade med den svarta pilen) som skulle kunna tyda på att de är mer sköra. Den svarta pilen visar området av celldöd. Bilden till höger är identiskt utan stödlinjer som döljer strukturerna.

I vår studie fann vi stora 'hål' i plack från den grupp av möss som behandlats med den typ av läkemedel som initialt stimulerar testosteronproduktionen för att sedan sjunka

(figur 3). 'Hålet' som uppstår bidrar till att placket blir skört eftersom vävnaden som kapslar in placket har blivit tunnare och det är större risk för att det spricker. Exakt vad läkemedlet gör som orsakar hålen i placken vet vi inte. Vi har utökat studien för att försöka förstå hur testosteronsänkande läkemedel påverkar befintliga plack i kärlen. Med vår studie hoppas vi kunna bidra till att patienter får den säkraste behandlingen vid prostatacancer med avseende på risken för hjärtkärlsjukdom.

Studie II

De glatta muskelcellerna i ett kärl (figur 1) har stor betydelse för läkningen av kärlskador. Precis som andra musklerceller i kroppen kan de spänna sig eller slappna av, och kan därmed hjälpa till med att reglera blodtrycket i kroppen. Vid en skada i ett kärl sker det en inflammatorisk process. Inflammationen påverkar de glatta muskelcellerna så att de tappar sina muskelegenskaper. Istället för att jobba som muskelceller, börjar de glatta muskelcellerna att producera bindväv för att reparera skadan och kapsla in inflammationen i kärlet. Man har tidigare bevisat att glatta muskelceller som behåller sina muskelegenskaper hindrar ett åderförkalkningsplack från att växa. Samtidigt som de glatta muskelcellerna kan läka och reparera skador, så kan de också påskynda placktillväxten genom att förvärra det inflammatoriska tillståndet i kärlet.

I vår studie har vi tittat på ett signalprotein som heter IL-22. IL-22 produceras av inflammatoriska celler och kommunicerar bland annat med glatta muskelceller. Man har tidigare sett att vid utebliven IL-22-utsöndring bildas det mindre åderförkalkningsplack. I vår studie ville vi undersöka om frånvaro av IL-22 påverkar placktillväxten av båda sköra och stabila plack. Vi upptäckte att IL-22 påverkar uppbyggnaden av sköra plack, men inte stabila plack. Vi upptäckte också att sköra plack utan IL-22 hade fler glatta muskelceller med sina muskelegenskaper i behåll. Detta tyder på att frånvaro av IL-22 gör det svårare för glatta muskelceller att tappa sina muskelegenskaper, vilket ger mindre placktillväxt.

Denna studie bidrar till att förstå hur olika celler som finns i plack samverkar och påverkar utvecklingen av hjärtkärlsjukdom.

Studie III

Diabetiker har en mycket högre risk att drabbas av hjärtkärlsjukdom jämfört med icke-diabetiker. I Sverige har 4.7% av befolkning någon form av diabetes. Tidigare studier har visat att diabetiker har en högre grad av inflammation i kroppen, vilket man har trott ökar risken för att plack ska bli sköra. En ny studie visar att plack från diabetiker innehåller mycket mindre bindväv som stabiliserar plack och skyddar dem från ruptur. Vi har gjort en studie där vi undersöker om höga blodsockervärden hos möss påverkar läkningen av en kärlskada samt bindvävens struktur vilket skulle kunna förklara varför diabetiker oftare än icke-diabetiker drabbas av hjärtkärlsjukdom. Intressant nog så fann vi inget sådant samband. För att undersöka saken vidare har vi har ytterligare en studie som pågår där vi upprepar studien om sårläkning i kärl hos diabetiska möss. Skillnaden i detta försök är att vi har inkluderat höga blodfetter eftersom detta ofta drabbar diabetiker och därmed bättre återspeglar miljön i kärlen hos en diabetiker.

Studie IV

Osteopontin är ett protein som har olika funktioner i kroppen varav en funktion är att reglera immunförsvaret. I en studie som föranledde studie III ville vi undersöka effekten på åderförkalkning vid diabetes vid avsaknaden av osteopontin. Betaceller kallas de insulinproducerande cellerna som finns i bukspottskörteln och tidigare studier tyder på att osteopontin är viktigt för att dessa celler ska fungera normalt, alltså producera insulin (figur 4). Osteopontin visade sig var så viktigt för betacellerna i möss med diabetes att det inte gick att genomföra studien. Detta fynd ledde till en ny studie där undersöker osteopontins betydelse för betacellernas funktion.

I samarbete med en forskargrupp i Malmö som är specialister på betaceller utförde vi studie IV. Vi fann att avsaknaden av osteopontin gav betacellerna defekter i sin struktur och funktion, som intressant nog inte gav utslag på insulinproduktionen. De förändringar vi hittade i betacellerna påverkade inte insulinproduktionen under normala förhållanden, men vid tror att den kan påverkas vid sjukdom, som till exempel kronisk inflammation. Detta måste undersökas vidare.

Denna studie är viktig därför att osteopontin är en av bovarna i dramat kring insulinresistens. Där undersöks det om man skulle kunna slå ut effekten av osteopontin för att öka insulinkänslighet. Vår studie visar att om man slår ut osteopontin kan betacellerna få defekter som eventuellt slår ut insulinproduktionen. Dessa motstridiga fynd behöver utforskas närmare.
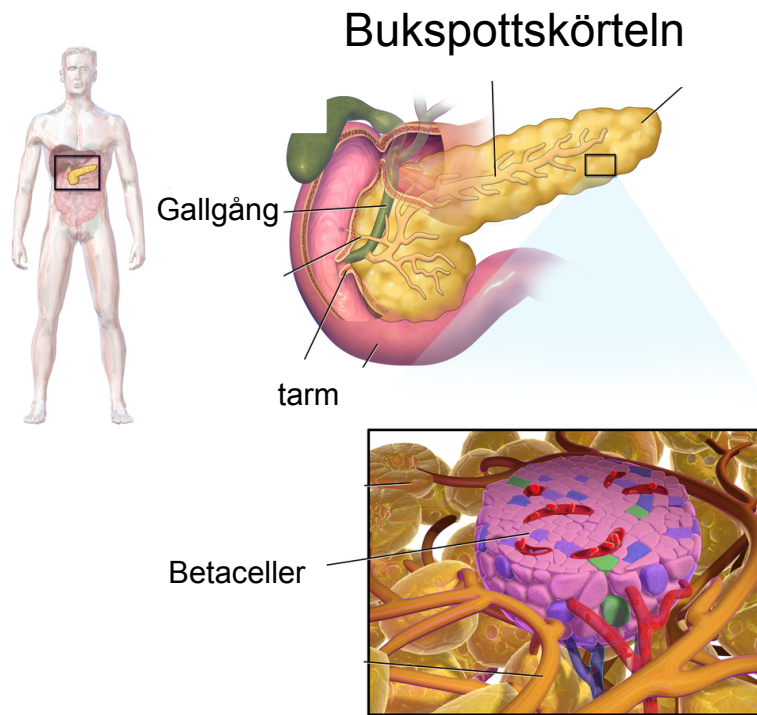
Figur 4.
Insulinproducerande betaceller visas i den nedersta bilden, som i sin tur finns i bukspottskörteln som visas i den övre bilden. Bild: Blausen 2014[1].

Forskning

Forskning om ateroskleros handlar om att hitta mekanismer som påverkar plackets tillväxt och uppbyggnad. I fokus finns förstås en önskan om att finna de mekanismer som ger placket dess benägenhet att ge symptom som till exempel hjärtinfarkt eller stroke.

Acknowledgements

Many minds, many hands and many hours are behind the papers in this thesis. At times, even blood, sweat and tears.

You know who you are!

As a student I lived at Helsingkrona Nation. One day I saw a note in the elevator that they were looking for people to wait tables for a private dissertation party at our nation. After just deciding to take a break from the biomedical program I signed up to earn some money. It turns out that the party was from **Anna Hultgårdh's** floor at BMC. I was in charge of Anna's table and at the time Anna was the head of the biomedical program at Lund University. As I kept serving wine we talked about my future and what I would do now that I had taken a break. After more wine, she asked me to come see her the following Monday. My immediate thought was that I had served too much wine. But as the weekend passed and Monday came along, I received a call from Anna asking me to come to BMC. It was decided later that day that I would come work for Anna for two semesters. Two semesters became 2 years, after which I went surfing around the world for 'some time' before returning to Lund to finish my masters. And now, some more years down the road, I am about to defend my thesis with Anna as my supervisor. I have had the freedom to work independently on this thesis but with Anna's door always being open or her various Apple-gadgets serving as a direct link at any time when I have needed help. Anna has the skill to make any sentence in a text sound ten times better then what it just did by just changing some words. I think this story sums up my gratitude.

Jan Nilsson can easily see the potential in study and convey his thoughts in a manuscript. I need to learn that. His input on our work and manuscripts would have been difficult to be without.

My co-supervisor **Madeleine Durbeej-Hjalt**, besides leading her own research group and being the Deputy Head of our department, has always taken the time when I have showed up at her office for a scientific discussion or to get tips on what TV series to start watching next. Madde is also an excellent baker and makes delicious cakes for fika! Much appreciated.

Pontus Dunér, my second co-supervisor, and I have a long list of experiments in the trashcan. I don't know if it is the combination of the two of us in the lab that brings bad luck, or if it is our ideas behind our experiments that are bad or that some

proteins just have a mysterious way. Either way, it has been fun and adventurous. Let's hope that we can finally put a lid on our osteopontin paper after all these years, once and for all.

Gunnel Roos, also known as Suckel, has taught me everything I know. I don't even know where to begin but be it pore sizes of gloves, tricks with paraffin sections and accurate markings on glass bottles for measuring. She is the real deal old school lab rat who is now retired. I do hope that life after BMC is everything she wished it would be and that she keeps recycling. Besides teaching me lab stuff she tried to educate me in the cultural areas of life too, like the stories of Hasse och Tage. Perhaps less successfully.

Uwe Rauch once said as a response to my whinging about another experiment landing in the trash: 'Now you are treading cream, soon you will be standing on-top of it'. Uwe is the Eeyore of our lab so when he is encouraging or positive towards something, you know it MUST be good. There is always a rodent bar (sugary-oatmeal-chocolate-berry-snack) or a can of coke in his office when your glucose levels drop below zero that is much appreciated. I forgot to mention that Uwe found the Holy Grail! Ask him about it!

Annelie Shami and I have been colleagues during most of our PhD-student years. We shared labs, offices, rooms at conferences (roomie) and developed our own hydrogen peroxide-freshness tests (Don't ask. Well, do, it can come in handy (literally)). She is the smart one, I am the strong one who gets to lift heavy lab equipment and we make an excellent 'remove-boxes-out-of-liquid-nitrogen-tank' team. She's the rational one that walks you through your panic attacks and calms you down. She has the fastest metabolism on earth if anyone reading this is looking for an experimental subject. I am anxiously waiting for her return from her postdoc. My Sci-Fi.

The Minions

Definition: Minions are small, yellow creatures who have existed since the beginning of time, evolving from single-celled organisms into beings that exist only to serve history's most despicable masters (Wikipedia). Minions can also be human examples of medical or biomedical students that come to a lab for summer projects or lab experience during the semesters.

Selvi Celik was the first minion. She later affectionately acquired the name Chihuahua for being the size xxs and the person that you always hear before you see! Selvi is a computer genius that always understands written instructions ten times faster than anyone else. Perhaps it is because she reads a lot of books. She wakes up rested at ungodly hours and goes to work early. Without Selvi I couldn't have managed all the histological work for my Ferring project. I am forever grateful.

Selvi's friend **Sabrina Hsiung** was looking for a lab to do her masters project and Selvi recommended our lab (thank you!). Selvi went off to the States (but is now working

on her PhD only one floor away and we still have lunch) and Sabrina stayed as a PhD student in our lab. Having the combo of Selvi and Sabrina in the lab was like a dream, very skilled individuals that made our projects move forward.

Remember in school when you had a lab partner? In real life there is no such thing, but Sabrina and I are lab partners. We work together on every project and I cannot remember how lab-life was before Selvi brought her to us. We agree on everything and have a similar way of thinking and working. We are equally useless; perhaps I am a little more useless, at solving quadratic equations. Thank you Google for finding places on the Internet where we can punch in our numbers and it solves the equation for us. We take the same classes at different gyms and somehow we have a lot to say about it because it consumes a lot of our conversations. Sabrina always has two items in her bag: chewing gum and a phone charger. Her battery lasts just a few hours and the charger has been used so much that even the cable had to be replaced. I think someone should get her a new phone for Christmas.

Jakob Larsson och Oscar van der Have, my mini pigs, Helan och Halvan.

Jakob and Oscar came to our lab in their first semester of medical school for a course lab. We had so much fun and they were ridiculously easy to teach so I asked them to apply for a summer project in our lab. For two summers now they have been working with us on the papers in this thesis. Oscar, Big Tuna, is our songbird and a member of Studentsångarna. He spends his days ducking the emergency showers and other signs that are placed lower then 207 cm from the floor. Jakob is the mathematician, chemist and always sick. One summer he had both Borrelia and a 'ruptured appendix'. I shall forever remember the pH 2.4 10xPBS that they made for me. Anyone working on Fridays knows that it's 'Theme Day' where we pick a topic and play music accordingly. It happens that we do sing along so that the PBS makes waves in their bottles. Or was that the base on 'hip-hop Friday' that I am confusing it with? Please return to Vessel Wall Biology...

Ellen Andersson and **Anna Roxå** are technically Sabrina's minions. But they are my lucky charms and my inspirational vegetarian friends.

Zandra Körner defended her thesis just recently and is now on maternity leave. We started our PhDs around the same-ish time so we have gone through it all together. At times when I have felt like opening the window to throw out my computer because of 'some files or programs' being evil, Zandra has been there to help me fix it. You can imagine the rage that Zandra so nicely put up with to help me. Zandra is the one who takes your food out of the microwave instead of telling you it's done. If a laundry bag in the changing room is full, Zandra will replace it. And Zandra, keep your horse.

Azra is not a minion, but a friend of the minions who used to work in Zandra's lab. What would Selvi be without Azra? And what would our lunches be without Selvi (Sylvi)? (answer:quiet) Azra is super intelligent and a super picky eater.

Eva Degerman, Ann-Ki and Måns The three musketeers. I don't even know where to start. I have been helping this trio with osmotic minipumps during which I have heard the most unbelievable stories that I don't think they want me to print. My first meeting with Måns ended at the doctor's office. That's a different story for another time. Eva and Ann-Ki, thank you for all the 'fat help' that you have given me. It is always a please and an adventure to be working with you.

Vignesh Besides taking swimming lessons on his free time, Vignesh makes the most tasty and complicated Indian sweets for fika. He said his mom is on Skype as support. One day he made something called milk sweets (I think), where he filtered 9 liters of milk through cheesecloth. A for effort! Vignesh, you need to acquire the taste for lakrits. I can imagine that would go well with some of the dishes you make.

Cibely and Bernardo are part of our collection of Brazilians. Cibely has dental braces like me and I am grateful that she takes the time to listen to me describing my pain. She knows what it is like to remove a molar because she removed two. Bernardo is the healthy guy who works out and feels bad for eating cake. I enjoy our conversations about resistance training and posture. And oh, Bernardo is a language genius. He speaks Swedish after being here just a short time.

Kinga is sometimes known as 'Laminina' for being the laminin expert and the person who can squeeze every drop out of an experiment and makes really pretty IF stainings. I have heard she is quite the card shark too. The real diamond child.

Johan Holmberg, besides being a scientist, is the rock star drummer of a ska band called Liberator. They used to tour Europe. It is very refreshing to have someone around that took the long road.

Sebastian aka GIGA knows everything about SLRPs. He is also an excellent chef and baker. Only crème de la crème products come into his kitchen or out of his kitchen. If you are nice, he brings you warm homemade croissants for breakfast in the lab. For Christmas one year I got a set of magnetic letters for the fridge so I could practice the alphabet. He calls me nano banano.

Åke Oldberg would be The King in the ant world (he can tell you about the ant hierarchy system) and he never washes his car.

Anna Wendt and **Lena Eliasson** are the brains behind our islet paper. I am grateful for getting the opportunity to learn how to inflate a pancreas. It really looks like Kosta Boda art! Lena invited me come along for the electron microscopy of our islets and Anna has taken a lot of her time to introduce me to her world of research, for this, I am thankful.

Karin Tran Lundmark is the PI of her own research group within the Vessel Wall Biology group. Karin works in the clinic as well as a pediatric cardiologist. Karin has two students, '**Christian** and **Christian**'. The Christians are friends of my minions and I believe that someone once called our lunches 'fritidsgård'. I don't know what they are talking about.

Sara, Daniel, Cat, Xenia and **Maria W** were the core of the PhD students at CRC, but have all moved on. They have been a great support when projects have landed in the trash and fun company at conferences. Sara, who would have thought that we would share love for IL-22 after I broke up with IL-5? A few years ago Xenias returned from a short visit to Greece and invited us all to an evening at CRC. She served Ouzo on ice. I love liquorice and didn't think. Pontus Dunér can tell you the story about the train ride home.

Ingrid Yao Mattisson has an Icelandic horse and saved me when I was in a tight situation and needed to send in a manuscript revision.

Lena Sundius is the IHC-genius and has a lot of tips and tricks to teach about histology. Please come work with us!

Matilda Sjöstrand is THE best animal caretaker you can imagine. She sees things before they happen, is super organized and a great friend.

Karin och Viveka always have the time and answers to your lab-questions. Rock solid people. Thank you for singing along with us on Fridays!

Catharina Müller once told me that she wrote her thesis in 2 months. I now understand it is possible but not something I would recommend. But it has been encouraging throughout this whole ordeal.

Kristin Holmgren and I take BodyBalance classes together and share the joy of mushroom picking.

Mea Pelkonen is an awesome, thoughtful and talented person. Forget me not.

Ricard Rinaldo aka Gadget can fix any machine or item that is broken. My cooling centrifuge that no one wanted to repair is now spinning happily again. He is also handy with 3D programs and can make you nice figures for your manuscripts. He snapped the cover photo on this thesis.

Borstahusens Hunddagis is a heaven-like place for dogs that is run by the two most loving people.

Ida aka Ape rescued our BioPix-inferno this spring. There would not be a thesis without Ape.

Carina and **Mimmi** know what hard academic work is and that is relieving.

References

1. Blausen gallery 2014. *Wikiversity Journal of Medicine* (2014).

2. Wight, T.N. The extracellular matrix and atherosclerosis. *Curr Opin Lipidol* **6**, 326-334 (1995).

3. Adiguzel, E., Ahmad, P.J., Franco, C. & Bendeck, M.P. Collagens in the progression and complications of atherosclerosis. *Vasc Med* **14**, 73-89 (2009).

4. Davis, E.C. Endothelial cell connecting filaments anchor endothelial cells to the subjacent elastic lamina in the developing aortic intima of the mouse. *Cell Tissue Res* **272**, 211-219 (1993).

5. Raines, E.W. The extracellular matrix can regulate vascular cell migration, proliferation, and survival: relationships to vascular disease. *Int J Exp Pathol* **81**, 173-182 (2000).

6. Tunon, J., Ruiz-Ortega, M. & Egido, J. Regulation of matrix proteins and impact on vascular structure. *Curr Hypertens Rep* **2**, 106-113 (2000).

7. Ross, R., Glomset, J. & Harker, L. Response to injury and atherogenesis. *Am J Pathol* **86**, 675-684 (1977).

8. Williams, K.J. & Tabas, I. The response-to-retention hypothesis of early atherogenesis. *Arterioscler Thromb Vasc Biol* **15**, 551-561 (1995).

9. Williams, K.J. & Tabas, I. The response-to-retention hypothesis of atherogenesis reinforced. *Curr Opin Lipidol* **9**, 471-474 (1998).

10. Chait, A. & Wight, T.N. Interaction of native and modified low-density lipoproteins with extracellular matrix. *Curr Opin Lipidol* **11**, 457-463 (2000).

11. Miller, Y.I.*, et al.* Oxidation-specific epitopes are danger-associated molecular patterns recognized by pattern recognition receptors of innate immunity. *Circ Res* **108**, 235-248 (2011).

12. Cominacini, L.*, et al.* The binding of oxidized low density lipoprotein (ox-LDL) to ox-LDL receptor-1 reduces the intracellular concentration of nitric oxide in endothelial cells through an increased production of superoxide. *J Biol Chem* **276**, 13750-13755 (2001).

13. Greaves, D.R. & Gordon, S. The macrophage scavenger receptor at 30 years of age: current knowledge and future challenges. *J Lipid Res* **50 Suppl**, S282-286 (2009).

14. Witztum, J.L. The role of oxidized LDL in atherosclerosis. *Advances in experimental medicine and biology* **285**, 353-365 (1991).

15. Witztum, J.L. & Steinberg, D. Role of oxidized low density lipoprotein in atherogenesis. *The Journal of clinical investigation* **88**, 1785-1792 (1991).

16. Nakashima, Y., Chen, Y.X., Kinukawa, N. & Sueishi, K. Distributions of diffuse intimal thickening in human arteries: preferential expression in atherosclerosis-prone arteries from an early age. *Virchows Archiv : an international journal of pathology* **441**, 279-288 (2002).

17. Nakashima, Y., Fujii, H., Sumiyoshi, S., Wight, T.N. & Sueishi, K. Early human atherosclerosis: accumulation of lipid and proteoglycans in intimal thickenings followed by macrophage infiltration. *Arterioscler Thromb Vasc Biol* **27**, 1159-1165 (2007).

18. Skalen, K.*, et al.* Subendothelial retention of atherogenic lipoproteins in early atherosclerosis. *Nature* **417**, 750-754 (2002).

19. Gustafsson, M., Flood, C., Jirholt, P. & Boren, J. Retention of atherogenic lipoproteins in atherogenesis. *Cell Mol Life Sci* **61**, 4-9 (2004).

20. Schwenke, D.C. & Carew, T.E. Initiation of atherosclerotic lesions in cholesterol-fed rabbits. I. Focal increases in arterial LDL concentration precede development of fatty streak lesions. *Arteriosclerosis* **9**, 895-907 (1989).

21. Schwenke, D.C. & Carew, T.E. Initiation of atherosclerotic lesions in cholesterol-fed rabbits. II. Selective retention of LDL vs. selective increases in LDL permeability in susceptible sites of arteries. *Arteriosclerosis* **9**, 908-918 (1989).

22. Schwenke, D.C. & St Clair, R.W. Influx, efflux, and accumulation of LDL in normal arterial areas and atherosclerotic lesions of white Carneau pigeons with naturally occurring and cholesterol-aggravated aortic atherosclerosis. *Arteriosclerosis and thrombosis : a journal of vascular biology / American Heart Association* **13**, 1368-1381 (1993).

23. Furnkranz, A.*, et al.* Oxidized phospholipids trigger atherogenic inflammation in murine arteries. *Arterioscler Thromb Vasc Biol* **25**, 633-638 (2005).

24. Nakashima, Y., Raines, E.W., Plump, A.S., Breslow, J.L. & Ross, R. Upregulation of VCAM-1 and ICAM-1 at atherosclerosis-prone sites on the endothelium in the ApoE-deficient mouse. *Arterioscler Thromb Vasc Biol* **18**, 842-851 (1998).

25. Libby, P. Inflammation in atherosclerosis. *Nature* **420**, 868-874 (2002).

26. Cheng, C.*, et al.* Atherosclerotic lesion size and vulnerability are determined by patterns of fluid shear stress. *Circulation* **113**, 2744-2753 (2006).

27. Kzhyshkowska, J., Neyen, C. & Gordon, S. Role of macrophage scavenger receptors in atherosclerosis. *Immunobiology* **217**, 492-502 (2012).

28. Heinecke, J.W. Oxidants and antioxidants in the pathogenesis of atherosclerosis: implications for the oxidized low density lipoprotein hypothesis. *Atherosclerosis* **141**, 1-15 (1998).

29. Bobryshev, Y.V. Monocyte recruitment and foam cell formation in atherosclerosis. *Micron* **37**, 208-222 (2006).

30. Raines, E.W. & Ross, R. Smooth muscle cells and the pathogenesis of the lesions of atherosclerosis. *Br Heart J* **69**, S30-37 (1993).

31. Gomez, D. & Owens, G.K. Smooth muscle cell phenotypic switching in atherosclerosis. *Cardiovasc Res* **95**, 156-164 (2012).

32. Ross, R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* **362**, 801-809 (1993).

33. Newby, A.C. & Zaltsman, A.B. Fibrous cap formation or destruction--the critical importance of vascular smooth muscle cell proliferation, migration and matrix formation. *Cardiovasc Res* **41**, 345-360 (1999).

34. Gosain, A. & DiPietro, L.A. Aging and wound healing. *World journal of surgery* **28**, 321-326 (2004).

35. Owens, G.K. Regulation of differentiation of vascular smooth muscle cells. *Physiol Rev* **75**, 487-517 (1995).

36. Rensen, S.S., Doevendans, P.A. & van Eys, G.J. Regulation and characteristics of vascular smooth muscle cell phenotypic diversity. *Neth Heart J* **15**, 100-108 (2007).

37. Naghavi, M.*, et al.* From vulnerable plaque to vulnerable patient: a call for new definitions and risk assessment strategies: Part I. *Circulation* **108**, 1664-1672 (2003).

38. Fernandez-Ortiz, A.*, et al.* Characterization of the relative thrombogenicity of atherosclerotic plaque components: implications for consequences of plaque rupture. *J Am Coll Cardiol* **23**, 1562-1569 (1994).

39. Hansson, G.K., Libby, P. & Tabas, I. Inflammation and plaque vulnerability. *J Intern Med* **278**, 483-493 (2015).

40. Galis, Z.S., Sukhova, G.K., Lark, M.W. & Libby, P. Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. *The Journal of clinical investigation* **94**, 2493-2503 (1994).

41. Newby, A.C. Metalloproteinases and vulnerable atherosclerotic plaques. *Trends in cardiovascular medicine* **17**, 253-258 (2007).

42. Guyton, J.R. Phospholipid hydrolytic enzymes in a 'cesspool' of arterial intimal lipoproteins: a mechanism for atherogenic lipid accumulation. *Arterioscler Thromb Vasc Biol* **21**, 884-886 (2001).

43. Guyton, J.R. & Klemp, K.F. Development of the lipid-rich core in human atherosclerosis. *Arterioscler Thromb Vasc Biol* **16**, 4-11 (1996).

44. Moore, K.J. & Tabas, I. Macrophages in the pathogenesis of atherosclerosis. *Cell* **145**, 341-355 (2011).

45. Edfeldt, K., Swedenborg, J., Hansson, G.K. & Yan, Z.Q. Expression of toll-like receptors in human atherosclerotic lesions: a possible pathway for plaque activation. *Circulation* **105**, 1158-1161 (2002).

46. Quillard, T.*, et al.* TLR2 and neutrophils potentiate endothelial stress, apoptosis and detachment: implications for superficial erosion. *European heart journal* **36**, 1394-1404 (2015).

47. Khalil, M.F., Wagner, W.D. & Goldberg, I.J. Molecular interactions leading to lipoprotein retention and the initiation of atherosclerosis. *Arterioscler Thromb Vasc Biol* **24**, 2211-2218 (2004).

48. Hegele, R.A. Plasma lipoproteins: genetic influences and clinical implications. *Nature reviews. Genetics* **10**, 109-121 (2009).

68

49. Daniels, T.F., Killinger, K.M., Michal, J.J., Wright, R.W., Jr. & Jiang, Z. Lipoproteins, cholesterol homeostasis and cardiac health. *International journal of biological sciences* **5**, 474-488 (2009).

50. Powell, L.M.*, et al.* A novel form of tissue-specific RNA processing produces apolipoprotein-B48 in intestine. *Cell* **50**, 831-840 (1987).

51. Greeve, J., Altkemper, I., Dieterich, J.H., Greten, H. & Windler, E. Apolipoprotein B mRNA editing in 12 different mammalian species: hepatic expression is reflected in low concentrations of apoB-containing plasma lipoproteins. *J Lipid Res* **34**, 1367-1383 (1993).

52. Fogelstrand, P. & Boren, J. Retention of atherogenic lipoproteins in the artery wall and its role in atherogenesis. *Nutrition, metabolism, and cardiovascular diseases : NMCD* **22**, 1-7 (2012).

53. Mahley, R.W. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science* **240**, 622-630 (1988).

54. Mahley, R.W. Heparan sulfate proteoglycan/low density lipoprotein receptor-related protein pathway involved in type III hyperlipoproteinemia and Alzheimer's disease. *Israel journal of medical sciences* **32**, 414-429 (1996).

55. Beisiegel, U., Weber, W., Ihrke, G., Herz, J. & Stanley, K.K. The LDL-receptor-related protein, LRP, is an apolipoprotein E-binding protein. *Nature* **341**, 162-164 (1989).

56. Bovenschen, N., Mertens, K., Hu, L., Havekes, L.M. & van Vlijmen, B.J. LDL receptor cooperates with LDL receptor-related protein in regulating plasma levels of coagulation factor VIII in vivo. *Blood* **106**, 906-912 (2005).

57. Zaiou, M.*, et al.* Apolipoprotein E;-low density lipoprotein receptor interaction. Influences of basic residue and amphipathic alpha-helix organization in the ligand. *J Lipid Res* **41**, 1087-1095 (2000).

58. Huggins, C. & Hodges, C.V. Studies on prostatic cancer: I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. 1941. *J Urol* **168**, 9-12 (2002).

59. Labrie, F. Adrenal androgens and intracrinology. *Semin Reprod Med* **22**, 299-309 (2004).

60. Cook, T. & Sheridan, W.P. Development of GnRH antagonists for prostate cancer: new approaches to treatment. *The oncologist* **5**, 162-168 (2000).

61. Belchetz, P.E., Plant, T.M., Nakai, Y., Keogh, E.J. & Knobil, E. Hypophysial responses to continuous and intermittent delivery of hypopthalamic gonadotropin-releasing hormone. *Science* **202**, 631-633 (1978).

62. Hiller-Sturmhofel, S. & Bartke, A. The endocrine system: an overview. *Alcohol health and research world* **22**, 153-164 (1998).

63. Naor, Z., Shacham, S., Harris, D., Seger, R. & Reiss, N. Signal transduction of the gonadotropin releasing hormone (GnRH) receptor: cross-talk of calcium, protein kinase C (PKC), and arachidonic acid. *Cellular and molecular neurobiology* **15**, 527-544 (1995).

64. Reiter, E. & Lefkowitz, R.J. GRKs and beta-arrestins: roles in receptor silencing, trafficking and signaling. *Trends Endocrinol Metab* **17**, 159-165 (2006).

65. Klotz, L*., et al.* The efficacy and safety of degarelix: a 12-month, comparative, randomized, open-label, parallel-group phase III study in patients with prostate cancer. *BJU Int* **102**, 1531-1538 (2008).

66. Bhatia, N*., et al.* Cardiovascular Effects of Androgen Deprivation Therapy for the Treatment of Prostate Cancer: ABCDE Steps to Reduce Cardiovascular Disease in Patients With Prostate Cancer. *Circulation* **133**, 537-541 (2016).

67. Tayek, J.A*., et al.* Nutritional and metabolic effects of gonadotropin-releasing hormone agonist treatment for prostate cancer. *Metabolism* **39**, 1314-1319 (1990).

68. Smith, J.C*., et al.* The effects of induced hypogonadism on arterial stiffness, body composition, and metabolic parameters in males with prostate cancer. *The Journal of clinical endocrinology and metabolism* **86**, 4261-4267 (2001).

69. Smith, M.R*., et al.* Changes in body composition during androgen deprivation therapy for prostate cancer. *The Journal of clinical endocrinology and metabolism* **87**, 599-603 (2002).

70. Smith, M.R. Changes in fat and lean body mass during androgen-deprivation therapy for prostate cancer. *Urology* **63**, 742-745 (2004).

71. Saylor, P.J. & Smith, M.R. Metabolic complications of androgen deprivation therapy for prostate cancer. *J Urol* **181**, 1998-2006 (2009).

72. Smith, M.R*., et al.* Metabolic changes during gonadotropin-releasing hormone agonist therapy for prostate cancer: differences from the classic metabolic syndrome. *Cancer* **112**, 2188-2194 (2008).

73. Nguyen, P.L*., et al.* Adverse effects of androgen deprivation therapy and strategies to mitigate them. *European urology* **67**, 825-836 (2015).

74. Conteduca, V., Di Lorenzo, G., Tartarone, A. & Aieta, M. The cardiovascular risk of gonadotropin releasing hormone agonists in men with prostate cancer: an unresolved controversy. *Critical reviews in oncology/hematology* **86**, 42-51 (2013).

75. Nguyen, P.L*., et al.* Androgen deprivation therapy reversibly increases endothelium-dependent vasodilation in men with prostate cancer. *J Am Heart Assoc* **4**(2015).

76. Albertsen, P.C*., et al.* Cardiovascular morbidity associated with gonadotropin releasing hormone agonists and an antagonist. *European urology* **65**, 565-573 (2014).

77. Rosario, D.J*., et al.* The role of gonadotrophin-releasing hormone antagonists in the treatment of patients with advanced hormone-dependent prostate cancer in the UK. *World J Urol* (2016).

78. Pedersen, T.R*., et al.* Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). 1994. *Atheroscler Suppl* **5**, 81-87 (2004).

79. Nanda, A., Chen, M.H., Braccioforte, M.H., Moran, B.J. & D'Amico, A.V. Hormonal therapy use for prostate cancer and mortality in men with coronary artery disease-induced congestive heart failure or myocardial infarction. *JAMA* **302**, 866-873 (2009).

80. Hayes, J.H*., et al.* Androgen-suppression therapy for prostate cancer and the risk of death in men with a history of myocardial infarction or stroke. *BJU Int* **106**, 979-985 (2010).

70

81. Hansson, G.K. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* **352**, 1685-1695 (2005).

82. Xia, Q.*, et al.* Characterisation of IL-22 and interferon-gamma-inducible chemokines in human carotid plaque. *Int J Cardiol* **154**, 187-189 (2012).

83. Zhang, L.*, et al.* Elevated frequencies of circulating Th22 cell in addition to Th17 cell and Th17/Th1 cell in patients with acute coronary syndrome. *PLoS One* **8**, e71466 (2013).

84. Rattik, S.*, et al.* IL-22 affects smooth muscle cell phenotype and plaque formation in apolipoprotein E knockout mice. *Atherosclerosis* **242**, 506-514 (2015).

85. Dumoutier, L., Louahed, J. & Renauld, J.C. Cloning and characterization of IL-10-related T cell-derived inducible factor (IL-TIF), a novel cytokine structurally related to IL-10 and inducible by IL-9. *J Immunol* **164**, 1814-1819 (2000).

86. Nagalakshmi, M.L., Murphy, E., McClanahan, T. & de Waal Malefyt, R. Expression patterns of IL-10 ligand and receptor gene families provide leads for biological characterization. *Int Immunopharmacol* **4**, 577-592 (2004).

87. Wolk, K.*, et al.* IL-22 increases the innate immunity of tissues. *Immunity* **21**, 241-254 (2004).

88. Huang, S.M.*, et al.* High-glucose-cultivated peripheral blood mononuclear cells impaired keratinocyte function via reduced IL-22 expression: implications on impaired diabetic wound healing. *Exp Dermatol* **24**, 639-641 (2015).

89. Han, X., Kitamoto, S., Wang, H. & Boisvert, W.A. Interleukin-10 overexpression in macrophages suppresses atherosclerosis in hyperlipidemic mice. *FASEB J* **24**, 2869-2880 (2010).

90. Zhang, W.*, et al.* Antiapoptotic activity of autocrine interleukin-22 and therapeutic effects of interleukin-22-small interfering RNA on human lung cancer xenografts. *Clinical cancer research : an official journal of the American Association for Cancer Research* **14**, 6432-6439 (2008).

91. Dudakov, J.A.*, et al.* Interleukin-22 drives endogenous thymic regeneration in mice. *Science* **336**, 91-95 (2012).

92. Kong, X.*, et al.* Interleukin-22 induces hepatic stellate cell senescence and restricts liver fibrosis in mice. *Hepatology* **56**, 1150-1159 (2012).

93. Radaeva, S., Sun, R., Pan, H.N., Hong, F. & Gao, B. Interleukin 22 (IL-22) plays a protective role in T cell-mediated murine hepatitis: IL-22 is a survival factor for hepatocytes via STAT3 activation. *Hepatology* **39**, 1332-1342 (2004).

94. Li, L.J., Gong, C., Zhao, M.H. & Feng, B.S. Role of interleukin-22 in inflammatory bowel disease. *World journal of gastroenterology* **20**, 18177-18188 (2014).

95. Sellau, J.*, et al.* IL-22 dampens the T cell response in experimental malaria. *Sci Rep* **6**, 28058 (2016).

96. Zheng, Y.*, et al.* Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. *Nature* **445**, 648-651 (2007).

97. Wolk, K.*, et al.* IL-22 and IL-20 are key mediators of the epidermal alterations in psoriasis while IL-17 and IFN-gamma are not. *J Mol Med (Berl)* **87**, 523-536 (2009).

98. Boniface, K.*, et al.* A role for T cell-derived interleukin 22 in psoriatic skin inflammation. *Clin Exp Immunol* **150**, 407-415 (2007).

99. Brito-Luna, M.J.*, et al.* Correlation of IL-12, IL-22, and IL-23 in patients with psoriasis and metabolic syndrome. Preliminary report. *Cytokine* **85**, 130-136 (2016).

100. da Rocha, L.F., Jr.*, et al.* Increased serum interleukin 22 in patients with rheumatoid arthritis and correlation with disease activity. *J Rheumatol* **39**, 1320-1325 (2012).

101. Geboes, L.*, et al.* Proinflammatory role of the Th17 cytokine interleukin-22 in collagen-induced arthritis in C57BL/6 mice. *Arthritis Rheum* **60**, 390-395 (2009).

102. Ikeuchi, H.*, et al.* Expression of interleukin-22 in rheumatoid arthritis: potential role as a proinflammatory cytokine. *Arthritis Rheum* **52**, 1037-1046 (2005).

103. Pickert, G.*, et al.* STAT3 links IL-22 signaling in intestinal epithelial cells to mucosal wound healing. *J Exp Med* **206**, 1465-1472 (2009).

104. McGee, H.M.*, et al.* IL-22 promotes fibroblast-mediated wound repair in the skin. *J Invest Dermatol* **133**, 1321-1329 (2013).

105. Avitabile, S.*, et al.* Interleukin-22 Promotes Wound Repair in Diabetes by Improving Keratinocyte Pro-Healing Functions. *J Invest Dermatol* **135**, 2862-2870 (2015).

106. Liang, S.C.*, et al.* Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med* **203**, 2271-2279 (2006).

107. Ciccia, F.*, et al.* Type 3 innate lymphoid cells producing IL-17 and IL-22 are expanded in the gut, in the peripheral blood, synovial fluid and bone marrow of patients with ankylosing spondylitis. *Annals of the rheumatic diseases* **74**, 1739-1747 (2015).

108. Liang, S.C.*, et al.* An IL-17F/A heterodimer protein is produced by mouse Th17 cells and induces airway neutrophil recruitment. *J Immunol* **179**, 7791-7799 (2007).

109. Volpe, E.*, et al.* Multiparametric analysis of cytokine-driven human Th17 differentiation reveals a differential regulation of IL-17 and IL-22 production. *Blood* **114**, 3610-3614 (2009).

110. Xie, M.H.*, et al.* Interleukin (IL)-22, a novel human cytokine that signals through the interferon receptor-related proteins CRF2-4 and IL-22R. *J Biol Chem* **275**, 31335-31339 (2000).

111. Kotenko, S.V.*, et al.* Identification of the functional interleukin-22 (IL-22) receptor complex: the IL-10R2 chain (IL-10Rbeta) is a common chain of both the IL-10 and IL-22 (IL-10-related T cell-derived inducible factor, IL-TIF) receptor complexes. *J Biol Chem* **276**, 2725-2732 (2001).

112. Li, J.*, et al.* Temporal associations between interleukin 22 and the extracellular domains of IL-22R and IL-10R2. *Int Immunopharmacol* **4**, 693-708 (2004).

113. Tachiiri, A.*, et al.* Genomic structure and inducible expression of the IL-22 receptor alpha chain in mice. *Genes and immunity* **4**, 153-159 (2003).

114. Lim, C. & Savan, R. The role of the IL-22/IL-22R1 axis in cancer. *Cytokine Growth Factor Rev* **25**, 257-271 (2014).

115. Justa, S., Zhou, X. & Sarkar, S. Endogenous IL-22 plays a dual role in arthritis: regulation of established arthritis via IFN-gamma responses. *PLoS One* **9**, e93279 (2014).

116. Ciccia, F.*, et al.* Interleukin (IL)-22 receptor 1 is over-expressed in primary Sjogren's syndrome and Sjogren-associated non-Hodgkin lymphomas and is regulated by IL-18. *Clin Exp Immunol* **181**, 219-229 (2015).

117. Martin, J.C.*, et al.* Interleukin-22 binding protein (IL-22BP) is constitutively expressed by a subset of conventional dendritic cells and is strongly induced by retinoic acid. *Mucosal immunology* **7**, 101-113 (2014).

118. Donath, M.Y. Targeting inflammation in the treatment of type 2 diabetes: time to start. *Nat Rev Drug Discov* **13**, 465-476 (2014).

119. Nguyen, B., Bauman, A. & Ding, D. Incident Type 2 Diabetes in a Large Australian Cohort Study: Does Physical Activity or Sitting Time Alter the Risk Associated With Body Mass Index? *Journal of physical activity & health* (2016).

120. Butalia, S., Kaplan, G.G., Khokhar, B. & Rabi, D.M. Environmental Risk Factors and Type 1 Diabetes: Past, Present, and Future. *Canadian journal of diabetes* (2016).

121. Sattar, N. Revisiting the links between glycaemia, diabetes and cardiovascular disease. *Diabetologia* **56**, 686-695 (2013).

122. Beckman, J.A., Creager, M.A. & Libby, P. Diabetes and atherosclerosis: epidemiology, pathophysiology, and management. *JAMA* **287**, 2570-2581 (2002).

123. Creager, M.A., Luscher, T.F., Cosentino, F. & Beckman, J.A. Diabetes and vascular disease: pathophysiology, clinical consequences, and medical therapy: Part I. *Circulation* **108**, 1527-1532 (2003).

124. Folsom, A.R.*, et al.* Prospective associations of fasting insulin, body fat distribution, and diabetes with risk of ischemic stroke. The Atherosclerosis Risk in Communities (ARIC) Study Investigators. *Diabetes care* **22**, 1077-1083 (1999).

125. de Jager, J.*, et al.* Endothelial dysfunction and low-grade inflammation explain much of the excess cardiovascular mortality in individuals with type 2 diabetes: the Hoorn Study. *Arterioscler Thromb Vasc Biol* **26**, 1086-1093 (2006).

126. Brownlee, M. The pathobiology of diabetic complications: a unifying mechanism. *Diabetes* **54**, 1615-1625 (2005).

127. Moreno, P.R.*, et al.* Coronary composition and macrophage infiltration in atherectomy specimens from patients with diabetes mellitus. *Circulation* **102**, 2180-2184 (2000).

128. Edsfeldt, A.*, et al.* Impaired fibrous repair: a possible contributor to atherosclerotic plaque vulnerability in patients with type II diabetes. *Arterioscler Thromb Vasc Biol* **34**, 2143-2150 (2014).

129. Schranz, D.B. & Lernmark, A. Immunology in diabetes: an update. *Diabetes/metabolism reviews* **14**, 3-29 (1998).

130. Scatena, M., Liaw, L. & Giachelli, C.M. Osteopontin: a multifunctional molecule regulating chronic inflammation and vascular disease. *Arterioscler Thromb Vasc Biol* **27**, 2302-2309 (2007).

131. Strom, A.*, et al.* Altered vascular remodeling in osteopontin-deficient atherosclerotic mice. *J Vasc Res* **41**, 314-322 (2004).

132. Bruemmer, D.*, et al.* Angiotensin II-accelerated atherosclerosis and aneurysm formation is attenuated in osteopontin-deficient mice. *The Journal of clinical investigation* **112**, 1318-1331 (2003).

133. Matsui, Y.*, et al.* Osteopontin deficiency attenuates atherosclerosis in female apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* **23**, 1029-1034 (2003).

134. Haluzik, M. & Nedvidkova, J. The role of nitric oxide in the development of streptozotocin-induced diabetes mellitus: experimental and clinical implications. *Physiol Res* **49 Suppl 1**, S37-42 (2000).

135. Mandrup-Poulsen, T. The role of interleukin-1 in the pathogenesis of IDDM. *Diabetologia* **39**, 1005-1029 (1996).

136. Eizirik, D.L. & Mandrup-Poulsen, T. A choice of death--the signal-transduction of immune-mediated beta-cell apoptosis. *Diabetologia* **44**, 2115-2133 (2001).

137. Tuck, A.B., Hota, C., Wilson, S.M. & Chambers, A.F. Osteopontin-induced migration of human mammary epithelial cells involves activation of EGF receptor and multiple signal transduction pathways. *Oncogene* **22**, 1198-1205 (2003).

138. Zhang, G.X., Zhao, Z.Q. & Lin, X.W. [EGF/PI3K signaling pathway regulates the expression of osteopontin in liver cancer HepG2 cells]. *Zhonghua Yi Xue Za Zhi* **83**, 1980-1983 (2003).

139. Kim, M.S.*, et al.* Hyaluronic acid induces osteopontin via the phosphatidylinositol 3-kinase/Akt pathway to enhance the motility of human glioma cells. *Cancer Res* **65**, 686-691 (2005).

140. Mazzali, M.*, et al.* Osteopontin--a molecule for all seasons. *QJM* **95**, 3-13 (2002).

141. Gong, Q., Chipitsyna, G., Gray, C.F., Anandanadesan, R. & Arafat, H.A. Expression and regulation of osteopontin in type 1 diabetes. *Islets* **1**, 34-41 (2009).

142. Arafat, H.A., Lada, E., Katakam, A.K. & Amin, N. Osteopontin deficiency impacts the pancreatic TH1/TH2 cytokine profile following multiple low dose streptozotocin-induced diabetes. *Exp Clin Endocrinol Diabetes* **114**, 555-562 (2006).

143. Kwon, N.S., Lee, S.H., Choi, C.S., Kho, T. & Lee, H.S. Nitric oxide generation from streptozotocin. *FASEB J* **8**, 529-533 (1994).

144. Corbett, J.A., Sweetland, M.A., Wang, J.L., Lancaster, J.R., Jr. & McDaniel, M.L. Nitric oxide mediates cytokine-induced inhibition of insulin secretion by human islets of Langerhans. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 1731-1735 (1993).

145. Eizirik, D.L. & Pavlovic, D. Is there a role for nitric oxide in beta-cell dysfunction and damage in IDDM? *Diabetes/metabolism reviews* **13**, 293-307 (1997).

146. Cabrera, O.*, et al.* The unique cytoarchitecture of human pancreatic islets has implications for islet cell function. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 2334-2339 (2006).

147. Ashcroft, F.M.*, et al.* Stimulus-secretion coupling in pancreatic beta cells. *J Cell Biochem* **55 Suppl**, 54-65 (1994).

148. MacDonald, P.E. Signal integration at the level of ion channel and exocytotic function in pancreatic beta-cells. *American journal of physiology. Endocrinology and metabolism* **301**, E1065-1069 (2011).

149. Kwan, E.P. & Gaisano, H.Y. New insights into the molecular mechanisms of priming of insulin exocytosis. *Diabetes, obesity & metabolism* **9 Suppl 2**, 99-108 (2007).

150. Gandasi, N.R. & Barg, S. Contact-induced clustering of syntaxin and munc18 docks secretory granules at the exocytosis site. *Nature communications* **5**, 3914 (2014).

151. Katakam, A.K.*, et al.* Streptozotocin (STZ) mediates acute upregulation of serum and pancreatic osteopontin (OPN): a novel islet-protective effect of OPN through inhibition of STZ-induced nitric oxide production. *J Endocrinol* **187**, 237-247 (2005).

152. Ahlqvist, E.*, et al.* A link between GIP and osteopontin in adipose tissue and insulin resistance. *Diabetes* (2013).

153. Lyssenko, V.*, et al.* Pleiotropic effects of GIP on islet function involve osteopontin. *Diabetes* **60**, 2424-2433 (2011).

154. Chiocchetti, A.*, et al.* The osteopontin gene +1239A/C single nucleotide polymorphism is associated with type 1 diabetes mellitus in the Italian population. *International journal of immunopathology and pharmacology* **23**, 263-269 (2010).

155. Marciano, R.*, et al.* Association of alleles at polymorphic sites in the Osteopontin encoding gene in young type 1 diabetic patients. *Clin Immunol* **131**, 84-91 (2009).

156. Jawien, J., Nastalek, P. & Korbut, R. Mouse models of experimental atherosclerosis. *Journal of physiology and pharmacology : an official journal of the Polish Physiological Society* **55**, 503-517 (2004).

157. Brown, M.L.*, et al.* Molecular basis of lipid transfer protein deficiency in a family with increased high-density lipoproteins. *Nature* **342**, 448-451 (1989).

158. Plump, A.S. & Breslow, J.L. Apolipoprotein E and the apolipoprotein E-deficient mouse. *Annual review of nutrition* **15**, 495-518 (1995).

159. Nakashima, Y., Plump, A.S., Raines, E.W., Breslow, J.L. & Ross, R. ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. *Arteriosclerosis and thrombosis : a journal of vascular biology / American Heart Association* **14**, 133-140 (1994).

160. Zhang, S.H., Reddick, R.L., Piedrahita, J.A. & Maeda, N. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science* **258**, 468-471 (1992).

161. Cunningham, K.S. & Gotlieb, A.I. The role of shear stress in the pathogenesis of atherosclerosis. *Lab Invest* **85**, 9-23 (2005).

162. Chyu, K.Y.*, et al.* Decreased neointimal thickening after arterial wall injury in inducible nitric oxide synthase knockout mice. *Circ Res* **85**, 1192-1198 (1999).

163. Thyberg, J., Blomgren, K., Hedin, U. & Dryjski, M. Phenotypic modulation of smooth muscle cells during the formation of neointimal thickenings in the rat carotid artery after balloon injury: an electron-microscopic and stereological study. *Cell Tissue Res* **281**, 421-433 (1995).

164. Strom, A.*, et al.* Inhibition of injury-induced arterial remodelling and carotid atherosclerosis by recombinant human antibodies against aldehyde-modified apoB-100. *Atherosclerosis* **190**, 298-305 (2007).

165. Dimayuga, P.*, et al.* Inhibitory effect on arterial injury-induced neointimal formation by adoptive B-cell transfer in Rag-1 knockout mice. *Arterioscler Thromb Vasc Biol* **22**, 644-649 (2002).

166. Chatzizisis, Y.S.*, et al.* Role of endothelial shear stress in the natural history of coronary atherosclerosis and vascular remodeling: molecular, cellular, and vascular behavior. *J Am Coll Cardiol* **49**, 2379-2393 (2007).

167. Cheng, C.*, et al.* Shear stress-induced changes in atherosclerotic plaque composition are modulated by chemokines. *The Journal of clinical investigation* **117**, 616-626 (2007).

168. Hung, M.J., Cherng, W.J., Hung, M.Y., Wu, H.T. & Pang, J.H. Interleukin-6 inhibits endothelial nitric oxide synthase activation and increases endothelial nitric oxide synthase binding to stabilized caveolin-1 in human vascular endothelial cells. *Journal of hypertension* **28**, 940-951 (2010).

169. Kelly, D.M. & Jones, T.H. Testosterone: a vascular hormone in health and disease. *J Endocrinol* **217**, R47-71 (2013).

170. Ohlsson, C.*, et al.* High serum testosterone is associated with reduced risk of cardiovascular events in elderly men. The MrOS (Osteoporotic Fractures in Men) study in Sweden. *J Am Coll Cardiol* **58**, 1674-1681 (2011).

171. Yoshida, S.*, et al.* Androgen receptor promotes sex-independent angiogenesis in response to ischemia and is required for activation of vascular endothelial growth factor receptor signaling. *Circulation* **128**, 60-71 (2013).

172. Ajayi, A.A., Mathur, R. & Halushka, P.V. Testosterone increases human platelet thromboxane A2 receptor density and aggregation responses. *Circulation* **91**, 2742-2747 (1995).

173. Horne, B.D.*, et al.* Which white blood cell subtypes predict increased cardiovascular risk? *J Am Coll Cardiol* **45**, 1638-1643 (2005).

174. Keating, N.L., O'Malley, A.J. & Smith, M.R. Diabetes and cardiovascular disease during androgen deprivation therapy for prostate cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **24**, 4448-4456 (2006).

175. Feil, S.*, et al.* Transdifferentiation of vascular smooth muscle cells to macrophage-like cells during atherogenesis. *Circ Res* **115**, 662-667 (2014).

176. Langer, C.*, et al.* Testosterone up-regulates scavenger receptor BI and stimulates cholesterol efflux from macrophages. *Biochemical and biophysical research communications* **296**, 1051-1057 (2002).

177. Ackers-Johnson, M*., et al.* Myocardin regulates vascular smooth muscle cell inflammatory activation and disease. *Arterioscler Thromb Vasc Biol* **35**, 817-828 (2015).

178. Orr, A.W*., et al.* Molecular mechanisms of collagen isotype-specific modulation of smooth muscle cell phenotype. *Arterioscler Thromb Vasc Biol* **29**, 225-231 (2009).

179. Lim, S. & Park, S. Erratum: Role of vascular smooth muscle cell in the inflammation of atherosclerosis. *BMB reports* **49**, 134 (2016).

180. Raines, E.W. & Ferri, N. Thematic review series: The immune system and atherogenesis. Cytokines affecting endothelial and smooth muscle cells in vascular disease. *J Lipid Res* **46**, 1081-1092 (2005).

181. Chang, Y*., et al.* Th17-associated cytokines promote human airway smooth muscle cell proliferation. *FASEB J* **26**, 5152-5160 (2012).

182. Chang, Y*., et al.* TH17 cytokines induce human airway smooth muscle cell migration. *The Journal of allergy and clinical immunology* **127**, 1046-1053 e1041-1042 (2011).

183. Chapman, J*., et al.* Osteopontin is required for the early onset of high fat diet-induced insulin resistance in mice. *PLoS One* **5**, e13959 (2010).

184. Zeyda, M*., et al.* Osteopontin is an activator of human adipose tissue macrophages and directly affects adipocyte function. *Endocrinology* **152**, 2219-2227 (2011).

185. Nomiyama, T*., et al.* Osteopontin mediates obesity-induced adipose tissue macrophage infiltration and insulin resistance in mice. *The Journal of clinical investigation* **117**, 2877-2888 (2007).

186. Gomez-Ambrosi, J*., et al.* Plasma osteopontin levels and expression in adipose tissue are increased in obesity. *The Journal of clinical endocrinology and metabolism* **92**, 3719-3727 (2007).