Targeting vascular remodeling in abdominal aortic aneurysm

To identify novel treatment strategies and drug candidates

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“Start by doing what’s necessary; then do what’s possible; and suddenly you are doing the impossible”

-Francis of Assisi

ABSTRACT

Abdominal aortic aneurysm (AAA) is a degenerative weakening of the aortic wall, mainly affecting elderly men with a prevalence of 4.4-7.7 %. AAA is characterized by medial and adventitial inflammatory cell infiltration associated with vascular remodeling of the extracellular matrix proteins such as collagen and elastin and with phenotypic modulation and loss of vascular smooth muscle cells (VSMCs). Although much research has been performed, the precise cellular and molecular pathways behind these processes are still poorly understood. The overall aim of this thesis was to target signaling pathways that affect vascular remodeling of AAA to potentially identify novel strategies and drug candidates for future treatment of aneurysmal diseases. In order to develop our understanding of the pathophysiology of AAA, we used the angiotensin (Ang) II-induced AAA animal model and human biopsies taken at end-stage of disease to recapitulate key aspects of disease formation.

Innate immune receptors such as toll-like receptors (TLRs) are known to regulate immunological processes leading to the formation and progression of vascular disease including AAA. In paper I, we aimed to investigate the role of TLR signaling under the control of the TRIF adaptor protein in the formation of AAA. Human, aneurysmal aortas displayed increased expression of TLR3 and TLR4 in surface of macrophages and T lymphocytes. AngII-induced aneurysm formation was attenuated in mice lacking the *Trif* gene (*ApoE^{-/-}Trif^{-/-}*), and these knockout mice presented with a more intact medial layer together with a reduced inflammatory response by macrophages and T lymphocytes and reduced levels of pro-inflammatory cytokines, chemokines, and proteases. Our results suggest an involvement of TRIF in the pathophysiology of AAA.

Current management of AAA fully depends on imaging and surgical techniques, and drug-based therapies are still mostly ineffective. In paper II, we aimed to investigate the potential protective role of the tyrosine kinase inhibitor imatinib on the molecular mechanism involved in AAA formation. In AngII-infused *ApoE^{-/-}* mice, 10 mg/kg imatinib per day affected several key features important in aneurysmal formation, including preservation of the medial layer of the VSMCs, reduced infiltration of CD3ε-positive T lymphocytes, and reduced gene expression of mast cell chymase, resulting in decreased aortic diameter and vessel wall thickness. These results highlight the importance of the tyrosine kinase inhibitor imatinib as a

potential drug in the treatment of pathological vascular inflammation and remodeling in conditions such as AAA.

In paper III, we aimed to investigate the role of adiponectin in experimentally induced AAA formation in mice. In mice with elevated adiponectin levels, AAA development was inhibited, and this was associated with reduced inflammatory cell infiltration, reduced medial degeneration of VSMCs and of elastin in the aortic vessel wall together with an improved systemic cytokine profile and the attenuation of periaortic adipose tissue (PVAT) inflammation. These results support the protective effect of adiponectin in the remodeling occurring in the aortic wall and in the prevention of AAA.

In paper IV, we performed a descriptive study investigating the composition of PVAT adjacent to the aneurysmal aorta. We used immunohistochemistry to identify neutrophils, macrophages, mast cells, and T lymphocytes surrounding necrotic adipocytes in PVAT together with increased gene expression of IL-6 and cathepsin K and S. We also determined the concentrations of pro-inflammatory ceramides in PVAT and found an association to T lymphocytes. These results suggest that inflamed adipose tissue might be a source of pro-inflammatory cells and mediators that contribute to aortic wall degeneration.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Pulsåderbråk är en sjuklig vidgning av ett blodkärl som vanligast drabbar stora kroppspulsådern i buken och kallas då för bukaortaaneurysm eller abdominalt aorta aneurysm (AAA). Ett AAA definieras utifrån en aortadiameter som är ≥ 30 mm. Sjukdomen förekommer oftast ihop med en underliggande åderförkalkning. Risken att drabbas av AAA är 4-6 gånger vanligare hos män än kvinnor med en prevalens på 4-7.7 % hos män över 65 år och 1.3 % hos kvinnor i samma ålder. De flesta som drabbas är omedvetna om det då aneurysm är symtomfria. Vidgningen av kärlet är exponentiellt som vid ett förvärrat tillstånd kan leda till bristning i kärlväggen vilket medför en mycket hög dödlighet. Eftersom AAA främst drabbar män erbjuds idag alla män vid 65 års ålder en ultraljudsundersökning, detta för att upptäcka AAA i tid och för att lägga in en förebyggande operation då en aortadiameter överskridit 50-55 mm.

AAA är en kronisk inflammatorisk sjukdom med inflammation i aortakärlväggen som bidrar till att beståndsdelar i kärlväggen försvagas och bryts ner vilket leder till att elasticiteten och hållfastheten i kärlet försvinner. Där kärlväggen är som svagast bildas succesivt en vidgning och därmed ett aneurysm. Den underliggande orsaken som ligger till grund för utvecklingen av AAA är ännu oklart och ingen medicinsk behandling finns att tillgå. För att bättre kunna studera sjukdomsförloppen för AAA finns välbeprövade djurmodeller att tillgå. Dessa efterliknar många viktiga processer som det mänskliga aneurysmet har samt ger oss möjligheten att studera sjukdomsförloppet i ett tidigt skede av aneurysmutvecklingen. Delarbete I-III är baserade på djurförsök där AAA experimentellt framkallades genom frisättning av det inflammatoriska proteinet angiotensin (Ang) II från en osmotisk pump som inopereras under nackskinnet på mössen.

Det övergripande syftet med denna avhandling riktar sig mot en öka förståelsen kring signalvägar involverade i omformning av kärlväggen som leder till utvecklingen av AAA. En ökad kunskap och förståelse kring mekanismerna bakom AAA ger oss förhoppningsvis möjligheten att identifiera nya behandlingsstrategier och läkemedelskandidater för framtida behandling av sjukdomen.

Det är känt att inflammation är en av de starkt bidragande faktorerna till förvärring av AAA. I delarbete I studerades genen TRIF, ett intracellulärt protein som reglerar signalerna från immunreceptorerna TLR3 och TLR4, och dess roll i utvecklingen av AAA. Inflammationens roll i sjukdomsutvecklingen studerades genom avsaknad av genen hos möss samtidigt som AAA framkallats experimentellt med AngII modellen. De möss som saknade TRIF-genen var skyddade från utvecklingen av AAA vilket var starkt kopplat till ett minskat inflammatoriskt svar, med färre inflammatoriska celler och mindre inflammationstriggande faktorer i aortaväggen. Delarbete I ger en ökad förståelse för inflammationens roll med avseende på genen TRIF, i utvecklingen av AAA.

Läkemedlet imatinib (Glivec®) är ett väl beprövat läkemedel som idag används vid behandling av maligna blodsjukdomar som kronisk myeloisk leukemi. Imatinib verkar genom att blockera processer som leder till utvecklingen av onormala celler. Studier visar att imatinib även har en inverkan på kroppens normala blodceller och har påvisat en förebyggande roll i utvecklingen av åderförkalkning hos möss. I delarbete II studerades behandlingseffekten av imatinib i AngII-framkallad AAA hos möss. De möss som behandlades med imatinib påvisade ett dämpat inflammatoriskt svar och en kärlstruktur som efterliknar den normala aortan. Delarbete II visar på att imatinib hämmar viktiga mekanismer och signaleringsvägar vid sjukdomsförloppet av AAA. En framtida strategi att använda läkemedlet för att bromsa inflammationsutvecklingen hos aneurysm patienter bör prövas i framtida kliniska studier.

Adiponektin är ett hormon som produceras av fettceller och finns normalt cirkulerande i blodplasman. Kliniska studier visar ett samband mellan ökade nivåer av adiponektin och förbättrad insulinkänslighet vid typ 2 diabetes samt minskad vaskulär inflammation. I delarbete III studerades adiponektinets verkan på aneurysmutvecklingen genom att studera effekten av höga cirkulerande nivåer av adiponektin under utvecklingen av AngII-framkallad AAA hos möss. Adiponektinet hade en skyddande effekt mot utvecklingen av AAA vilket var starkt kopplat till minskad infiltration av inflammatoriska celler och inflammationstriggande faktorer i aortaväggen men även i fettcellerna som omger kärlväggen, samt bidrog till en stabilare kärlstruktur av bindvävsproteinerna elastin och kollagen. Delarbete III resulterade i en ökad förståelse av mekanismen bakom adiponektinets rollen i utvecklingen av AAA.

Våra inre organ täcks av fettvävnad som består av fettceller vars främsta funktioner är att lagra energi, reglera kroppstemperaturen samt bidra till en hormonell funktion däribland att utsöndra hormonet adiponektin. Men om fettvävnaden kring våra organ ökar i massa kan detta leda till en inflammerad och dysfunktionell fettvävnad som tidigare kopplats till en ökad tillväxt av AAA diametern. I delarbete IV studerades mänskliga kärl och kompositionen av inflammatoriska celler och inflammationstriggande faktorer i fettvävnaden som omger aortans yttre kärlvägg. Hos aneurysmpatienter förekom en ökad mängd fettvävnad som karaktäriserades av nekrotiska döda fettceller vilka var omgivna av inflammatoriska celler som neutrofiler, makrofager, mast celler och T celler. Hos aneurysmpatienterna påvisades även ökade nivåer av bindvävsnedbrytande enzymer. Resultaten i delarbete IV indikerar på en eventuellt bidragande roll av inflammerad fettvävnad i sjukdomsprocessen vid AAA.

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* In paper III the two first authors share first authorship.

ABBREVIATIONS

AAA	Abdominal aortic aneurysm
ACE	Angiotensin-converting enzyme
AdipoR	Adiponectin receptor
AMPK	Adenosine monophosphate-activated protein kinase
AngII	Angiotensin II
ApoE	Apolipoprotein E
AT1R	Angiotensin type-1 receptor
BMI	Body mass index
CCL	Chemokine C-C motif ligand
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CXCL	Chemokine C-X-C motif ligand
DAMP	Damage associated molecular pattern
dsRNA	Double-stranded ribonucleic acid
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
Erk	Extracellular regulated kinase
GFP	Green fluorescence protein
HMGB	High-mobility group box
HMW	High- molecular weight
HSP	Heat shock protein
IFN	Interferon
IL	Interleukin
ILT	Intraluminal thrombus
IRF3	Interferon regulatory factor-3
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LELE	Leading-edge to leading-edge
LOX	Lysyl oxidase
Mal	MyD88 adaptor-like protein
MAPK	Mitogen-activated protein kinase
MIP1	Macrophage inflammatory protein 1a

MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MyD88	Myeloid differentiation factor-88
NF- κ B	Nuclear factor kappa B
PAMP	Pathogen associated molecular pattern
PDGF	Platelet-derived growth factor
PI3K	Phosphoinositide 3-kinase
PPAR	Peroxidase proliferator-activated receptor
PVAT	Perivascular adipose tissue
qPCR	Quantitative polymerase chain reaction
RAS	Renin-angiotensin system
S1P	Sphingosine-1 phosphate
SCF	Stem cell factor
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SMC	Smooth muscle cells
Taq	<i>Thermus aquaticus</i>
TCR	T-cell receptor
TGF	Transforming growth factor
T _H 1	T-helper type 1
TIMP	Tissue inhibitors of metalloproteinase
TIR	Toll-interleukin-1 receptor
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor protein including IFN- β
VSMC	Vascular smooth muscle cell
WAT	White adipose tissue

INTRODUCTION

Aneurysm

The word aneurysm is derived from the Greek word “άνευρυσμα” – aneurysma – meaning “widening”. An aneurysm is the irreversible widening of blood vessels that is caused by segmental weakening of all three layers in the vascular wall. Aneurysms are generally without clinical symptoms, and large aneurysms can rupture causing extensive internal bleeding, a life threatening condition with potentially fatal consequences. The most common location of an aneurysm is in the infrarenal aorta, distal to the renal arteries and proximal to the iliac bifurcation, thus the name abdominal aortic aneurysm (AAA) (Figure 1). There are several other common locations of aneurysm development, including the intracranial, iliac, femoral, and popliteal arteries, and these sometimes occur simultaneously with AAA.(Norman and Powell, 2010)

Figure 1. Location of an abdominal aortic aneurysm.

Abdominal aortic aneurysm

Definition

There are several proposed definitions of AAA in clinical use, all based on the abdominal aortic diameter. The most common definition is that the abdominal aortic diameter below the renal arteries is 30 mm, or more.(McGregor et al., 1975, Wanhainen and Bjorck, 2011) Another definition of AAA is an increase in abdominal aortic diameter by 1.5 times or more compared to the diameter of the adjacent normal aorta in the patient. This definition is probably the most scientifically correct definition because the size of the aorta varies with body size, gender, and age.(Sonesson et al., 1994) This definition is not commonly used in clinical practice, however, because the necessary information for the normal abdominal aortic diameter from these patients is often not available. Because the normal abdominal aortic diameter is considered to be <25 mm and an aneurysm is set to 30 mm or more, patients whose abdominal aorta measures in-between these values are classified as a separate subgroup. Hafez *et al.* showed that 2.5% of all patients screened for AAA have an aortic diameter between 25 and 29 mm and that 65% of those went on to develop AAA at the rescreening 5 years after the initial “normal” screening. Men with an initial aortic diameter of 25–29 mm are at a higher risk of developing an aneurysm later in life, and these patients are therefore classified as having an “aneurysm in formation” and a 5-year follow-up is recommended for this subgroup.(Hafez et al., 2008)

In most AAAs an intraluminal thrombus (ILT) is present, which is a fibrin clot that adheres to the aortic wall.(Hans et al., 2005) The ILT arises as a result of altered blood flow in the aorta and is composed of platelets, erythrocytes, and inflammatory cells.(Adolph et al., 1997) ILT has been shown to be a source of proteolytic activity, and it promotes the degradation of the underlying aneurysmal wall.(Vorp et al., 2001)

Prevalence and risk factors

Cardiovascular disease is the major cause of premature death in western society.(Golledge and Norman, 2010) Large screening studies have demonstrated the prevalence of AAA in men 64–80 years old to be 4.0–7.7%, while in women the prevalence is much lower at around 1.3% for the same age range.(Lindholt et al., 2005, Norman et al., 2004, Ashton et al., 2002)

The overall mortality rate of ruptured AAA is 90%. AAAs rupture posteriorly into the retroperitoneal cavity in approximately 80% of the patients. This rupture clinically manifests as back pain with or without abdominal pain. Retroperitoneal ruptures commonly remain sealed for a few hours, allowing the patient time to be transferred to the hospital for diagnosis and surgery. AAAs can also rupture anteriorly into the intraperitoneal cavity, which occurs in approximately 20% of the patients. This tear results in rapid bleeding into the peritoneal cavity, and death usually occurs before the patient reaches the hospital.(Sakalihasan et al., 2005)

Although the pathogenesis of AAA is still unknown, various important risk factors have been suggested to alter the development of AAA including smoking, male gender, older age, atherosclerosis, family history, high blood pressure, inflammation and obesity.(Golledge et al., 2007, Shibamura et al., 2004)

By far, smoking is the most important environmental risk factor for AAA, and smoking increases the growth rate of AAA by 15–20%.(MacSweeney et al., 1994, Brady et al., 2004) Current smokers are more than 7 times more likely to develop AAA compared with nonsmokers (Wilmsink et al., 1999), and 87% of all individuals with AAA are or were smokers (Svensjo et al., 2011).

There is a strong link between atherosclerosis and AAA, and the majority of patients with AAA suffer co-morbidity with atherosclerosis. Approximately 9–16% of all patients with atherosclerotic aorta develop AAA.(Reed et al., 1992, Guo et al., 2006) Because the majority of AAA patients have underlying atherosclerosis, it was initially considered to be the leading cause of AAA. This theory has, however, been revised due to clear histological differences between the two diseases. Johnsen et al. suggested that atherosclerosis develops in parallel with AAA formation and that multiple mechanisms are responsible for AAA development.(Johnsen et al., 2010)

A family history of AAA increases the risk of developing AAA and genetic influence has been shown to have an important role in the etiology of AAA with a higher prevalence in siblings to patients with AAA compared to the prevalence in the general population.(Linne et al., 2012) First-degree relatives of aneurysm patients have an approximately doubled risk of

developing AAA (Larsson et al., 2009), and approximately 15% of individuals with AAA have a positive family history of AAA (Darling et al., 1989).

Although not considered a traditional risk factor for AAA, several studies have found an association between obesity or increased visceral fat and AAA. Some studies showed an association between waist circumference and the presence of AAA (Golledge et al., 2007), while other studies demonstrated an association between body mass index (BMI) and the presence of AAA or with increased AAA diameter.(Stackelberg et al., 2013, Cronin et al., 2013) Allison and colleagues used body fat percentage to measure obesity and showed that increased body fat percentage was associated with increased aortic diameter.(Allison et al., 2008) Recently, studies in obese mice have shown that the perivascular adipose tissue (PVAT) surrounding their abdominal aorta has increased macrophage accumulation, which promotes AAA formation. It has been suggested that PVAT might contribute to the association between obesity and vascular disease by promoting vascular inflammation, matrix remodeling and angiogenesis. In addition, visceral adipose tissue has been proposed to be a driver of PVAT inflammation through paracrine secretion to the adjacent vessel wall.(Police et al., 2009) In addition, PVAT has been associated with abdominal aortic diameter after adjustment for BMI, visceral adipose tissue volume and cardiovascular risk factors.(Thanassoulis et al., 2012)

Diagnosis of AAA

In Sweden, a nationwide screening program has been implemented to reduce morbidity and mortality related to AAA. Because the condition is less prevalent in women, screening has centered primarily on men. Men aged 65 years are therefore routinely invited to undergo screening for early diagnosis of the possible occurrence of AAA. AAA is detected with ultrasound where the maximum infrarenal anteroposterior diameter is measured according to the “leading edge to leading edge” (LELE) principle. Today, more than 90% of all 65-year-old men in Sweden are included in a screening program.(Wanhainen and Bjorck, 2011, Hultgren et al., 2013) Because AAA is asymptomatic, the present clinical challenge is to diagnose the aneurysm at an early stage in order to prevent sudden aortic rupture.

Small aneurysms in men (<55 mm) and in women (<50 mm) are subjected to a surveillance program with regularly scheduled ultrasounds, and surveillance is performed at certain

intervals based on the size of the aneurysm. With increased aortic diameter, the patients are under more frequent observations because the increase is associated with increased risk of rupture. Currently there is no therapeutic treatment approved for AAA, and surgical repair is the primary treatment for AAA. Patients with an AAA diameter >55 in men or >50 mm in women are generally offered elective surgery, either as open repair surgery in which the abdominal aorta is replaced with a synthetic graft or as endovascular aortic repair in which a catheter is introduced via the femoral artery followed by insertion of a stent or synthetic graft. Open surgical repair is effective in preventing aneurysmal rupture but has a higher perioperative mortality of 5.2 % compared with endovascular repair which provides an early survival advantage at the time of surgery, with a mortality rate of 1.6%. However, within 5 years the outcome of endovascular repair is similar to traditional open repair since endovascular repair is associated with higher risk of additional interventions and complications due to endoleak as well as continued risk of aneurysm rupture.(Blankensteijn et al., 2005, Schermerhorn et al., 2015, De Bruin et al., 2010) The surgical choice is based on several factors, including AAA diameter, expansion rate, patient age, and risk of open surgery.

In the coming years, increased awareness of AAA due to increased screening could dramatically increase the number of small AAA patients seeking treatment options for early-stage aneurysmal disease. However, because elective surgery is highly expensive together with the prohibitive risks of surgical and post-surgical complications, these patients are not considered as candidates for elective surgery. As the aorta expands, the risk of rupture also increases. Generally, AAAs smaller than 40 mm expand slowly and are likely to require surgical repair within 5 years, while AAAs larger than 40 mm expand faster and are expected to require surgical repair in 2 years.(Vega de Ceniga et al., 2006) A mean aneurysmal growth rate of 2.21 mm per year, independent of age and sex, has been reported in a meta-analysis as the normal rate of enlargement of the aneurysmal sac.(Sweeting et al., 2012) The aneurysmal growth rate is very individual and varies between 1 to 6 mm/year.(Brady et al., 2004)

Anatomy of the aorta

The aorta is the largest and strongest artery in the body and consists of three distinct layers, the *tunica intima*, *tunica media* and *tunica adventitia* (Figure 2). The innermost component of

the *tunica intima* is a monolayer of endothelial cells resting on a basal lamina composed of type IV collagen and laminin. The more complex layer, the *tunica media*, mainly consists of vascular smooth muscle cells (VSMCs) embedded in an extracellular matrix (ECM) composed of elastic fibers, multiple types of collagen and proteoglycans. Surrounding the *tunica media* is the *tunica adventitia*, which mostly consists of connective tissue including fibroblasts, collagens and elastin.(Fuster Valentin 2004)

Figure 2. Structure of an artery. Permission obtained from Oxford University Press. Modified from (Raffort et al., 2016).

Components of the aortic wall

The major constituents of the vessel wall consist of the ECM, which is composed mainly of elastin and collagen fibers as well as other matrix components such as proteoglycans (hyaluronan) and glycoproteins (fibronectin), all of which are crucial for vessel wall function and integrity. Components of the ECM do not just provide the structural integrity and mechanical properties required for vessel function, and these proteins also modulate cell function by interacting with matrix receptors on cells. This interaction is important in directing the development that occurs, for example, in response to injury. VSMCs are the dominant cell type in the vessel wall and are essential for the proper performance of the aortic wall. VSMCs maintain blood pressure through relaxation and contraction, and they play a major role in synthesizing the components of the ECM.

Elastin

Elastin provides the elasticity and compliance of the aorta and also plays a critical role in supporting and maintaining vascular cells. Elastin constitutes 40% of the total dry weight of the aorta and is therefore the most abundant protein in the aorta wall. Elastin is encoded by only one gene, the *ELN* gene and it is synthesized by cells in the vessel wall through cross-linkage of its soluble precursor tropoelastin which is initiated by lysyl-oxidase (LOX). Tropoelastin is then introduced into microfibrils in the ECM to form insoluble mature elastin with a very stable and persistent structure that provides elastic recoil in the aorta.(Rosenbloom et al., 1993)

In early stages of postnatal development, elastin is a major synthetic product. However, synthesis and accumulation of elastin generally peaks early during postnatal growth of arteries, decreases rapidly after further development, and essentially ends in adult tissue. This almost non-existent level of synthesis of new elastin in adult aortas explains why elastin does not contribute to repair processes in the vessel.(Bendeck and Langille, 1991)

Collagen

Several types of collagens have been identified in the aortic wall, but collagen type I and III are the two most abundant collagen fibers representing 80–90% of the total collagen. In the media and adventitia, the structural collagen network provides the tensile and mechanical strength of the vascular wall.(Burgeson and Nimni, 1992) collagens are synthesized principally by VSMCs and fibroblasts, and the biosynthesis of the unique molecular structure of collagen involves several steps. The collagen molecule is formed by three polypeptides twisted together to form a triple helix. Covalent cross-linking between the collagen helices and aggregation of several subunits forms fibrils. Multiple fibrils are then packed together to form a collagen fiber, which is the main component of the ECM.(Rizzo et al., 1989, Carmo et al., 2002) Collagens also play an important role in stabilizing the VSMC phenotype and might facilitate the maintenance of VSMCs in a contractile phenotype.(Glukhova, 1995)

Vascular smooth muscle cells

VSMCs are a major component of the tunica media, and they provide the main support for the structural integrity of the vascular wall and regulate the vascular tone in order to maintain

intravascular pressure. VSMCs also have an important role in synthesizing and repairing the structural elements in the ECM such as collagen and elastin. Under normal and healthy conditions, contractile VSMCs are the predominant SMC phenotype and are essential in regulating vessel diameter and blood flow.(Zalewski et al., 2002) In this condition, contractile VSMCs proliferate at an extremely slow rate and only produce small amounts of ECM proteins. However, under pathological conditions, or in response to injury, contractile VSMCs undergo phenotypic modulation and differentiate to the synthetic phenotype and migrate into the intima. These cells exhibit a rapid increase in proliferation, migration, and production of ECM components such as collagen and fibronectin, among others, which play a critical role in vascular repair and maintaining the vessel wall integrity.(Owens et al., 2004, Beamish et al., 2010)

Pathogenesis of AAA

The pathologic characteristics of AAA are highly heterogeneous (Figure 3). AAA is a chronic inflammatory disease associated with phenotypic modulation and increased apoptosis of VSMCs and with degradation of collagen and elastin in the ECM.(Ailawadi et al., 2009, Kazi et al., 2003, Lopez-Candales et al., 1997, Michel et al., 2011, Freestone et al., 1995) It has been suggested that rapid loss of elastin fibers might be the initiating event in AAA and that degradation of elastin leads to AAA expansion. Loss of collagen might in turn be the responsible factor for AAA rupture.(Dobrin and Mrkvicka, 1994) During AAA formation, collagen turnover is important for vessel wall repair and regeneration. It has been suggested that increased collagen synthesis might occur as a response to increased wall tension as a consequence of elastin loss and aneurysmal dilatation. Studies have reported that collagen synthesis increases during the early stages of aneurysm formation, suggesting that repair processes are occurring but in later stages of the disease collagen degradation exceeds its synthesis.(Knox et al., 1997, Baxter et al., 1994) The increased collagen synthesis is a consequence of increased elastin degradation together with an expanding aorta.(Menashi et al., 1987) It is believed that the rupture of AAA is associated with increased degradation of collagen fibers and that impaired collagen networks might reduce the mechanical strength of the aortic wall. This emphasizes the important role of compensatory collagen synthesis in maintaining the strength of the aortic wall and its structural integrity during AAA progression. (Tanios et al., 2015) Elastin degradation products in the aortic wall might serve

as the primary chemotactic attractant for infiltrating immune cells. The major components of the cellular infiltrates present in AAA are T lymphocytes, macrophages, mast cells and neutrophils, all producing a spectrum of proinflammatory cytokines, chemokines and ECM proteases such as matrix metalloproteinases (MMPs) and neutrophil elastase, both of which are involved in the progression of AAA.(Koch et al., 1990, Pearce and Koch, 1996, Shimizu et al., 2006, Cohen et al., 1991) The pathophysiological background of AAA is poorly understood, and whether the inflammation in the aortic wall represents a primary event or a response to tissue destruction remains unclear.

Figure 3. A simplified overview of the pathogenesis of AAA.

Chronic inflammation and immune response in AAA

The immune system consists of complex processes and a variety of immune cells and molecules that are important for maintaining homeostasis in the body. It is organized into innate immunity, which forms the first line of defense against pathogens, and adaptive immunity, which develops later and can acquire an immunological memory.(Abbas A.K, 2014) Degradation products from ECM fragmentation such as elastin and collagen in the aortic wall might serve as primary chemotactic attractants for infiltrating immune cells, causing an innate immune response that attempts to resolve the damage. In AAA, it is believed that the inflammation is poorly regulated resulting in progressive tissue damage and aneurysmal progression.(Dale et al., 2015) The inflammatory response is transmural in distribution, and the primary site for infiltrating cells appears to be located in the media and adventitia.(Hellenthal et al., 2009) T lymphocytes and macrophages are the most prominent cell types in the aneurysmal wall, but mast cells and neutrophils are also present. The majority of the infiltrating lymphocytes are cluster of differentiation 4-positive ($CD4^+$) T lymphocytes; however, there is an ongoing controversy in the literature as to the contribution of T-helper type 1 (T_H1) or T-helper type 2 (T_H2) cells in AAA.(Ocana et al., 2003) Schönbeck *et al.* specified the subpopulation of T lymphocytes as an anti-inflammatory T_H2 -predominant immune response with T_H2 -associated cytokines, including interleukin (IL)-4, -5 and -10, while little or no expression of the pro-inflammatory T_H1 -associated cytokines IL-2, -12, -15 and interferon (IFN)- γ was observed.(Schonbeck et al., 2002) However, Galle *et al.* demonstrated that human aneurysmal tissue expressed high levels of INF- γ but not IL-4, a typical T_H2 marker. They suggested the presence of a large number of T_H1 lymphocytes with minimal T_H2 involvement in the late stages of human AAA.(Galle et al., 2005) Xiong et al. investigated the role of $CD4^+$ T lymphocytes and INF- γ in experimentally induced AAA in mice. Deficiency of either CD4 or INF- γ prevented $CaCl_2$ -induced aneurysm, but the aneurysm could be reconstituted in $CD4^{-/-}$ mice with INF- γ injections, suggesting an essential role of T_H1 lymphocytes in AAA formation.(Xiong et al., 2004) In addition, T_H2 produced IL-10 that promotes apoptosis of T_H1 cells (Ayala et al., 2001) and activation of anti-inflammatory M2 macrophages. Production of IFN- γ by T_H1 cells can in turn activate macrophages and stimulate production of pro-inflammatory cytokines, such as those produced by the M1 macrophages, e.g. IL-12 and IL-23, and these are involved in tissue injury and promote further activation of T_H1 lymphocytes. Macrophages play a critical role in both the innate and the adaptive immunity, and consists of two major phenotypes, the M1 and

M2 macrophages. Initial arterial injury recruits the pro-inflammatory M1 macrophages to sustain the ongoing inflammation. Normally, these macrophages would later convert to anti-inflammatory M2 macrophages and promote tissue repair and wound healing through the production of anti-inflammatory IL-10 and transforming growth factor- β 1 (TGF)- β 1.(Murray and Wynn, 2011) If M1 macrophages continue to dominate, chronic inflammation occurs.

Among inflammatory cells types, neutrophils and mast cells have also been identified in the wall of AAA, although they are not as prominent as T lymphocytes and macrophages. Neutrophils are quickly recruited to the site of injury, and in AAA neutrophils are most commonly found in the intraluminal thrombus.(Folkesson et al., 2007) In the aortic wall of AAA, neutrophils have been highlighted as important mediators in AAA development and neutropenia limited AAA development after elastase perfusion in mice.(Eliason et al., 2005) Further, mast cells have been found to be present within atherosclerotic and aneurysmal aortas.(Metzler and Xu, 1997, Mayranpaa et al., 2009) Upon activation, mast cells produce a spectrum of proinflammatory cytokines and chemokines such as IL-1, -3, -4, -5 and -6, tumor necrosis factor (TNF)- α , IFN- γ and granulocyte-colony stimulating factor as well as serine proteases, chymase, tryptase and cathepsin G, which induces MMP activation and might thereby actively participate in disease progression.(Lindstedt et al., 2007) Further, the role of mast cells in experimentally induced AAA was demonstrated with two different aneurysm models in rodents. Sun et al. showed that mice that were deficient in mast cells were protected from AAA formation for 56 days following elastase perfusion (Sun et al., 2007), and Tsuruda et al. demonstrated that mast cell deficiency effectively suppressed AAA in rats for 14 days after periaortic application of CaCl_2 .(Tsuruda et al., 2008) Mast cell secretion of various inflammatory mediators is capable of activating T lymphocytes (Nakae et al., 2005) and macrophages (Wei et al., 1986).

Proteases in AAA

It is clear that the prominent inflammatory response identified in AAA has a role in promoting aneurysmal expansion. This inflammatory response is thought to account for the increased expression of proteolytic enzymes that are released in response to the increased levels of cytokines that are produced by infiltrating immune cells. AAA exhibits increased local production of enzymes capable of degrading collagen and elastin. MMPs, serine

proteases, and cysteine proteases are all localized in the aneurysmal aorta at higher concentrations than are seen in the normal aorta. Given the importance of elastin and collagen fibers in aortic wall structure and the unique loss of medial elastin and collagen that occurs in AAA, proteases with elastolytic and collagenolytic activity are of high interest.

MMPs are proteins with a zinc-binding motif in their catalytic domain, and they have been suggested to play a critical role in inflammation as well as in degradation of components such as elastin and collagen in the aortic wall. Degradation of collagens depends on the action of the collagenases MMP-1, MMP-8 and MMP-13 through destabilization of the triple helix of the native fibrillary collagen. (Abdul-Hussien et al., 2007) Destabilized collagen can further be degraded by proteases such as the gelatinases MMP-2 and MMP-9, and cathepsin K, L and S. MMP-2 and MMP-9 are the two most studied MMPs implicated as having a pivotal role in AAA development and together with MMP-12 degrade elastic fibers. (Sakalihasan et al., 1996) Their inhibitors – tissue inhibitors of metalloproteinases (TIMPs) – are suppressed in the aneurysmal wall. (Freestone et al., 1995)

The serine proteases chymase and tryptase are produced by mast cells during degranulation and are abundant during aneurysmal formation, and they are involved in degradation of the ECM by activating MMP-1, MMP-2 and/or MMP-9. (Tchougounova et al., 2005) Chymase might also induce apoptosis in VSMCs. (Leskinen et al., 2001, Johnson et al., 1998) Neutrophil elastase is also a serine protease that is produced by neutrophils and stored in their azurophilic granules. This enzyme is released during inflammation and has the capacity to degrade components of the ECM, especially elastin, but also collagen III, fibronectin and proteoglycans. (Cohen et al., 1991) The major inhibitor of neutrophil elastase is the serine protease inhibitor α 1-antitrypsin, which is essential for regulating the activation of this protease. Neutrophil elastase can contribute to a pro-inflammatory state by cleaving pro-IL1 β into its active form thereby stimulating the production of MMPs. (Owen and Campbell, 1999)

The cysteine proteases cathepsin K, L and S also play a role in AAA formation (Sukhova et al., 1998), while their inhibitor cystatin C has been shown to be decreased in AAA. (Shi et al., 1999) All three cathepsins have been found to be expressed in human AAA, and cathepsin K is the most potent elastolytic enzyme known. The inflammatory factors TNF- α and IFN- γ can induce the secretion of these cathepsins from immune cells as well as from vascular cells. (Abisi et al., 2007, Lohoefer et al., 2012)

Toll-like receptors

The toll-like receptor (TLR) family was first discovered in *Drosophila*, and in the late 1990s TLRs were identified in humans. Today the TLR family comprises ten members (TLR1–TLR10), all of which are a major focus of research within the field of immunology. TLRs are classified as type I transmembrane glycoproteins containing leucine-rich repeats in their extracellular recognition domain and a cytoplasmic domain named toll-interleukin-1 receptor (TIR) that is essential for downstream signaling.(Bell et al., 2003, Xu et al., 2000) The TLRs are a family of pattern-recognition receptors, and each TLR recognizes specific pathogen-associated molecular patterns (PAMPs), which are exogenous ligands used for recognizing microbial structures, or damage-associated molecular patterns (DAMPs), which are endogenous ligands released upon tissue damage and tissue remodeling. Detection of these ligands by TLRs allows the host to initiate the innate immunity and to develop the adaptive immunity. TLR1, -2, -4, -5, -6, and -10 are positioned at the cellular surface and are primarily involved in recognizing lipoproteins and polysaccharides from bacteria. In contrast, TLR3, -7, -8, and -9 are localized on the intracellular endosomes and recognize nucleic acids from viruses.(Akira and Hemmi, 2003) TLR3 is essential for recognition of the double-stranded (ds) RNA (Alexopoulou et al., 2001) that is produced by many viruses during replication and for recognition of the mRNA released from necrotic cells.(Kariko et al., 2004) TLR4 is essential for the detection of lipopolysaccharide, which is the major component of the outer membrane of gram-negative bacteria. TLR4 further recognizes a broad spectrum of endogenous ligands including heat shock proteins (HSPs) (Johnson et al., 2002), high-mobility group box 1 (HMGB1) (Park et al., 2004) and fragments from fibronectin (Okamura et al., 2001) and hyaluronan (Noble et al., 1996).

Toll-like receptor signaling

TLR recognition of exogenous or endogenous ligands leads to activation of the TLR signaling pathways starting from the cytoplasmic TIR domain. The TLR signaling cascades largely depends on the adaptor molecule that associate with it, downstream of the TIR domain, including myeloid differentiation factor-88 (MyD88), TIR domain-containing adaptor protein including IFN- β (TRIF), TRIF-related adaptor molecule (TRAM), TIR domain-containing adaptor protein (TIRAP) and MyD88 adaptor-like protein (Mal) (Figure

4). The most commonly used signaling pathways involve the MyD88-dependent pathway, which is utilized by all TLRs except TLR3. A complex series of events results in the activation of nuclear factor (NF)- κ B, activating protein-1, mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K), all of which are essential in controlling the expression of genes involved in the inflammatory response through induction of proinflammatory immune mediators such as TNF- α , IL-1 β , IL-6, IL-12 and macrophage inflammatory protein 1a (MIP1a).(Arancibia et al., 2007, Frantz et al., 2007) In association with MyD88, the adaptor proteins TIRAP and Mal are required in the signaling pathways that are initiated by TLR2 and TLR4.(Yamamoto et al., 2002) In contrast, the TRIF-dependent pathway requires TRIF as an adaptor, and this is essential for the TLR3 and TLR4-mediated signaling pathways. Like the adaptors TIRAP/Mal, TRAM is important in the TLR4-mediated response by acting as a bridge to couple TRIF with TLR4.(Yamamoto et al., 2003b) Activation of this pathway initiates signaling through interferon responsive elements, and this leads to the activation of the transcription factors, interferon regulatory factor 3 (IRF3) and NF- κ B. This favors the expression of immune mediators such as IFN- α , IFN- β and IL-12 as well as the activation of the chemokines chemokine C-C motif ligand (CCL) 2, CCL5 and chemokine C-X-C motif ligand (CXCL) 10.(Akira and Takeda, 2004, Yamamoto et al., 2003a)

Figure 4. Simplified view of the TLR signaling pathways. TLR3, 7, 8 and 9 are expressed in endosomes (not shown) while TLR2, 4, 5, 6 and 10 are expressed on the cell surface.

TLRs in animal models of aneurysm disease

Several studies have shown that TLRs are potential mediators of immunological processes leading to the formation and progression of atherosclerosis.(Michelsen et al., 2004, Lundberg et al., 2013, Zimmer et al., 2011) In AAA, alteration of the ECM due to tissue destruction can stimulate the innate immune response through activation of TLRs, and thus the detection of endogenous ligands by TLRs could be an important link between AAA and activation of the immune response. More recently, several studies have implicated the importance of TLRs in aneurysmal disease, but these have mainly focused on MyD88-dependent signaling. Owens and coworkers demonstrated that whole-body deficiency of either MyD88 or TLR4 attenuated angiotensin (Ang) II-induced AAA and atherosclerosis in mice. They also demonstrated that depletion of MyD88 in hematopoietic cells had similar effects as whole-body deficiency, but this was not the case for TLR4.(Owens et al., 2011) This was further confirmed by Lai *et al.* who demonstrated that TLR4 exerts its actions in AAA through non-hematopoietic cells and that TLR4, which is derived mainly from VSMCs, promotes the release of immune mediators and thereby contributes to AAA formation.(Lai et al., 2016) Moreover, Yan and colleagues demonstrated that blockage of TLR2 using neutralizing monoclonal antibodies diminishes AngII-induced inflammation and promotes the reconstruction of the aneurysmal wall.(Yan et al., 2015)

Platelet-derived growth factor

Platelet-derived growth factor (PDGF) is a potent mitogen for cells of mesenchymal origin such as SMCs, fibroblasts and monocytes, and it is one of numerous growth factors that are involved in cell growth and division. In both mice and humans, the PDGF family consists of four monomeric variants that dimerize to form five different isoforms: PDGF-AA, -AB, -BB, -CC and -DD. Their receptors, PDGFR- α and - β are tyrosine kinase receptors that exist in three isoforms composed of the homodimers PDGFR- $\alpha\alpha$ and - $\beta\beta$, or the heterodimer - $\alpha\beta$. PDGF-AA binds only to the $\alpha\alpha$ receptor, PDGFR-BB binds to all three receptor isoforms, PDGFR-AB and -CC bind both the $\alpha\alpha$ and $\alpha\beta$ receptors and PDGFR-DD binds to the $\alpha\beta$ and $\beta\beta$ receptors.(Fredriksson et al., 2004). Upon ligand binding, PDGFR undergoes dimerization that leads to receptor auto-phosphorylation that in turn leads to increased receptor tyrosine kinase activity and binding affinity for signaling molecules. This further activates the

downstream signaling pathways including extracellular regulated kinase (Erk), MAPK, and PI3K. The signal is transduced in the cell to promote cell survival, migration and differentiation.(Heldin et al., 1998, Heldin and Westermark, 1999)

The α -granules of platelets are a major storage site for PDGF, but PDGF can also be synthesized by a number of different cell types including SMCs, fibroblasts, endothelial cells and macrophages. PDGF synthesis is often increased in response to external stimuli such as cytokines, chemokines and thrombin. The physiological roles of PDGF and PDGFR have been investigated using gene knockout mouse models. Whole body deletion of any of the receptors is embryonic lethal with severe phenotypic defects.(Kaminski et al., 2001, Soriano, 1997) In adult mice, both *in vivo* and *in vitro* studies have demonstrated that inhibition of either PDGF-A or PDGF-B reduces the proliferation and migration rate of VSMCs leading to reduced neointimal formation.(Kotani et al., 2003, Deguchi et al., 1999, Ross et al., 1990) PDGF-C and PDGF-D have been implicated in cardiovascular disease by stimulating monocyte migration and invasion and by affecting MMP production.(Wagsater et al., 2009) Imatinib is a potent inhibitor of both PDGFR- α and - β .(Buchdunger et al., 2000)

Adiponectin – an adipokine

Adiponectin is an adipokine that is mainly secreted from adipocytes and is abundantly present in the circulating blood.(Scherer et al., 1995) Adiponectin is synthesized as a 244 amino-acid polypeptide of approximately 30-kDa that assembles and circulates in plasma in three different isoforms: a high molecular weight (HMW) multimer, a middle molecular weight hexamer and a low molecular weight trimer.(Ouchi et al., 2003) Adiponectin has previously been shown to have important metabolic and cardiovascular effects. Experimental studies have demonstrated that adiponectin suppresses the development of atherosclerosis, macrophage lipid accumulation and foam cell formation, endothelial cell apoptosis and stimulates angiogenesis in response to ischemia.(Okamoto et al., 2002, Tian et al., 2012, Kobayashi et al., 2004, Shibata et al., 2004) Further, low levels of plasma adiponectin in patients have been associated with obesity, myocardial infarction, type-2 diabetes and hypertension.(Han et al., 2009, Arita et al., 1999, Lihn et al., 2004, Pischon et al., 2004, Persson et al., 2010, Iwashima et al., 2004) Adiponectin is the only adipokine whose levels

decrease with increased body fat mass. It also has been demonstrated that males have lower circulating levels of adiponectin compared to females.(Ryo et al., 2004)

The HMW adiponectin has been suggested to be the most bioactive of the three isoforms.(Pajvani et al., 2004) Adiponectin exerts its effects through two transmembrane receptors, adiponectin receptor (AdipoR) 1 and AdipoR2 (Yamauchi et al., 2007), but also via the cell-surface glycoprotein T-cadherin.(Denzel et al., 2010) Interaction of adiponectin with AdipoR1 and AdipoR2 leads to the activation of adenosine monophosphate-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor (PPAR)- α primarily in skeletal muscle and liver, but it has also been found in various tissues, including macrophages (Chinetti et al., 2004). Adiponectin can thereby reduce lipid levels through increased fatty acid oxidation and can reduce glucose levels through glucose uptake in muscle and inhibition of gluconeogenesis in liver.(Yamauchi et al., 2002) Furthermore, activation of the two adiponectin receptors reduces inflammation through suppression of NF- κ B and thereby reduces the production and activity of TNF- α and IL-6.(Wulster-Radcliffe et al., 2004) Adiponectin also stimulates the production of anti-inflammatory cytokine IL-10 by macrophages.(Kumada et al., 2004) Through induction of ceramidase activity, adiponectin might also decrease caspase-8-mediated apoptosis (Figure 5).(Holland et al., 2011) These data suggest an important protective anti-inflammatory role for adiponectin.

Figure 5. Pleiotropic effects of adiponectin.

Involvement of perivascular adipose tissue in vascular inflammation

The role of adipose tissue is more than just a storage depot for triglycerides. The adipose tissue is considered to be an endocrine and paracrine organ that contributes to the maintenance of energy homeostasis and can mediate biological effects in energy metabolism, insulin sensitivity and immune responses by secreting adipokines. The two most abundant depots for adipose tissue are visceral and subcutis. In addition, adipocyte depots can also be found throughout the body in association with multiple organs including, lungs, heart, and kidneys and in the adventitia of large blood vessels. Virtually all arteries are surrounded by PVAT, which varies in amount and content of white and brown fat in different anatomical locations and are highly vascularized.(Villacorta and Chang, 2015, Gu and Xu, 2013) PVAT was long assumed to provide the mechanical strength of the vessels from neighboring tissue, but in addition to its structural role PVAT also play many roles in vascular function. Through both endocrine and paracrine functions, PVAT regulates vascular tone in both humans and rodents (Figure 6).(Chang et al., 2013) Under normal physiological conditions, the balance between the pro- and anti-contractile activities of PVAT is essential for maintaining vascular homeostasis and normal blood pressure. PVAT exerts its anti-contractile effect via direct action on VSMCs through the activation of potassium channels (Lohn et al., 2002) and subsequent vasorelaxation. Adiponectin is another essential anti-contractile factor released from PVAT.(Fesus et al., 2007) Inflamed PVAT contributes to vascular dysfunction through multiple mechanisms. With increased adipose mass, a local hypoxic environment develops that triggers the infiltration of immune cells into the adipose tissue. In this state, inflamed PVAT results in impaired secretion of the protective anti-contractile factors such as adiponectin and the paracrine effects of PVAT are shifted to vasoconstriction.(Greenstein et al., 2009) In addition, PVAT starts to secrete a large number of chemokines such as IL-18, CCL2 and CCL5 which induces the recruitment of macrophages, neutrophils and lymphocytes together with increased secretion of cytokines and adipokines (IL-6, TNF- α , leptin and resistin) that might cause endothelial dysfunction(Ketonen et al., 2010), induce VSMC proliferation and migration and promote neointimal formation (Manka et al., 2014).

Figure 6. Content and expression in normal and increased adipose tissue.

Sphingolipid metabolites – ceramide and sphingo-1-phosphate

Sphingolipids, a family of membrane lipids, are bioactive molecules that play a significant role in cellular processes such as cell division, differentiation and death. Sphingolipid metabolism is a complex network that produces biologically active molecules including ceramide, sphingosine, sphingosine-1-phosphate (S1P), ceramide-1-phosphate and others. The sphingolipid precursor sphingomyelin and its metabolite ceramide have been shown to be independent risk factors for coronary artery disease and to be involved in human atherosclerosis.(Jiang et al., 2000, Schissel et al., 1996) Low density lipoprotein (LDL) is enriched with ceramide to a higher degree in atherosclerotic lesions than plasma LDL, and ceramide-enriched LDL is only found in aggregated forms of lesion LDL, which can be induced with increased sphingomyelinase activity.(Schissel et al., 1996)

Sphingolipid metabolites, particularly ceramide and S1P, are important molecules in the regulation of pro-inflammatory pathways and cell migration and proliferation, and they play key roles in apoptosis, inflammation and angiogenesis.(Clarke et al., 2007, Hait et al., 2006, Peters and Alewijnse, 2007, Hannun and Obeid, 2008) Ceramide mediates many cell stress responses including apoptosis, differentiation and cell senescence (Geilen et al., 1997) whereas S1P has a critical role in cell survival, growth, proliferation and migration,

inflammation and in protection from apoptosis.(Cuvillier et al., 1996, Hait et al., 2006, Peters and Alewijnse, 2007) Ceramide and S1P formation can be induced by several factors including cytokines TNF- α (Dbaibo et al., 1993), IL-1 and hypoxia (Hannun and Obeid, 2008). Sphingolipids have a rapid turnover, and sphingolipid homeostasis is controlled by the balance between synthesis and degradation. The ratio between ceramide and S1P determines cell fate.(Takabe et al., 2008)

Medical treatment for AAA

Medical treatments for patients with AAA seek to decrease the expansion rate and thereby the risk of rupture. Development of novel treatment strategies and drug candidates is essential to decrease the risk of cardiovascular events including AAA. The reduction or stabilization of AAA growth would provide advantages for patients with small AAA and patients with prohibitive surgical risks, and the AAA screening program would be improved if there were a drug treatment that could slow or arrest aneurysmal growth and expansion.

Anti-inflammatory strategies such as use of statins, angiotensin-converting enzyme (ACE) inhibitor and doxycycline in quenching aneurysmal progression has long been proposed. Pre-clinical studies have shown that statins and ACE inhibitors suppress aneurysmal formation through pleotropic activity, including anti-inflammatory activity and anti-proteolytic activity.(Steinmetz et al., 2005, Xiong et al., 2014) Clinical evaluation showed the interference of statins, ACE inhibitors and doxycycline with vascular inflammation and protease activity.(van der Meij et al., 2013, Kortekaas et al., 2014, Lindeman et al., 2009) A number of small retrospective reports demonstrated that statins decrease aneurysmal expansion rate (Mosorin et al., 2008, Schouten et al., 2006), yet analysis in much larger cohort failed to confirm this.(Ferguson et al., 2010) The effects of ACE inhibitors on aneurysmal progression are inconsistent, whereas a population-based case-control study showed that patients with AAA were less likely to rupture.(Hackam et al., 2006) Contradictory, one study indicated an association between ACE inhibition and increased aneurysmal growth.(Sweeting et al., 2010) Doxycycline has been proven as beneficial in elastase induced AAA in rat by suppressing the MMP-9 activity and thereby quenching AAA formation.(Petrinec et al., 1996) Although promising effects in clinical trials, antibiotics do

not produce any lasting alteration on the expansion rate of small AAA.(Vammen et al., 2001, Mosorin et al., 2001)

There is a negative association between diabetes mellitus and AAA prevalence and progression.(Lederle et al., 1997) However in patients with established AAA, diabetes is associated with reduced growth rate and rupture risk compared to non-diabetic AAA patients.(Sweeting et al., 2012) Pre-clinical studies reported pleiotropic effects of metformin and PPAR- γ agonist (thiazolidinedione) with anti-inflammatory properties in aneurysmal disease.(Vasamsetti et al., 2015, Jones et al., 2009) To date, no clinical studies have been performed.

Even though some potential effects have been documented and major discoveries involving the role of the immune system have been acknowledged, none of the drugs available to date have proven optimal for AAA treatment. Tobacco smoking is a specific risk factor for AAA and smoking cessation is the most important strategy to slow the aneurysmal progression.

AIMS

The overall aim of this thesis was to target signaling pathways that affect vascular remodeling in AAA to potentially identify novel strategies and drug candidates for future treatment of aneurysmal diseases.

More specific aims:

- The aim of Paper I was to investigate the potential role of TLR signaling, under the control of TRIF, and its effects on the inflammatory response and AAA development.
- The aim of Paper II was to characterize the potential protective role of imatinib in AAA development and the molecular mechanisms involved.
- The aim of Paper III was to investigate the role of adiponectin and its potential benefit in suppressing aortic and perivascular adipose inflammation and ECM degradation in the aortic wall to prevent AAA development.
- The aim of Paper IV was to investigate the cellular and cytokine/protease composition of PVAT in AAA.

METHODOLOGICAL CONSIDERATIONS

Angiotensin II induced abdominal aortic aneurysm model in mice

Animal models of disease are used to mimic the cellular and biochemical characteristics and the progression of human diseases. AngII induced AAA is one of three commonly used mouse models used to gain insights into the mechanisms of AAA pathogenesis. Chronic subcutaneous infusion of AngII through osmotic pumps to induce AAA and atherosclerosis was initially reported in low density-lipoprotein receptor (LDLR)-deficient mice that were fed a saturated fat-enriched diet (Daugherty and Cassis, 1999), and later also demonstrated in apolipoprotein (Apo) E-deficient mice fed a normal diet (Daugherty et al., 2000).

The initial event in AngII-induced AAA formation in mice is the accumulation of macrophages in the media and adventitia of the suprarenal aorta, which occurs 1 to 3 days after infusion. This accumulation is speculated to give rise to pro-inflammatory cytokines and ECM-degrading proteases such as MMPs that lead to medial elastin destruction. The breakage of elastin fibers is controlled by a rapid thickening of the adventitial layer. In some cases, an intramural thrombus is formed due to aortic dissection, and this becomes fibrous and accumulates more macrophages. A pronounced inflammatory response occurs, which stimulates adhesion molecules, chemokines, and cytokines and ultimately leads to infiltration by T and B lymphocytes. Over time, the aorta gradually expands, and the increase in aortic diameter coincides with evident remodeling of aneurysmal tissue.(Saraff et al., 2003, Manning et al., 2002)

Owens and coworkers demonstrated that AngII increases medial thickness through VSMC hyperplasia in the ascending aorta, while VSMC hypertrophy occurs in all other aortic regions.(Owens et al., 2010) The embryological origins of the SMCs differ along the aortic length. SMCs in the descending and abdominal aorta originate from splanchnic mesoderm, while SMCs in the ascending aorta originate from the neural crest.(Majesky, 2007) The region-specific difference in AAA formation might be due to the phenotypic diversity of embryological origin as well as the cytokines, chemokines, and growth factors that are present. The difference in embryological origin might explain the differences of SMC

responsiveness to AngII, and AngII infusion *in vivo* is known to promote changes in medial VSMCs via mechanisms that are independent of increased systolic blood pressure.(Su et al., 1998)

It is noteworthy that infusion of AngII at a rate of $1000 \text{ ng}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ into hypercholesterolemic mice has maximal effects on AAA development. The incidence of AngII-induced AAA is 80% in hypercholesterolemic mice compared to less than 30% in normocholesterolemic mice. Further, aortic rupture occurs in approximately 10–30% of both hyper- and normocholesterolemic mice during infusion with AngII.(Lu et al., 2015, Daugherty et al., 2000, Daugherty and Cassis, 1999, Manning et al., 2002) AngII is a peptide that plays an essential role in the maintenance of vascular homeostasis, and it has cellular functions under physiological conditions. AngII induces the development of AAA through mechanisms that are independent of blood pressure, and this suggest that other effects of AngII - presumably those related to inflammation - are responsible for theses diverse pathologies. (Cassis et al., 2009) However, this is debatable since another study shows that aneurysmal formation depend on hypertension.(Kanematsu et al., 2010)

The effects of AngII are primarily manifested via two receptor subtypes, angiotensin 1 receptor (AT1R) and AT2R, that belong to the G-protein coupled receptor superfamily. In mice and rats, the AT1R is split into the two subgroups of AT1a and AT1b receptors. AT1R regulates vascular constriction, aldosterone synthesis and secretion, cell growth, and cell proliferation, and it increases blood pressure. While AT1R mediates most of the physiological effects of AngII, AT2R binds AngII and exerts anti-proliferative and anti-apoptotic effects on VSMCs. Both receptors are expressed in most tissues including macrophages (Scheidegger et al., 1997), endothelial cells (Grafe et al., 1997) and VSMCs (Chen et al., 1998). Subsequently studies demonstrated that co-infusion of AngII with the AT1R antagonist losartan completely attenuated AngII-induced AAA formation (Daugherty et al., 2001) which was later demonstrated to be mediated by the receptor subtype AT1a in AT1aR-deficient *LDLR*^{-/-} mice (Cassis et al., 2007). They found that AT1aR deficiency in bone marrow-derived cells failed to influence AngII-induced AAA, inferring that AngII induced changes in resident cells play an important role in the initiation of AngII-induced AAA formation.(Cassis et al., 2007) In contrast, co-infusion with the AT2R antagonist PD123319 resulted in a pronounced increase in the severity of AngII-induced AAA in *ApoE*^{-/-} mice.(Daugherty et al., 2001) This suggests that AngII promotes vascular pathology

and subsequent AAA formation via AT1R, and that this receptor is required for both AngII-induced atherosclerosis and AAA.

Similar to the human AAA profile, the AngII model causes elastic fiber degradation, proteolytic destruction of medial connective tissue, transmural inflammation and aortic expansion. An interesting difference between human and AngII-induced mouse AAA is the location of the aneurysm. In humans, AAA occurs in the infrarenal region of the aorta while AngII-induced AAA in mice is localized in the suprarenal aorta. One explanation could be the heterogeneity of the embryological origin of VSMCs in the different regions of the aorta could explain the difference in VSMC responsiveness to AngII. Another explanation for the difference in AAA location is hemodynamics caused by altered mechanical properties of the aorta as a result of regional differences in the ratio of collagen to elastin. Compared to the ascending aorta, the descending and abdominal aorta has significantly lower amounts of elastin and collagen and a lower collagen to elastin ratio (2:1). It is likely that having fewer lamellar units makes the abdominal aorta more prone to aneurysmal degeneration.(Manning et al., 2002, Halloran et al., 1995) Another similar characteristic of these mice to human AAA is that male mice are more susceptible to AAA formation than female mice.(Manning et al., 2002)

In paper I, II and IV, osmotic pumps were implanted subcutaneously into the right flank of mice releasing AngII ($1000 \text{ ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) to induce AAA or saline which served as controls, over a course of 28 days. In paper I, we infused *ApoE*^{-/-}*Trif*^{-/-} mice (n = 11) and *ApoE*^{-/-} mice (n = 12) with AngII and a group of *ApoE*^{-/-} mice with saline (n = 6). In paper II, we used *ApoE*^{-/-} mice to study the impact of imatinib on AAA development. Two groups received oral gavage treatment with 10 mg/kg imatinib mesylate (STI571, Novartis, Stockholm, Sweden), given daily throughout the study starting two days prior implantation of osmotic pumps. Two groups received oral gavage treatment with tap water and served as control mice. Groups were divided into following; NaCl and tap water (n=7), AngII and tap water (n = 9), NaCl and imatinib (n = 9) and AngII and imatinib (n = 8). In paper III, osmotic pumps were implanted subcutaneously into the right flank of *LDLR*^{-/-} mice to release AngII ($1500 \text{ ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) or saline over a course of 56 days. After 4 weeks of AngII-infusion, a new replacement 4-week osmotic pump was implanted to continue AngII infusion. A recombinant adenoviral vector encoding full-length mouse adiponectin (2×10^8 pfu) was injected intravenously into AngII-infused mice (n = 8). A recombinant adenovirus expression

green fluorescence protein (GFP, 2×10^8 pfu) was used as a control vector in AngII-infused mice ($n = 12$). A group of $LDLR^{-/-}$ mice were infused with saline ($n = 8$) and served as controls. Mice were fed high-fat diet throughout the study. In paper IV, aortas from $ApoE^{-/-}$ mice infused with AngII ($n = 10$) or saline ($n = 10$) were used to demonstrate PVAT. In all studies, mice used were male and osmotic pumps were implanted at 8-10 weeks of age. In paper I, II and IV, mice were fed with normal chow diet and in paper III mice received high-fat western diet. Microscopic and histological view of control aortas and characteristics of AngII-infused aneurysmal aorta are demonstrated in figure 7.

Figure 7. Macroscopic view of mice aortas after saline infusion in a normal aorta and after AngII infusion demonstrating a small and large AAA indicated with arrows (A). Histological characteristics of aneurysmal aortas after AngII infusion for 28 days in $ApoE^{-/-}$ mice as compared with a saline infused normal aorta (B). Small aneurysmal aorta with considerable thickening of the vessel wall (C). Aneurysmal dilated aorta considerable thickening of the vessel wall (D). Aneurysmal aorta with normal luminal diameter, intact elastic structure and a thrombus present in the adventitia surrounded by a thick region of ECM (E).

Determination of mouse abdominal aortic diameter

No clear definition of AAA in mouse models has been established, but several studies define AAA as an increase in abdominal aortic diameter by 50% or more.(Cao et al., 2010,

Daugherty et al., 2011) In compliance with these studies, we set the definition of an aneurysm as a 50% or greater enlargement of the aortic diameter. In the studies included in this thesis, we presented three methods for measuring the size of the abdominal aorta including the outer adventitial diameter, the vessel wall thickness and the LELE. All aortic diameter measurements were performed histologically. After 28 days of AngII or saline infusion, mice were sacrificed and the aorta was cut out, either at the macroscopically thickest aortic area or approximately 4–5 mm above the right renal artery. After dehydration, paraffin embedment and sectioning, the aortas were stained with Masson's trichrome stain and the aortic circumference was measured for the outer adventitial area and for the inner luminal area. In papers I and III, we used the outer adventitial diameter to represent the abdominal aortic diameter. However, both the outer adventitial diameter and the vessel wall thickness are of high relevance because both take the aneurysmal structure into account. AngII-induced aneurysmal aortas display an enlarged aortic thickness together with more disorganized medial and adventitial structures due to medial degeneration, increased adventitial remodeling, and in some cases the presence of a thrombus. The vessel wall thickness was determined from the outer adventitial diameter and the inner luminal diameter. In the clinic, the LELE method is used today as a standard technique in population-based screening in Sweden.(Wanhainen et al., 2010, Hultgren et al., 2013) With ultrasound LELE measures the aortic diameter from the beginning of the anterior wall, the adventitia, to the beginning of the posterior wall, the intima (Figure 8). In paper IV we adapted this method when measuring the mouse aortic diameter so as to resemble the method used for human screening approaches. Because the measurements were performed on histological sections, LELE was measured as the outer adventitial diameter with the vessel wall thickness subtracted. After evaluating the different methods, we believe that the LELE method is the most relevant method to use because this method takes both the outer adventitial circumference and the vessel wall thickness into account.

Figure 8. Mouse aortic size measurements based on outer adventitial diameter, inner luminal diameter, vessel wall thickness and LELE on histological aortas. $A\sigma$ =adventitial diameter, $L\sigma$ =luminal diameter, T =thickness and LELE=leading-edge to leading-edge.

Adenoviral gene transfer

Adenoviral vectors can be used to transfer genomic material to a wide variety of cell types without integrating into the host genome and being replicated during division. The viral genome remains extrachromosomal which minimizes the risk of insertional mutagenesis. In paper III, mouse adiponectin cDNA was inserted into the pAxCawT plasmid (TAKARA Biomedical, Shiga, Japan) to generate pAxCawT-mouse adiponectin. The resulting plasmid, which contains the adiponectin cDNA under the control of the CAG promoter (CMW enhancer, chicken β -actin promoter, and part of an untranslated region of rabbit β -globin), was transferred into HEK 293 cells. The virus was further expanded by ViraQuest, Inc.

(North Liberty, IA, USA). Eight weeks after injection, adiponectin levels were elevated by 10-fold. The vectors used in paper III were third-generation vectors that produce better and prolonged expression of the transgene adiponectin. In contrast, many of the early vectors only produce transient and low level transgene expression due to the ensuing immune response after viral injection.

Study population

Patients undergoing elective aneurysmal repair and demonstrating an abdominal aortic diameter of >55 mm, as determined with computed tomography scan, were included in the studies. In papers I and II, biopsies from aneurysmal patients were obtained from Karolinska University Hospital in Stockholm, Sweden. In paper IV, biopsies from aneurysmal patients were obtained from Linköping University Hospital in Linköping, Sweden. Informed consent was obtained from all study participants before surgical repair. Aneurysmal biopsies were divided into intima/media and adventitia by adventicectomy or collected as whole AAA wall (without PVAT). PVAT surrounding the aneurysmal vessel wall and subcutaneous fat, which was used as a control, was collected for paper IV. For RNA extraction and gene expression analysis, tissue biopsies were fixated in RNAlater for 24 h and thereafter stored at -80°C . For immunohistological analysis, tissue biopsies were fixed in 4% zinc formaldehyde for 24 h and thereafter stored in 70% ethanol until dehydrated. Papers I and II were approved by the local ethical committee at Karolinska Institutet in Stockholm, Sweden. Paper IV was approved by the regional ethical review board in Linköping, Sweden.

Aortas collected from organ donors (layers not separated) without clinical or macroscopic signs of aortic atherosclerosis or aneurysm served as non-aneurysmal control aortas. The sections were immediately snap frozen in liquid nitrogen for RNA extraction and gene expression analysis or fixed in 4% formaldehyde for light microscopy. In papers I and II, autopsies of control aorta samples were performed at the Department of Forensic Medicine, University of Helsinki. The use of organ donor and autopsy tissues was approved by the National Authority for Medicolegal Affairs of Finland. In paper IV, organ donors were obtained from Lund, Sweden which was approved by the regional ethical review board in Lund, Sweden.

Paper I and II

The study described in paper I and II included 12 AAA subjects demonstrating an aortic diameter > 55 mm with computed tomography (age 66 ± 5.5 years, male $n = 12$). These biopsies were used for gene expression analysis and histological examination. Control aorta biopsies were obtained from 8 organ donors for gene expression studies, and from 14 organ donors for histological examination (age 57 ± 6.2 years, male $n = 5$ and female $n = 7$).

Paper IV

In paper IV, biopsies were taken from AAA subjects who demonstrated an aortic diameter > 55 mm with computed tomography (age 70 ± 5.2 years, male $n = 16$ and female $n = 3$). We included 19 AAA subjects for histological examination, of which 12 biopsies were used for gene expression analysis of the aneurysmal aorta and PVAT was taken from 9 AAA subjects for gene expression analysis. For measuring the sphingolipid content in PVAT and peripheral fat, PVAT from 12 subjects and peripheral fat from 4 AAA patients were included. Aorta biopsies were obtained from 9 organ donors for gene expression studies and from 6 organ donors for histological examination (age 53 ± 8.5 years, male $n = 8$ and female $n = 1$).

Real-time quantitative polymerase chain reaction

Real-time quantitative polymerase chain reaction (qPCR) provides sensitive, specific, and reproducible detection and measurement of products generated during each cycle of the PCR.(Heid et al., 1996) To be able to quantify the messenger (mRNA) from aortic tissues, the mRNA was reverse-transcribed to complementary DNA (cDNA), and the synthesized cDNA served as a template for the real-time qPCR. There are three major steps in each real-time qPCR cycle – the denaturation phase that “melts” the double-stranded DNA into single strands, the annealing phase that allows hybridization of probe/primer to the template DNA strand, and the extension phase during which elongation of the single DNA strand occurs. The TaqMan probe has a fluorescent FAM dye attached to its 5′ end and a quencher minor groove binder (MGB) dye at its 3′ end. During the annealing phase, if the target sequence is present on the template cDNA, the primer and TaqMan probe will hybridize to this sequence. As long as the probe is intact, the close proximity of the 3′ quencher will inhibit the fluorescent signal from the 5′ reporter fluorochrome. A fluorescent signal is only emitted

upon cleavage of the probe. During the extension phase, the 5' to 3' exonuclease activity of the thermostable enzyme *Thermus aquaticus* (Taq) DNA polymerase cleaves the hybridized probe, releasing the reporter dye from the quencher and allowing the fluorescence signal to be detected.(Holland et al., 1991) During the qPCR cycles, the exponential increase in fluorescent signal is directly proportional to the amplified material. This makes it possible to quantify the amount of starting material by relating it to a standard curve.

In papers I–IV, we used TaqMan probe-based real-time qPCR to examine the expression level of several genes involved in AAA formation in humans and in mouse models. Normalization genes were used as controls for experimental variability. We used 3 or 4 reference genes in each project to determine the most stable expressed reference gene for normalization of our data.

Paraffin embedding and sectioning

To maintain tissue architecture, cell morphology, and antigenicity of target epitopes, complete preparation of the sample is critical. Paraffin embedding is a standard technique that most research histopathology laboratories use, and this allows tissue morphology to be maintained and the samples to be stored for long periods of time. When obtaining human or mouse aortic biopsies, the tissue was preserved immediately by fixation in 4% zinc formaldehyde for 24 hours and then kept in 70% ethanol until further dehydration procedures. Tissues were then dehydrated in increasing concentrations of ethanol before being submerged in Tissue-Clear, which is miscible with paraffin, and then embedded into melted paraffin wax and cooled down to room temperature to obtain paraffin blocks for sectioning. Aortic tissue was sectioned in 5 μ m thick sections and incubated at 56 °C for 4 hours to dry the sections on glass slides. When performing histological staining, paraffin-embedded sections were rehydrated in Tissue-Clear and several changes of ethanol before proceeding with the desired histological staining.

Immunohistochemistry

Immunohistochemistry was performed in papers I–IV to visualize the distribution and localization of specific cellular components within human and mouse aneurysmal and non-

aneurysmal aortic walls. The basic principal of immunohistochemistry is to identify discrete tissue components through the specific interaction of labeled antibodies with the antigen of interest. There are multiple labeling approaches in immunohistochemistry. Epitope unmasking was performed using the antigen retrieval solution DIVA, and background staining was minimized by quenching the endogenous peroxidase activity with 3% hydrogen peroxide. Although different labeling approaches were used, the sample preparation was equivalent in all papers.

Avidin and biotin detection

In all papers, immunohistochemistry was performed using an avidin and biotin detection system. Non-specific binding between the primary antibodies and tissue samples was blocked with normal goat or horse serum prior to incubating with primary antibodies at 4°C overnight and secondary antibodies for 1 hour. Detection of the antibody-antigen complex is based on the avidin and biotin complex followed by visualization with 3,3-diaminobenzidine tetrahydrochloride substrate. Sections were then counterstained with hematoxylin to give a contrast to the primary stain before being dehydrated and mounted for light microscopy visualization.

MACH II detection

In paper IV, immunohistochemistry was also performed using the MACH II detection system. Non-specific binding between the primary antibodies and tissue was blocked with Punisher background blocker followed by incubation with primary antibodies for 1 hour. Secondary alkaline phosphatase-conjugated antibodies were applied for 1 hour and detected with the Wrap Red chromogen. Sections were counterstained with hematoxylin and dehydrated before being mounted for light microscopy visualization.

Quantification by immunohistochemistry

Quantification of cells was defined as the number of cells per vessel wall area or measured as the total number of cells in the aortic section. In paper IV, the quantification of phosphorylated PDGFR- β was performed blinded using a scoring system with 1 defined as no or low expression, 2 defined as expression in the aortic media, 3 defined as expression in the aortic media and adventitia, and 4 defined as strong expression in all layers of the aorta.

Masson's trichrome staining

In papers I and II, Masson's trichrome staining was used to differentiate between collagen and VSMCs in the aortic vessel wall and to observe histologic changes in connective tissue disease. As the name implies, a three-color staining is used to selectively stain collagen fibers in blue, muscle and erythrocytes in red and nucleus in black/brown. Masson's trichrome staining starts with mordanting the tissue section with a picric acid such as Bouin's solution to enhance the intensity of the trichrome stain. The general principle behind trichrome staining is that the less porous tissue is colored by the small molecule dyes, and if a larger molecule penetrates the same tissue element then it will replace the small molecule. Muscle cells have an open structural network with large pores while collagen has a dense network of small pores (Figure 9). (Bancroft JD, 2008)

Quantification of collagen was performed blinded under light microscopy, and a scoring system was used with 1 defined as a small amount of collagen in all layers of the aorta, 2 as a small amount of collagen in the aortic media or adventitia, 3 as a large amount of collagen in the aortic media or adventitia, and 4 as a large amount of collagen in all layers of the aorta.

Figure 9. Masson's trichrome staining of a normal saline-infused mouse aorta (A) and aneurysmal AngII-infused mouse aortas (B and C). Collagen is stained blue while muscle and elastic fibers are red.

Verhoeff's van Gieson staining

Verhoeff's van Gieson staining is used for identifying elastic fibers in tissue sections, and it was used in papers I and II to demonstrate degradation and loss of elastic fibers upon AAA formation. Verhoeff's is a regressive method, requiring the sections to be overstained and

then differentiated. Tissue sections are overstained with a hematoxylin-ferric chloride-iodine solution. The two latter components act as mordants to fix hematoxylin dye to the tissue and also act as oxidizing agents to convert hematoxylin to hematein. Because the elastic fibers have a strong affinity for the iron-hematoxylin complex, they will retain the dye longer than other tissue elements and allow the elastic fibers to remain stained while all other tissue elements are decolorized upon differentiation using excess ferric chloride. Elastic fibers are stained black while other elements are counterstained with van Gieson to give a yellow-pink color (Figure 10).(Cook, 1974)

Quantification of elastin was performed blinded under light microscopy, and a scoring system was used with 1 defined as intact elastin, 2 as low degradation of elastin, 3 as intermediate degradation of elastin, and 4 as high degradation of elastin.

Figure 10. *Verhoeff's van Gieson staining of a normal saline-infused mouse aorta (A) and aneurysmal AngII-infused mouse aortas (B and C). Elastic fibers are stained black. Breaks in elastic fibers can be seen in C, indicated with an arrow.*

Picro-sirius red staining

Picro-sirius red staining is a histological technique used to visualize the thick and thin collagen fibers in tissue sections. Sirius red is a strong cationic dye that reacts with sulfonic acid and basic amino groups present in the collagen molecules. The richer in basic amino acids, the more strongly collagen molecules react with the acidic dye.(Junqueira et al., 1979a) Sirius red is an elongated dye molecule that promotes the enhancement of collagen birefringence due to the fact that many dye molecules are aligned in parallel with the axis of each collagen molecule. In polarized light microscopy, thick collagen fibers consequently

present an intense birefringence with a red-orange color. Thin collagen fibers display weak birefringence resulting in a yellow-green color (Figure 11).(Junqueira et al., 1979b, Montes et al., 1980)

Picro-sirius red was used in paper III to visualize collagen fibers. Quantification of sirius red was performed under polarized light using LeicaQWin image analysis software to measure the amount of red-orange color and yellow-green color relative to the aortic area.

Figure 11. *Picro-sirius red staining of a normal saline-infused mouse aorta (A) and aneurysmal AngII-infused mouse aortas (B and C). Thick collagen fibers are stained red-orange and thin fibers are stained yellow-green.*

Toluidine blue

Toluidine blue is an acidophilic dye that selectively stains acidic tissue components and has a high affinity for nucleic acids; therefore, it binds to the nuclear material in tissues with a high DNA and RNA content.(Kumar GL, 2010) Toluidine blue stains tissues based on the principle of metachromasia in which the dye absorbs light at different wavelengths depending on how it binds chemically with various tissue components and produces a color different from the original color. Toluidine blue is highly selective, and only certain tissues can be stained metachromatically. Mast cells contain metachromatic granules composed of heparin and histamine, and when it attaches to the glycosaminoglycans in mast cell granules, the dye displays a red-purple color while the background tissue elements are stained in blue.(Drupy RA, 1980)

In paper II, we used toluidine blue to detect mast cells in the aortic vessel wall. Quantification of mast cells was performed blinded and normalized to the aortic vessel wall area.

Statistical analysis

Statistical analyses were performed in IBM SPSS Statistics 23 or GraphPad Prism software. All measurements are presented as medians or means with standard deviations. Depending on normal distribution of the data, either the non-parametric Mann–Whitney *U*-test or the parametric Student's *t*-test was used to determine the statistical significance of the quantitative data between two groups, and ANOVA (Newman–Keuls post-test) was used for comparison between more than two groups. Multiple test correction was performed using the Holm–Bonferroni method. Correlation between genes was performed using Pearson's rank correlation. In all studies, *p*-values < 0.05 were considered to be statistically significant where * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$. Quantitative results are represented as mean values with error bars representing the standard deviation.

RESULTS AND DISCUSSION

TRIF adaptor signaling is important in abdominal aortic aneurysm formation (Paper I)

Due to the ability of TRIF to regulate inflammatory-mediated activation we investigated whether blockage of TRIF-mediated TLR signaling could inhibit the pathologies associated with AAA development, including ECM degeneration and inflammatory response in mice infused with AngII.

Because TRIF signaling is associated with activation of TLR3 and TLR4, we analyzed the expression pattern of these receptors in human AAA tissue. In this study, we used immunostaining to show increased expression of TLR3 and TLR4 in the aneurysmal wall compared with non-aneurysmal control aortas. In AAA tissue, expression of these receptors was associated with T lymphocytes and macrophages, and to a lesser extent with SMCs and endothelial cells. Increased synthesis of *TLR4* was also observed on the gene-expression level.

The critical event controlling the initiation of inflammation in response to high concentrations of AngII is not associated with infection, but with the production of danger signals from damaged, stressed or dying cells in the local tissue.(Kono and Rock, 2008) A number of studies have clearly indicated the importance of endogenous DAMPs in triggering the immune response through interaction with TLRs including mRNA (Kariko et al., 2004), fibronectin (Okamura et al., 2001), hyaluronan (Noble et al., 1996), HSP (Johnson et al., 2002) and HBMG-1 (Park et al., 2004). AngII-infusion in *ApoE*^{-/-} mice over the course of four weeks results in histological features such as increased aortic diameter, markedly expanded aortic mass, and a more disorganized adventitial tissue.

To study the role of TRIF in abdominal aneurysmal disease genetic blockage of the *Trif* gene was manifested in male mice with an *ApoE*^{-/-} background and AngII was infused to develop AAA. All histological features associated with AngII-infusion were attenuated in *ApoE*^{-/-} *Trif*^{-/-} mice, and these mice presented with a more organized vessel wall and only

9% of the mice developed AAA compared to 55% of the *ApoE*^{-/-} mice. Of the *ApoE*^{-/-}*Trif*^{-/-} mice, only one mouse developed AAA. This aorta displayed an aortic structure and inflammatory response equal to the aneurysmal *ApoE*^{-/-} mice. This outlier affected the statistical outcome of several genes, among these *cathepsin K*, *CCL2* and *SM22α*. When removing this outlier from the group, expression of these genes differed significantly between *ApoE*^{-/-} and *ApoE*^{-/-}*Trif*^{-/-} mice infused with AngII.

Histological findings were associated with reduced vascular inflammation in mice lacking *Trif*. In this study, both macrophages and T lymphocytes were identified with CD68-positive and CD3ε-positive immunostaining, respectively, in the aneurysmal aortas of *ApoE*^{-/-} mice. *ApoE*^{-/-}*Trif*^{-/-} mice were found to have reduced numbers of macrophages and T lymphocytes together with reduced levels of the pro-inflammatory cytokine *TNF-α*, the proteases *MMP-12* and *cathepsin K* and the chemokine *CCL2*. We also saw an upregulation of anti-inflammatory adiponectin in *ApoE*^{-/-}*Trif*^{-/-} mice, which is described in paper III as protective against AAA development. This suggests that TRIF-dependent signaling increases AAA formation by promoting recruitment of macrophages to the aorta. Infiltration of macrophages and elastin degradation are two early events occurring in AngII-induced AAA formation. We observed a variation in the degree of elastin degradation in AngII-infused mice, but we were not able to detect differences between *ApoE*^{-/-} and *ApoE*^{-/-}*Trif*^{-/-} mice. As elastin degenerates during AAA formation, collagen synthesis increases to stabilize the weakened aorta. We observed increased synthesis of *pro-collagen I* as well as *LOX* in AngII-infused *ApoE*^{-/-} mice as a response to aneurysmal formation compared with saline-infused control mice. These effects were not observed in *ApoE*^{-/-}*Trif*^{-/-} mice. These findings suggest that TRIF is essential in early stages of AAA development by regulating the progression of the innate immune response by inhibiting the infiltration of macrophages and thereby decreasing the levels of pro-inflammatory *TNF-α*, *MMP-12*, *cathepsin K* and *CCL2*, which initiates the adaptive immune response with T lymphocyte infiltration.

Lundberg and colleagues observed a reduction in atherosclerotic lesion size in *LDLR*^{-/-} mice with hematopoietic deficiency of either TRIF or TRAM. These effects were accompanied by lower aortic infiltration of macrophages and T lymphocytes together with reduced levels of pro-inflammatory mediators such as *TNF-α* and *IL-6*. Their findings further suggested that TRIF mediates its pro-atherogenic role downstream of TLR3 in hematopoietic immune cells (Lundberg et al., 2013) and TRIF deletion affects both TLR3 and TLR4 receptor signaling. In

our study, TRIF deficient mice had reduced gene expression of TLR4 with a tendency in reduction of TLR3, reflecting the reduced infiltration of immune cells into the aorta and the decreased expression of pro-inflammatory *TNF- α* , *MMP-12*, *cathepsin K* and *CCL2*. It is important to note that TLR4 can induce both MyD88- and TRIF-signaling cascades. Blockage of TRIF might be compensated for by induction of MyD88-signaling and subsequent activation of TLR4. Because the TRIF adaptor protein is the only signaling protein for TLR3, our results indicate the importance of TLR3 as a mediator of AngII-induced AAA formation and suggest that blockage of this receptor results in attenuation of AAA. However, we cannot exclude the influence of TLR4 as a contributor to our results. TLR4-initiated TRIF signaling or MyD88 signaling has recently been indicated to depend on certain TLR4 ligands, with different ligands initiating different signaling cascades.(Bowen et al., 2012) In aneurysm development, degeneration of the ECM generates the expression of several TLR ligands that can initiate both the MyD88 and TRIF signaling cascades. Nevertheless, activation of TRIF signaling is needed to mediate important signals such as those activated via IRF-3. This is in line with findings from Goossens and coworkers who demonstrated that type I IFNs that are induced by IRF-3 promote atherosclerosis by stimulating recruitment of macrophages to the lesion site by enhancing macrophage adhesion to endothelial cell.(Goossens et al., 2010)

A limitation in our study is that we have no direct evidence showing that these effects resulted from macrophage and T lymphocyte-specific TLR3 and TLR4 rather than TLRs that are expressed by resident cells in the aorta. Nevertheless, in human biopsies, the majority of TLR3 and TLR4 are expressed by macrophages and T lymphocytes and to a much lesser extent by VSMCs and endothelial cells.

In summary, the results from paper I show that mice deficient in TRIF have a reduced inflammatory response with reduced levels of macrophages and T lymphocytes and reduced levels of the pro-inflammatory cytokine *TNF- α* , chemokine *CCL2* and proteases *MMP-12* and *cathepsin K*, together with more organized aortic wall morphology. Taken together, our results suggest that TRIF is an important regulator in the immune response associated with AngII-induced AAA and that mice deficient in TRIF show reduced vascular inflammation and attenuated AAA formation.

Imatinib treatment attenuates growth and inflammation of angiotensin II-induced abdominal aortic aneurysm (Paper II)

By increasing our understanding of the pathological and molecular mechanism of AAA, we can more easily identify novel therapeutic strategies and drug candidates to treat early stages of AAA or slow its progression.

Imatinib (STI571, Glivec®, Gleevec®) is a potent selective tyrosine kinase inhibitor that is currently approved by the Food and Drug Administration for the treatment of chronic myeloid leukemia and gastrointestinal stromal tumors due to its inhibitory effects on the growth of malignant cells.(Demetri et al., 2002, Druker et al., 2001) This potent tyrosine kinase inhibitor has specific activity for Abl, PDGFR and c-kit.(Buchdunger et al., 1995, Druker et al., 1996, Buchdunger et al., 2000) Imatinib functions by occupying the ATP binding site of the tyrosine kinase such that substrate phosphorylation does not occur and the subsequent downstream signaling pathway is switched off. Consequently, imatinib causes the arrest of cell proliferation and growth and results in apoptosis of malignant cells.(Schindler et al., 2000) However, the mechanism of imatinib might be broader than originally anticipated, and it might also target non-malignant disorders such as pulmonary arterial hypertension, hyperlipidemia, hypercholesterolemia, and atherosclerosis.(Hoeper et al., 2013, Grimminger et al., 2010, Lassila et al., 2004) Given the fact that imatinib affects key features in the formation of AAA including T lymphocytes (Seggewiss et al., 2005) and mast cells (Juurikivi et al., 2005), we aimed to characterize the molecular mechanisms behind the protective role of imatinib in AAA development.

AngII-infused male *ApoE*^{-/-} mice were administered 10 mg/kg imatinib via oral lavage daily throughout the study. In this study, we started by showing that AngII-infused mice had aortic enlargement and thickening of the vessel wall, which is a typical aneurysmal aortic phenotype. These mice also had a reduced expression of SMC gene indicating to some parts loss of VSMC due to apoptosis, which is a feature of AAA progression. However, immunohistochemical staining of SM22 α -positive VSMCs revealed that the VSMC in these vessels exhibited a synthetic phenotype characterized by increased proliferation and migration of resident cells.(Rensen et al., 2007, Jiao et al., 2010) Synthetic VSMCs increase their production of ECM components such as collagen, which we also could demonstrate in

these mice. Nevertheless, AngII-infused mice treated with imatinib lacked this aneurysmal phenotype and had VSMC gene levels equal to control mice.

In the non-aneurysmal aortic vessel wall, PDGFR- β is highly expressed by VSMCs. We hypothesized that imatinib could inhibit PDGFR activation in VSMCs and thereby regulate cellular growth and migration. Indeed, we demonstrated reduced expression of phosphorylated PDGFR- β in the aortas of imatinib treated mice, and this expression was mainly associated with VSMCs. Notably, we cannot exclude that the effect of imatinib might be through inhibition of other tyrosine kinases such as c-kit and Abl. For example, Lassila and coworkers demonstrated minimal expression of the Abl kinase in the atherosclerosis vasculature.(Lassila et al., 2004) In contrast, Abl has been found to be expressed by VSMCs and to be involved in cell proliferation, migration and contraction.(Cleary et al., 2014) The tyrosine kinase c-kit is highly expressed in de-differentiated VSMCs in the aortic wall (Hollenbeck et al., 2004) suggesting that attenuation of VSMC proliferation and migration after imatinib treatment could partially be mediated via Abl and c-kit as well as by PDGFR- β .

Imatinib has also been linked to immunosuppression.(Seggewiss et al., 2005) The Abl tyrosine kinase is required in T-cell receptor (TCR) signaling to regulate T lymphocyte development and activation, and mice lacking the Abl kinase in T lymphocytes exhibit reduced proliferation and cytokine production in response to TCR stimulation.(Gu et al., 2007, Zipfel et al., 2004) The tyrosine kinase receptor, c-kit, and its ligand, the stem cell factor (SCF), are essential for the development and survival of mast cells, (Iemura et al., 1994) and treatment with imatinib has been demonstrated to induce mast cell apoptosis through inhibition of the c-kit receptor.(Juurikivi et al., 2005) In this study, preserved medial VSMC content was associated with reduced vascular inflammation after imatinib treatment and reduced numbers of CD3 ϵ -positive T lymphocytes were observed together with reduced levels of the *mast cell chymase* marker in aortas of imatinib-treated *ApoE*^{-/-} mice. The chemokine CCL5 is important for recruitment of T lymphocytes, and a strong correlation was found between both markers. In our animal model, imatinib did not increase the expression of the pro-inflammatory *TNF- α* and *IL-6*, as was observed in control mice without imatinib treatment. This could to some extent depend on the reduced recruitment of T lymphocytes and mast cells along with preserved VSMC content in the aortic wall. Because imatinib targets several tyrosine kinases, it would be of interest to employ alternative strategies such as conditional knockout mice lacking specific tyrosine kinases in distinct cell types to define

the relevant molecular targets of imatinib. To elucidate the role of Abl kinase and c-kit activity in various cellular processes, further studies are needed.

Prior to this study, the expression and localization of PDGF-D in AAA was unknown. We demonstrated that PDGF-D was predominantly expressed by VSMCs and endothelial cells and to some extent by macrophages in human AAA. PDGF-D has previously been detected in macrophages and endothelial cells in atherosclerotic lesions (Wagsater et al., 2009). Interestingly, both PDGF-D and its receptor PDGFR- β were expressed to a lower degree compared to non-aneurysmal control aortas. So why would we use a tyrosine kinase inhibitor such as imatinib to block a pathway that is already reduced in this disease? We need to point out that human biopsies are taken at a late stage of aneurysmal disease in which the expression profile might differ from early stages of the disease. We also saw that *PDGFR- β* expression seemed to be differentially regulated in humans and mice. Mouse models of AAA sacrificed after 4 weeks of AngII infusion represent early stage of aneurysmal disease and might instead give us an insight into the progression of human small AAAs. However, the biological role of PDGF-D and its receptor in AAA needs further investigation.

Imatinib is generally a well-tolerated drug, and clinical side effects mainly appear as mild to moderate.(Demetri et al., 2002) The most common side effects that patients experience are edema, nausea, diarrhea, muscle cramps, skin rash and headache. The most common manifestation of edema is periorbital edema and this condition requires no further treatment. Most cases with skin rashes as side effects of imatinib are mild and easily manageable. Hematological effects involve neutropenia and thrombocytopenia, and anemia occurs in some cases.(Mughal and Schrieber, 2010, Sneed et al., 2004) On rare occasions, patients might develop more severe pathological events such as cardiac toxicities, heart failure or myocardial infarction, all of which are associated with long-term treatment. Other cardiac complications associated with imatinib are hypo/hypertension, tachycardia and pericarditis but the prevalence of these appears to be less than 1%.(Atallah et al., 2007, Ribeiro et al., 2008) These side effects are also dose dependent and can easily be managed by lowering the imatinib dosage.

A limitation to this study is that we only studied one relatively low dosage of imatinib in mice. It would be of interest to study the effects of imatinib also with a higher dosage. We administered a dose of 10 mg/kg, which was the dose used in a previous animal

study.(Boucher et al., 2003) We did not see any macroscopically observable side effects such as gain or loss of weight, edema or death. If we take mouse metabolism in to consideration, a dose of 10 mg/kg daily is relatively low because mouse metabolic rate is approximately seven to eight times more rapid than in humans. Although we used relatively low doses of imatinib, we still saw effects such as preserved VSMC content and decreased inflammatory response. The purpose with initiating therapeutic treatment in patients with AAA is to give them a drug that inhibits AAA progression without any adverse side effects. We believe that by lowering the dosage of imatinib, which today is given at a dose of 400 mg daily to cancer patients, we can provide AAA patients with a therapeutic drug that attenuates AAA progression without any severe side effects. Further clinical trials are needed to evaluate imatinib as a potential drug for AAA.

In summary, we found that imatinib, a tyrosine kinase inhibitor, affects several key features that are important in AAA formation, including preservation of medial VSMCs and inhibition of T lymphocyte and mast cell recruitment to the aortic wall. Our data suggest that blockage of tyrosine kinase signaling with imatinib could be a novel therapeutic option to hinder the development of AAA in patients due to its potential to impair features that are associated with AAA development.

Adiponectin inhibits angiotensin II induced abdominal aortic aneurysm formation (Paper III)

Low circulating adiponectin levels have been associated with metabolic and cardiovascular complications, and the vast majority of studies have shown that adiponectin exerts anti-inflammatory effects. We therefore investigated the possible therapeutic role of adiponectin in AngII-induced AAA.

To investigate the therapeutic role of adiponectin mice were injected with a recombinant adenovirus expressing mouse adiponectin, which induce elevated adiponectin plasma levels that were sustained throughout the study. More importantly, adenoviral adiponectin expression resulted not only in increased total adiponectin levels, but also increased HMW adiponectin. HMW adiponectin has been suggested to be a better biomarker than total adiponectin levels and to be more biologically active in metabolic syndrome.(Pajvani et al., 2004, Fisher et al., 2005) We demonstrated that infusion of AngII for 8 weeks resulted in advanced AAA expansion and aortic remodeling with an AAA incidence of 75%. In *LDLR*^{-/-} mice infused with AngII, elevated plasma adiponectin levels resulted in inhibition of AAA formation. Along with AAA inhibition, we also demonstrated a reduction in atherosclerotic lesions in the aortic wall, as previously shown by van Stijn and coworkers who demonstrated that elevated adiponectin levels inhibit atherosclerosis throughout the aorta in *LDLR*^{-/-} mice.(van Stijn et al., 2014) Okamoto et al. also showed that overexpression of adiponectin reduces atherosclerosis in *ApoE*^{-/-} mice by attenuating the endothelial inflammatory response and the transformation of macrophage to foam cells *in vivo*.(Okamoto et al., 2002) However, the role of adiponectin in atherosclerosis remains controversial because some studies have found no effect of adiponectin in atherosclerosis development.(Nawrocki et al., 2010)

Further, *LDLR*^{-/-} mice overexpressing adiponectin also display a more organized aortic wall with preserved elastic fibers and VSMC content. Infusion with AngII causes transmural inflammation, disruption of the ECM and loss and/or differentiation of medial VSMCs. We found that adiponectin suppressed *mast cell chymase* and *MMP-9* expression, which exhibit elastolytic activity that might be a potential mechanism contributing to preserved elastic fibers. Together with these results, we also demonstrated increased *LOX* expression, which has previously been demonstrated to prevent AAA development.(Maki et al., 2002) Mast cell chymase is also known to induce VSMC apoptosis by disrupting NF-κB-mediated

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signaling.(Leskinen et al., 2006) The reduced expression of this enzyme could contribute to the inhibition of AngII-induced VSMC loss by apoptosis in *LDLR*^{-/-} mice overexpressing adiponectin. Adiponectin has also previously been demonstrated to suppress VSMC proliferation.(Matsuda et al., 2002) We found that adiponectin increased the gene expression of both *SM22α* and *α-actin* compared with the aneurysmal mice which might suggest that mice overexpressing adiponectin have an VSMC phenotype that resembles the contractile state.

To further address the preventive actions of adiponectin on AAA development, we examined aortic and systemic inflammation. We found that adiponectin substantially reduced infiltration of both macrophages and T lymphocytes, and this was accompanied by the suppression of the mast cell proteases chymase and tryptase in the mouse abdominal aorta. Macrophage recruitment to the aortic wall is one of the early events in AngII-induced AAA and is also involved in the advanced stages of AAA and contributes to the expansion of the aneurysmal artery.(Rateri et al., 2011) Adiponectin is an important regulator of macrophage polarization and has been shown to promote activation of macrophages to resemble the anti-inflammatory M2 cells with increased IL-10 levels rather than the pro-inflammatory M1 cells producing increased levels of TNF-α, IL-6 and CCL2.(Ohashi et al., 2010, Lovren et al., 2010) The anti-inflammatory mechanism of adiponectin in macrophages consequently inhibits T lymphocyte recruitment by suppressing T lymphocyte chemoattractants such as CXCL10.(Okamoto et al., 2008) In addition, adiponectin has also been shown to inhibit monocyte adhesion to endothelial cells by lowering the expression of endothelial adhesion molecules.(Ouedraogo et al., 2007) In this study, analysis of the systemic plasma cytokines and chemokines revealed a reduction of pro-inflammatory mediators including the cytokines IL-1α, IL1β, IL-6, IL-12 IL-17 and TNF-α and the chemokines CXCL10 and CCL2 that are, important for recruitment of T lymphocytes and macrophages. We also demonstrated elevated levels of anti-inflammatory IL-10 in mice overexpressing adiponectin. Taken together, these results suggest that adiponectin favors the predominance of an anti-inflammatory profile in the aortic wall.

While this manuscript was being prepared, Yoshida and coworkers reported similar effects on the role of adiponectin in AAA formation. Using *ApoE*^{-/-} *Adiponectin*^{-/-} double knockout mice, they demonstrated large aortic diameters and a higher incidence of AAA together with an augmented inflammatory response.(Yoshida et al., 2014) Their results were consistent

with ours, which further confirms the protective effects of adiponectin against AAA development.

Several studies have linked obesity to AAA and shown that increased infiltration of macrophages contributes to both PVAT and white adipose tissue (WAT) and that inflammation contributes to AAA pathogenesis.(Police et al., 2009) We found that adiponectin regulates the inflammatory status of both PVAT and WAT through inhibition of macrophage and T lymphocyte infiltration by decreasing the production of *TNF- α* , *CCR2* and *CCL2* as well as components of the renin-angiotensin system (RAS), *ACE* and *AT1aR*. RAS is an important regulator of blood pressure and vascular function, and RAS products such as ACE, angiotensinogen and AT1 and AT2 receptors have been identified in human and murine adipocytes. The role of PVAT in human AAA is further discussed in paper IV.

The contributing action behind AngII-mediated aortic wall inflammation and damage is known to be mediated through stimulation of AT1aR (Cassis et al., 2007) and the available evidence emphasizes the importance of this receptor in both human and murine AAA. In humans, AT1aR polymorphisms are associated with AAA, (Jones et al., 2008) and blockers of AT1aR in rats inhibit experimentally induced AAA.(Fujiwara et al., 2008, Kaschina et al., 2008) In paper III, we demonstrated that adiponectin inhibited AT1aR in *LDLR*^{-/-} mice infused with AngII, which might result in halting macrophage migration to the aortic wall and thereby lead to decreased MMP secretion. Thus, suppression of this receptor might be one of the potential mechanisms by which adiponectin protects against AngII-induced aortic inflammation, vessel wall damage and AAA formation.

Increased levels of adiponectin have been demonstrated in human AAA suggesting that adiponectin is a potential biomarker for early AAA development.(Acosta-Martin et al., 2011, Gollledge et al., 2007) These findings could respond as a compensatory mechanism to inhibit the progression of the disease. We also need to address the fact that many older patients including AAA patients are currently under therapeutic treatments such as statins, ACE-inhibitors or β -blockers which might influence the expression of plasma adiponectin.

Adipocytes secrete a number of pro- and anti-inflammatory factors including adiponectin, leptin and resistin as well as cytokines such as *TNF- α* , IL-6 and IL-18. The unbalanced production of pro- and anti-inflammatory adipokines in obese individuals has been suggested

to contribute to increased cardiovascular and metabolic disorders. The vast majority of studies have shown that adiponectin exerts anti-inflammatory effects and that high plasma levels of adiponectin are associated with better outcome compared to lower levels, which are associated with increased prevalence of obesity-linked disorders. Conflicting studies make it difficult to determine whether adiponectin is a promising predictor or risk factor for cardiovascular diseases; however, the favorable effects associated with adiponectin make it a promising candidate as a therapeutic molecule for future treatment strategies of AAA.

The limitation of this study was that the adenoviral-induced overexpression of circulating adiponectin was significantly higher than what is normally expressed in plasma. Because adiponectin is an anti-inflammatory adipokine, we do not know how these high levels would affect other processes, such as innate immunity, in the animals. Another perspective we need to take into account is if the protective action of adiponectin is accomplished through blockage of AT1aR. One way to study this is to use another mouse model of AAA such as CaCl₂ or elastase-perfusion models.

In summary, we demonstrated that adiponectin prevents AngII-induced AAA in *LDLR*^{-/-} mice and that these protective actions are mediated through multiple mechanisms, including inhibition of aortic wall and PVAT inflammation, suppression of systemic inflammatory mediators, and inhibition of RAS components together with a more organized vessel wall structure by preventing the degeneration of elastin and medial VSMCs.

Inflammatory cells, ceramides and expression of proteases in perivascular adipose tissue adjacent to human abdominal aortic aneurysms (Paper IV)

The majority of research performed on cardiovascular disease has focused on the luminal surface of vessels in direct contact with flowing blood as the initiating line in the pathogenesis of vascular inflammation. In the setting of AAA development, new evidence supports an “outside-in” hypothesis in which the vascular inflammation is initiated in the adventitia and then advances towards the intima. Inflamed and dysfunctional PVAT modulates the balance between pro- and anti-inflammatory mediators, which might impact vascular disease in the underlying vessel and ultimately lead to aortic remodeling. The volume of PVAT has previously been associated with abdominal aortic diameter, and it has been suggested that alterations in PVAT function that allow for an increase in PVAT mass lead to the release of pro-inflammatory mediators and AAA formation.(Thanassoulis et al., 2012) In this study we aimed to investigate the composition of PVAT in AAA. We provide a descriptive study demonstrating the content of immune cells and immune mediators found in human PVAT adjacent to the AAA wall.

Sterile inflammation occurs as a result of intracellular proteins (DAMPs) being released upon cell damage or necrosis. Necrotic adipocytes induce a strong inflammatory response leading to the recruitment of macrophages to the damaged tissue.(Cinti et al., 2005, Kolak et al., 2007) In this study, we found that these dying adipocytes in PVAT are surrounded by neutrophils and macrophages. We also showed the presence of T lymphocytes and mast cells in PVAT adjacent to AAA. Even though most of the examined proteases were elevated in AAA media and/or adventitia, we found that *cathepsin K* and *S* levels were higher in PVAT from AAA compared with control aortas. Macrophages have the capacity to synthesize cathepsin S, and the accumulation of CD68-positive macrophages in the adipose tissue might explain the increased expression of this protease. Deficiency of either cathepsin K or S has been demonstrated to attenuate experimentally induced AAA formation in mice (Sun et al., 2012, Qin et al., 2012), demonstrating an essential role in the pathogenesis of AAA. The pro-inflammatory cytokine *IL-6* was expressed at a significantly higher in PVAT compared with adjacent AAA and non-aneurysmal control aorta. Inflamed PVAT is known to secrete high levels of IL-6 and is associated with stiffness of the aorta.(Du et al., 2015)

There are likely multiple mechanisms that promote the infiltration of vascular cells into PVAT including cell death, hypoxia and chemotactic regulation. Several studies have shown that PVAT has strong chemotactic activity that is mainly mediated by secretion of IL-6, IL-8 and CCL2 and that these factors are likely to contribute to the infiltration of immune cells at the interface between PVAT and the adventitia that subsequently lead to the development of atherosclerosis.(Henrichot et al., 2005, Chatterjee et al., 2009) In addition, a high-calorie diet can promote the development of a pro-inflammatory phenotype in these regions.(Chatterjee et al., 2009) Evidence suggests that perivascular factors might play an important role in the pathogenesis of AAA (Police et al., 2009) and this has led to the hypothesis that the inflammatory response might be initiated from the adventitial side of the vessel wall.

Kolak and coworkers found an association between high liver fat adipose tissue and increased levels of ceramide, suggesting that ceramides could contribute to inducing inflammation.(Kolak et al., 2007) We demonstrated that ceramide in PVAT correlated positively with the T lymphocyte marker *CD3e* at gene expression level. Ceramides have been demonstrated to be involved in inhibition of T lymphocyte proliferation and to contribute to CD95-mediated cell death.(Adam et al., 2002) Further, a positive correlation was observed for S1P in PVAT and the neutrophil marker *CD66b*. It has previously been demonstrated that S1P induces IL-8 secretion from airway SMCs and that this enhances neutrophil chemotaxis in vitro.(Rahman et al., 2014) Thus we hypothesized that necrotic adipocytes might release ceramides that in turn can recruit immune cells, indicating ceramides as a potential driver associated with leukocyte infiltration into the PVAT adjacent to the aneurysmal wall. The limitation with these results is however, that we calculated a correlation between sphingolipid concentrations in PVAT based solely on mRNA expression of cell markers rather than on protein expression of cell markers. Nonetheless, protein expression in immune cells was confirmed and its location in the adipose tissue was established by immunohistochemistry.

Recently an interesting link has surfaced between sphingolipid metabolism and the adipocyte hormone adiponectin. By binding to its receptors AdipoR1-mediated and AdipoR2, adiponectin decreases inflammation and increases insulin sensitivity.(Yamauchi et al., 2002) AdipoR1- and AdipoR2-mediated signaling stimulates ceramidase activity that degrades ceramide to sphingosine that is further converted to S1P thereby decreasing caspase-8-

mediated apoptosis. This suggests that the anti-inflammatory actions of adiponectin might be directly associated with ceramide depletion and that adiponectin and its receptors might play a fundamental role in the physiological regulation of the balance between ceramide and S1P.(Holland et al., 2011)

The limitation with this study is the lack of proper control tissue for PVAT. The optimal control would be PVAT from non-aneurysmal patients; however, the healthy aorta is surrounded by little PVAT, which makes such tissue difficult to obtain. Alternatively the surrounding peripheral arteries or WAT from the same patient as the aneurysmal PVAT could be used as a control tissue; however, we had limited access to this as well. It should be pointed out here that this is a descriptive study showing the infiltration and localization of immune cells, immune mediators, and the sphingolipid content, and future studies are needed to explain and evaluate the functional roles of PVAT, sphingolipids and inflammation.

In summary, we have shown that PVAT surrounding the aneurysmal aorta is characterized by necrotic adipocytes, immune cells and the expression of proteases. PVAT might therefore represent a local source of proinflammatory cells and mediators that promote aortic wall degeneration and aortic dilatation.

CONCLUDING REMARKS

AAA is a complex disease involving medial degeneration of connective tissue and VSMCs along with chronic inflammation that leads to the enlargement of the vessel wall and eventual aortic rupture. This thesis aimed to investigate novel molecular mechanisms involved in the remodeling of AAA to get a better insight of some of the many factors involved in the development and progression of AAA. The increased knowledge of the mechanisms behind AAA development provides a basis for developing novel treatment strategies and identifying drug candidates that will hopefully lead to better outcomes for patients. The main findings in this thesis and their potential role in AAA development are summarized in Figure 12.

Figure 12. Summary of main findings in the four papers.

The ability to develop new therapeutic treatments and ultimately prevent AAA development lies partially in our understanding of the critical signals and molecular mechanisms associated with vascular remodeling. Knowledge of human AAA pathology is primarily derived from biopsies of aneurysmal tissue obtained during open surgical repair of profoundly dilated aortas. It is noteworthy, therefore, that most of the information we have today is of a late-stage profile of the disease with major deformation of tissue architecture. Opportunities to obtain human AAA tissue biopsies are becoming more limited as open repair surgery is being replaced by endovascular repair surgery. Unfortunately, we lack fundamental knowledge of the natural history and characteristics of human AAA formation and stages of progression, and so we must rely at this time on experimental animal models to provide us with possibilities to investigate pharmacological interventions to prevent the initiation and progression of AAA. Noteworthy, a critical question is whether and how preclinical findings translate into the clinical context.

There are currently no validated medical therapies for AAA, and the current management of AAA is restricted to surgical repair. Therefore, there is an urgent need to determine the pathogenic mechanisms of AAA so as to develop effective pharmacological therapies to inhibit AAA growth and reduce the need for AAA repair. Modification of risk factors such as cessation of cigarette smoking are essential, however, strategies to slow the growth or stabilize small AAAs once they are present are needed.

In paper I, we demonstrated the importance of Trif-mediated signaling in attenuating AAA formation by reducing the level of inflammation, providing increased knowledge to the mechanism of this signaling pathway. These results may suggest Trif as a potential target for future therapy by reducing the inflammatory response during AAA formation. Today, various TLR drugs have been developed to target individual receptors for treatment of specific diseases. Most of them are agonists; however interest has been drawn into the prospective use of TLR antagonists to reduce inflammatory responses involved in diseases such as rheumatoid arthritis and lupus. The development of TLR antagonist could potentially be used as target therapy in AAA formation and would be an interesting aspect to take into consideration in future.

In paper II, we demonstrated that imatinib damps vascular inflammation and preserves VSMC in the aortic wall and attenuates aneurysmal growth. As imatinib already is an established therapeutic drug on the market, a clinical study could be taken under consideration to evaluate the therapeutic novelty of imatinib in human AAA progression. Further, the use of other tyrosine kinase inhibitors with different selectivity may be studied.

In paper III, we demonstrated a vascular protective role for adiponectin which attenuated AngII induced AAA in mice. However, in order to say that adiponectin can be used as a therapeutic compound to prevent or delay AAA development it would be of interest to evaluate adiponectin's ability to inhibit further growth.

In paper IV, we demonstrate that aneurysmal aortas are surrounded by inflamed adipose tissue. Further knowledge of the role of PVAT in AAA formation would provide us with a new insight into the disease progression.

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