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## Pharmaceutical stabilization of abdominal aortic aneurysms : changing its natural history

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### Citation

Kokje, V. B. C. (2017, June 28). *Pharmaceutical stabilization of abdominal aortic aneurysms : changing its natural history*. Retrieved from <https://hdl.handle.net/1887/50085>

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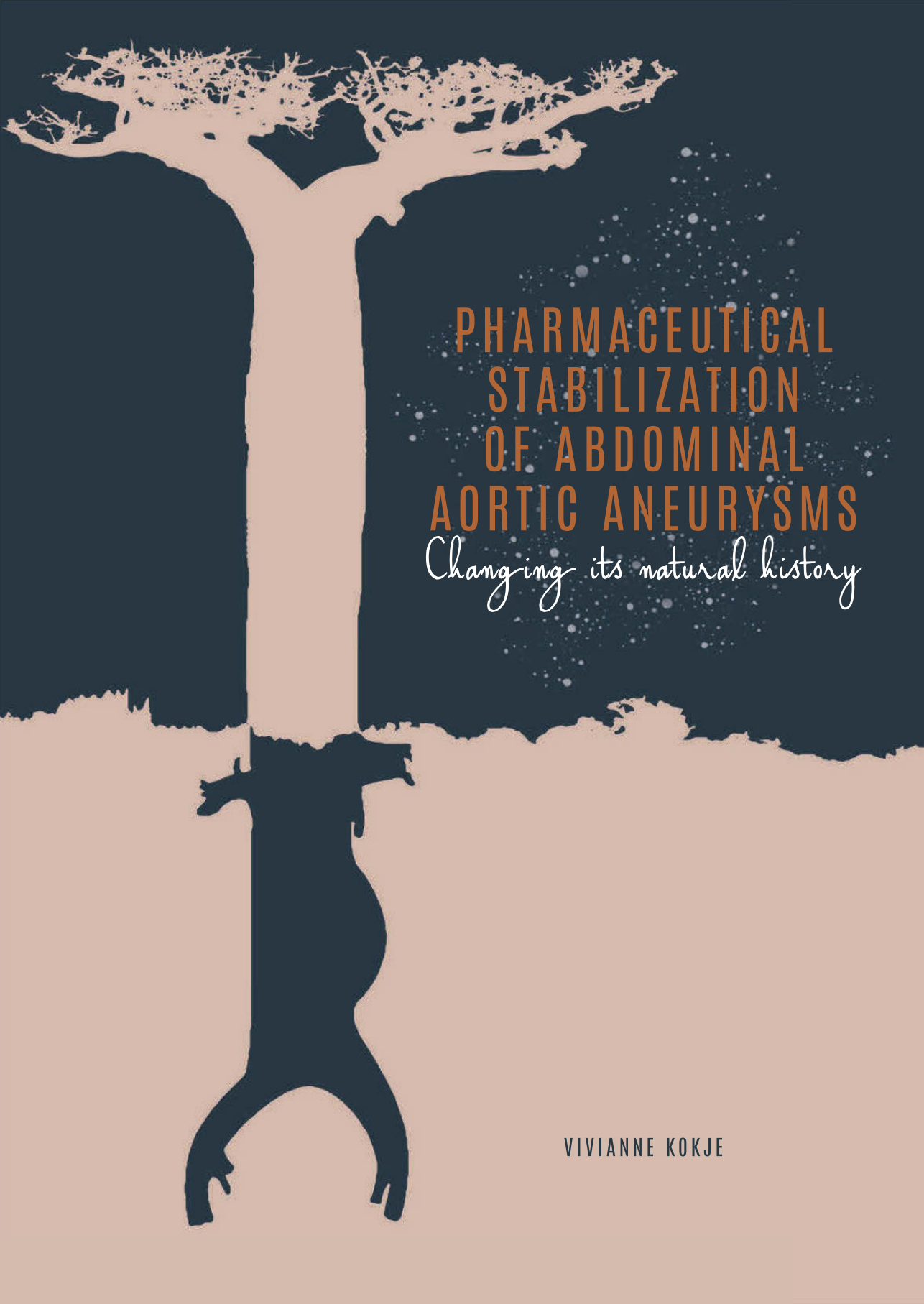


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**Author:** Kokje, Vivianne

**Title:** Pharmaceutical stabilization of abdominal aortic aneurysms : changing its natural history

**Issue Date:** 2017-06-28



PHARMACEUTICAL  
STABILIZATION  
OF ABDOMINAL  
AORTIC ANEURYSMS  
*Changing its natural history*

VIVIANNE KOKJE



**PHARMACEUTICAL STABILIZATION OF  
ABDOMINAL AORTIC ANEURYSMS**

Changing its natural history

Vivianne B. C. Kokje

Layout, cover and printing by: Off Page, Amsterdam

ISBN: 978-94-6182-798-2

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**PHARMACEUTICAL STABILIZATION OF  
ABDOMINAL AORTIC ANEURYSMS**

Changing its natural history

Proefschrift

ter verkrijging van  
de graad van Doctor aan de Universiteit Leiden, op  
gezag van Rector Magnificus prof. mr. C.J.J.M. Stolker,  
volgens besluit van het College voor Promoties te  
verdedigen op 28 juni 2017  
klokke 15.00 uur

door

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geboren te Delft  
in 1987

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Financial support by the Dutch Heart Foundation for the publication of this thesis is gratefully acknowledged.

The research described in this thesis was performed at the department of Vascular Surgery of the Leiden University Medical Center, Leiden, The Netherlands and the Stanford University, California, United States of America.

'Life is not waiting for the storm to pass, it's about learning how to dance in the rain.'

Anonymous

*Voor mijn ouders*



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# Chapter

INTRODUCTION

1



## ABDOMINAL AORTIC ANEURYSM

An abdominal aortic aneurysm (AAA) is a dilatation of the infrarenal aorta. The most accepted definition of an AAA is based on its diameter. A diameter of 30 mm or more is considered to be aneurysmal, which usually is more than two standard deviations above the mean diameter for both men and women<sup>1,2</sup>. To compensate for individual variation, other researchers have proposed a diameter 1.5 times larger than the expected diameter<sup>3,4</sup>. The prevalence of AAA is 3-5% in the adult population over 60 years and mainly affects men<sup>5,6</sup>. AAA itself is life-threatening problem due to spontaneous rupture, a risk that increases with the diameter of the AAA. The threat of ruptured AAAs has captured the attention of physicians for centuries. The term 'aneurysm' was written down for the first time by Rufus of Ephesus and is derived from the Greek 'ana' (towards outside) and 'eurunō' (widening). However, until the 16<sup>th</sup> century only small post-traumatic aneurysms were described. Antoine Saporta (1507-1573, University of Montpellier) was the first to report aortic aneurysms and described it as a pulsatile swelling. He also reported the symptoms of a rupture resulting in death. In Western countries the incidence of ruptured AAAs ranges between 5.6-17.5 per 100,000 person/year<sup>7,8</sup>. Most AAAs do not cause symptoms until the moment of rupture. A rupture can lead to intense abdominal and lower back pain and patients often present to the hospital with hypovolemic shock. Ruptured AAAs have an estimated overall mortality rate greater than 90% and probably most patients never reach the hospital because of the eminent massive abdominal blood loss<sup>9</sup>. The operative mortality rates of ruptured AAA are improving over the years, possibly due to new endovascular treatment options and better perioperative care<sup>10,11</sup>. AAAs with a diameter less than 40 mm have a negligible risk of rupture whereas the risk of rupture of AAAs with a diameter of 55 mm is approximately 10% per year. When the AAA diameter exceeds 55 mm the risk of rupture exponentially increases<sup>12</sup>. In a screening study of US veterans (n=73,451) the highest prevalence of AAAs (5.9%) was found in white male smokers between the age of 50 and 79. This and other studies indicate that important risk factors for AAA disease are advanced age, smoking and male gender<sup>13-15</sup>. Several more factors have been identified to be associated with AAA development such as hypertension and atherosclerosis<sup>15-17</sup>. However, the importance of these factors is still subject to scientific debate.

## CURRENT AAA TREATMENT

The treatment of AAA started with the description of wrapping the aneurysms in the early 1940s. Harrison and Chandy used cellophane around a subclavian aneurysm<sup>18</sup>. In 1949 Abbott reported a large series of wrapping AAA, describing the benefit of relieving pain in about 50% of the patients with advanced aneurysm disease but not reducing the risk of rupture<sup>19</sup>. In 1951 the first effective means to treat AAA was described<sup>20</sup>. Freeman used venous autografts for aortic replacement<sup>21</sup> and in 1952 Voorhees reported the use of synthetic grafts for aortic replacement<sup>22</sup>. It was not until the beginning of the nineties that Parodi et al described endoluminal approaches for elective AAA repair<sup>23</sup>. Currently, both operative and endovascular (EVAR) techniques are common practice and subject to continuous research and improvement. The timing of AAA repair currently depends on the diameter of the aneurysm and is a balance between the risk of rupture and the operative mortality for aneurysm repair. Due to the global

consensus based on clinical trials that the risk of rupture is negligible for small aneurysms and patients have no complaints, elective repair is currently recommended for patients with AAAs larger than 55 mm<sup>24, 25</sup>. Due to this consensus, a considerable amount of patients are under surveillance for years until their AAA reaches the cut-off point of 55 mm for surgical or endovascular repair. However the benefit of repair in patients with limited life expectancy or patients with serious co-morbidity and frail general condition remains uncertain. It has been estimated that up to 70% all AAA patients will eventually require surgical intervention due to the continued expansion of their AAA<sup>26</sup>. Outcome of elective open aneurysm repair has improved over the years. Between 1980 and 2000 an overall 30-day mortality rate between 5.3% and 27.1% was reported. In 2014, a Cochrane review on clinical trials reported a 30-day mortality of 4.2%<sup>27</sup>. In 2015, Chang et al reported a 30-day mortality rate of 7.8% after open AAA repair in a population-based study<sup>28</sup>. 30-day mortality rates of endovascular repair, in clinical trials and in population-based studies, are found to be significantly lower (0.5% - 1.54%) compared to the open surgical repair ( $p < 0.001$ ). In clinical trials the immediate (up to 4 years) and long term (>4 years) mortality rates of open and endovascular repair are not significantly different between the two (respectively for open 17.0% vs EVAR 15.8% and open 37.8% vs EVAR 37.3%)<sup>27</sup>. However, in a recent population based study the long-term mortality of EVAR is higher compared to open repair. This might be explained by the inclusion of high-risk patients in the population-based study<sup>28</sup>. EVAR is also increasingly used for ruptured AAAs (rAAAs) but controversy exists about the results of emergency EVAR of the ruptured aneurysm. A Cochrane review from 2014 reports no clear difference in short term mortality between open en EVAR repair ( $p = 0.52$ )<sup>29</sup>. In contrast to a recent retrospective observational study including and matching 10.998 patients that found a significant lower long-term mortality rate using EVAR to treat ruptured AAAs<sup>30</sup>.

## OTHER TREATMENT STRATEGIES FOR AAA

AAA is a disease of the ageing population and often presents in patients with several co-morbidities and these co-morbidities have a significant effect on the outcome of AAA repair<sup>31</sup>. Pharmacological intervention reducing or inhibiting progression of small AAA, and thus the eventual need for surgical repair could have major advantages; both from a patients' as from socio-economical perspective<sup>32</sup>.

Currently there are no means to intervene with the natural history of the aneurysm. Studies using human AAA tissues have helped to identify several molecular mediators and matrix-degrading proteases, which seem contribute to aneurysm disease and might be potential pharmacological targets<sup>33-35</sup>. These tissues are obtained during surgical open repair of (r)AAAs and therefore represent only the end stage of the disease<sup>36</sup>. They provide no insight in the processes driving the smaller aneurysms towards the 55mm diameter. Small animal models have been developed to allow more detailed investigations on the cellular and molecular mechanisms of the disease in a controlled manner. The models play a key role in the screening of potential therapeutics.

## AAA PATHOPHYSIOLOGY

In order to find new therapeutic strategies for AAA, a better understanding of the pathophysiological processes involved in the aneurysm development and progression at cellular and molecular levels is necessary. Current knowledge indicate that the pathophysiological process of AAA disease is

distinct from atherosclerosis and dominated by degeneration of the vascular wall<sup>12, 37, 38</sup>. Proteolytic degradation of the aortic wall has been postulated to be a key factor involved in the pathophysiology of AAA disease. Both elastase and collagenase activity have been found in aortic aneurysms and both have been correlated with aneurysm size. In particular, matrix-metallo proteases (MMPs) are considered to be the predominant proteases. MMP-2 and MMP-9 are able to degrade elastic fibers, interstitial collagen and denatured collagen. The increase in MMP2 and MMP9 has been clinically correlated with aneurysm size<sup>37-39</sup>.

Besides the formation and progression of AAA has been associated with chronic transmural inflammation. The majority of infiltrates contain invading monocytes and macrophages, plasma cells, B cells and T cells. These infiltrates have been correlated with abundant pro-inflammatory cytokines such as IL-6, CXCL8 and PGE2<sup>34, 40</sup>. Maximum AAA diameter has been found to correlate with increased circulating inflammatory markers, such as IL-6 and CRP<sup>41, 42</sup>.

Another key feature of AAA disease is neovascularization in the arterial aortic wall. In the healthy human aortic vessel wall the media is devoid of vasa vasorum and the adventitial layer has less vasa vasora compared with other mammals<sup>43</sup>. Investigation of human AAA tissue reveals prominent neovascularization in the aneurysm wall<sup>44-47</sup>. Experimental studies suggest that the neovascularization of the aortic wall might enhance aneurysm rupture<sup>48, 49</sup>.

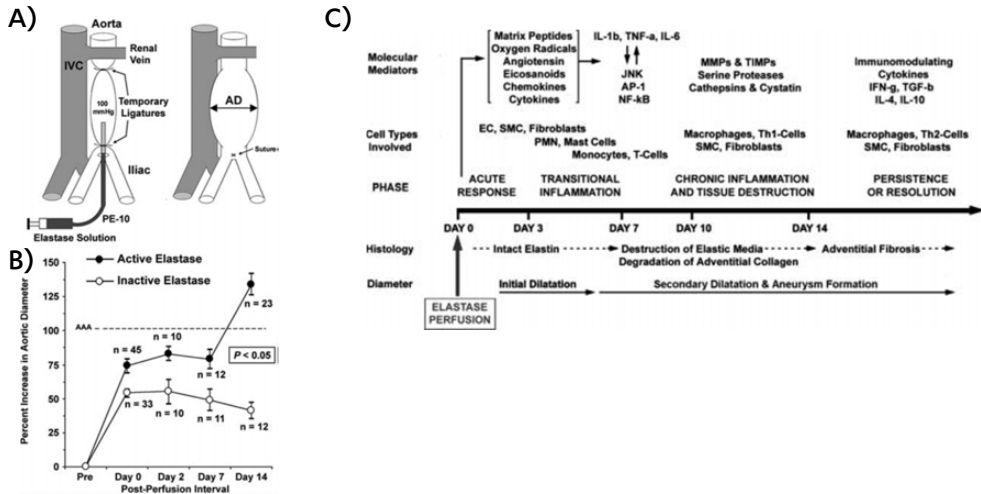
## ABDOMINAL AORTIC ANEURYSM MOUSE MODELS

A basic premise of animal models of disease is that they mimic the cellular and biochemical characteristics in the progression of human disease. Mice have become dominant in biomedical research due to their small size, their well-documented genetic backgrounds and the ability to delete or over-express specific genes. To date, no murine model that mimics the pathophysiology of human AAA disease exists and thus conclusions from commonly used animal models are based on the fact that they share several similar characteristics of those seen in human AAA disease. There are advantages and disadvantages associated with each of the models used in aneurysm disease. There are three categories of murine AAA models: genetically modified mice models<sup>39, 50</sup>, models with surgically induced AAAs<sup>51, 52</sup> but the most frequent used murine models use chemical induction of AAAs. Three AAA models using chemical induction have been described; the elastase model, the angiotensin-II model and the calciumchloride model.

### The elastase mouse model

One of the most used animal models for aneurysm disease is the transient intraluminal perfusion of the abdominal aorta of mice with porcine pancreatic elastase<sup>53</sup>. This model was first described in rats by Anidjar et al<sup>54</sup>. Currently the murine version of the model is widely used. The aortic diameter and structure remain stable for up to 7 days, after which rapid and significant increase in diameter begins to occur. The delayed onset of aortic dilatation in the model is associated with transmural aortic infiltration of monocyte, macrophages and T-cells along with increased activity of several matrix degrading proteases. The effect of the latter causes the elastin and collagen in the aortic wall to degrade resulting in a rapid secondary dilatation and aneurysm formation at day 14. The transmural aortic inflammation is a distinctive feature of this model and resembles the human

AAA. However, this model does not display rupture of the aneurysm as well as thrombus formation, both characteristic features of human aneurysms.



**Figure 1.** Elastase-induced model of AAAs [R.W. Thompson, Ann.N.Y.Acad.Sci. 1085:59-73 (2006)]. (A) Technique used for intraluminal perfusion of the infra-renal aorta. (B) Aortic dilatation at various days after aortic perfusion of elastase (black), saline (white). (C) Overview of cellular and molecular events involved in elastase-induced AAAs.

### The angiotensin II model

Angiotensin II is the primary bioactive peptide of the renin angiotensin system that plays a critical role in cardiovascular diseases. Due to the infusion of angiotensin II in mice, abdominal aortic aneurysms are induced<sup>55</sup>. In the model constant infusion of angiotensin II, for 4 weeks, is performed by implanting osmotic mini-pumps into the subcutaneous area of the mice. Angiotensin II infusion in mice reveals progressive changes in the vascular wall as well as considerable heterogeneity in the appearance of the AAAs. The heterogeneous characteristics of angiotensin II induced AAAs are considered to be beneficial for research properties as the characteristics of human AAAs are also considerably heterogeneous. Pathological features of aneurysms generated in this way include degeneration of the aortic media, dilatation of the lumen, prominent neovascularization and presence of a wall thrombus; all hallmarks of human AAA disease. It is found that the first reaction to angiotensin II infusion is medial macrophage accumulation in the region that is prone to AAA formation. At this stage elastin degradation is found. By day 3 to 10 gross dissections of the aortas were seen leading to prominent vascular hematomas, influx of T and B cells is seen. The dilated region gradually regains elastin fibers and re-endothelializes. The remodeled tissue prominently reveals neovascularization.

Besides the comparison in pathological features, a comparable gender preference to the human condition is seen, as male mice are more prone to aneurysm formation. However, there are some disadvantages to this model. Firstly not all infused mice develop AAA. Also, the angiotensin II induced AAAs are all located to the suprarenal aorta and no AAA's are formed. Besides, the inflammatory

response is thought to be provoked by the thrombus and therefore the aneurysms are formed secondary to an aortic dissection rather than gradual aneurysm development. Therefore, there is a discrepancy from the human situation in AAA location and morphology and therefore less comparable with the human AAA. Noteworthy further is that when infusing the angiotensin II into hypercholesterolemic mice, the AAA incidence is 3-4 fold higher than in normocholesterolemic mice<sup>56</sup>, while there is no evidence that there is an association between hypercholesterolemia and AAA development in humans.

### Calcium Chloride model

A third commonly used chemically induced model of AAA formation is the calcium chloride model. This model involves perivascular application of calcium chloride (CaCl<sub>2</sub>) onto the infra-renal aorta of mice to induce extracellular matrix remodeling. Gertz et al were the first to apply CaCl<sub>2</sub> to the adventitia of carotid arteries of rabbits to induce aneurysm formation<sup>57</sup>. They found an increase of the luminal diameter of 61% in three weeks. First CaCl<sub>2</sub> application to the aorta in mice was reported by Chio et al; an AAA formed 3 weeks after surgery<sup>58</sup>. The application of CaCl<sub>2</sub> leads to the development of luminal dilatation without the preceding mechanical effects that are noted in the elastase-model. The inflammation occurs on the luminal and medial aspect of the artery. Histological examination demonstrated that aortic dilatation was accompanied by vascular smooth muscle cell depletion, elastin degradation and infiltration of T-cells and macrophages. However, the CaCl<sub>2</sub>-induced AAAs do not display the transmural inflammation, rupture and thrombus which are important features of human AAA.

## AIM AND OUTLINE OF THE THESIS

The aim of this thesis was to gain more insight to the complex pathophysiology of human AAA disease and consequently identify new possible pharmacological therapies for the stabilization of growing AAA. It specifically assesses the effectiveness of specific immunomodulatory therapies on the inhibition of aneurysm growth for potentially pharmaceutical targets and interference with aneurysm growth in clinical setting. Using a murine model, the possibilities of new and unforeseen pharmacological means to intervene with AAA growth were investigated. As a result of the close resemblance between the created mouse aneurysm and the human aneurysm, the established elastase model was used. This model is the only model where true chronic transmural inflammation is observed.

Several potential pharmacological ways to intervene with aneurysm formation have been used in murine models, such as statins, anti-hypertensive agents and doxycycline. Some have been translated into human studies. **Chapter 2** provides an up-to-date systematic review on clinical human studies exploring the pharmacological therapies for stabilizing or impeding AAA growth. **Chapter 3** explores the possible parallels between AAAs and chronic obstructive pulmonary disease (COPD), as the identification of common mechanistic pathways is relevant to detect new targets for pharmacological stabilizing therapies.

Current concepts indicate that the pathophysiological process of AAAs is dominated by degeneration of the vascular wall. Four specific pathophysiological hallmarks of human aneurysm disease have been described: chronic inflammation, medial neovascularization, a decrease in

vascular smooth muscle cells and alterations in elastin and collagen. In **Chapter 4**, the anti-inflammatory potential of a vitamin D receptor agonist (Paricalcitol) was investigated. In vitro and in vivo studies have identified the vitamin D receptor (VDR) as a potent immune regulatory factor. To investigate its effect on human AAA, a small proof-of-concept study was conducted in which aneurysm patients received Paricalcitol 2-4 weeks prior to surgery. Degeneration of collagen and elastin is caused by several proteases. Literature states that the cysteine proteases, cathepsin K, L and S are prominent collagenases. We hypothesized that inhibiting these compounds might lead to stabilization of aneurysm growth. In **Chapter 5** the role of cathepsin-inhibitor E64, a broad-spectrum cysteine protease inhibitor described in two different murine models ; the elastase model and the angiotensin 2 model. The obtained aortic wall samples were matched with control aneurysm aortic wall samples and prepared for further investigation. **Chapter 6** describes the study of the potential contribution of CXCL8 to the inflammatory process seen in human AAAs. CXCL8 contributes to the extreme neutrophil content and the extensive neovascularisation that hallmarks AAAs. Human AAA samples were used to validate the previous reports of high CXCL8 content and the activation of the CXCL8-pathway. The role of the CXCL8-axis was tested in the murine elastase model via the neutrophil receptor (CXCR2) antagonist DF2156A. An additional interesting candidate for diminishing neutrophil chemotaxis, besides DF2156A, is colchicine. This already clinically available compound was investigated in **Chapter 7**. The effect of colchicine on neutrophil chemotaxis might be secured via either an indirect or direct pathway. Both pathways were investigated in human aneurysm samples. Next, the role of colchicine was identified in the murine elastase model. Besides the abundance in neutrophils, CXCL8 and cysteine proteases our research group previously reported an abundance of IL6 in human AAA samples. In fact, IL6 was found to be a discriminative factor between AAA and atherosclerotic disease. Because IL6 is a potential critical factor in aneurysm disease its role in aneurysm formation is investigated in **Chapter 8**. Using human aneurysm tissue samples we evaluated the IL6 pathway. Next, after induction of aneurysms via elastase perfusion, mice were treated with anti-IL6 injections to test the potential of IL6 in aneurysm formation.

Strong clinical and molecular associations exist between AAA and popliteal artery aneurysms (PAA). Yet, while the natural history of AAA is that of rupture, the primary concern in PAA's is thrombosis and rupture of PAA is rare. A patho-histological (re-)examination of human AAA samples and popliteal aneurysm wall samples was made in **Chapter 9** to provide clues towards auxiliary processes contributing to AAA wall rupture.

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# Chapter

# 2

## PHARMACEUTICAL MANAGEMENT OF SMALL ABDOMINAL AORTIC ANEURYSMS, A SYSTEMATIC REVIEW OF THE CLINICAL EVIDENCE

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## ABSTRACT

### Background

Management of abdominal aortic aneurysms (AAAs) fully relies on surgical repair of larger AAAs. Consequently, it has been pointed out that medical interventions inhibiting AAA progression could greatly reduce the need for surgical repair. A spectrum of pharmaceutical strategies has been reported, albeit conclusions often appear contradictory. Given the long-standing interest in pharmaceutical AAA stabilization we considered a systematic review of the available literature relevant.

### Objectives

To provide an up-to-date systematic review of the available data on pharmaceutical therapies for stabilizing or impeding AAA growth.

### Methods

A search using Pubmed, Embase, Web of science, Cochrane, CINAHL, Academic Search Premier and Science Direct identified 27 eligible papers that clinically studied the effect of the pharmaceutical therapy on AAA diameter growth.

### Results

This review shows that there is currently no pharmaceutical strategy that reduces AAA growth. Most studies are of poor methodological quality. Initial promising reports are often not confirmed in subsequent larger studies, raising the possibility of selective reporting.

### Conclusion

There is currently no pharmaceutical means that quenches AAA growth.

## INTRODUCTION

The risk of rupture of an abdominal aortic aneurysm (AAA) progressively increases in larger AAAs, i.e. aneurysms larger than 55mm. Four large clinical trials do not show a benefit of earlier repair<sup>1</sup> (i.e. for aneurysms smaller than 55 mm). Therefore, the therapeutic approach to AAAs is surveillance of small aneurysms and prophylactic surgical open or endovascular aneurysm repair (EVAR) in AAAs over 55 mm<sup>2</sup>. Yet, while open repair has excellent long-term outcome, it has a significant peri-operative morbidity and mortality. Although EVAR comes with a significantly lower peri-operative morbidity and mortality, its cost-effectiveness is being questioned. Consequently, it has been pointed out that pharmaceutical means slowing down or stabilizing progression of small AAAs, and thus postponing or obviating the need for surgery could have major advances<sup>3</sup>. In fact, pharmaceutical stabilization of AAA is now considered an unmet medical need.

A large body of preclinical evidence shows that interference with aspects of vascular inflammation and/or proteolytic activity alleviates AAA formation in rodent models of the disease<sup>4,5</sup>. Clinical studies on the other hand are limited and their conclusion often inconsistent<sup>6-8</sup>. There are currently 78 reviews (this literature search) on pharmaceutical AAA stabilization, yet a comprehensive systematic review is missing. Given the renewed interest in pharmaceutical AAA stabilization we considered a systematic review of the available evidence on pharmaceutical interventions for stabilizing or impeding AAA growth in humans relevant.

## METHODS

### Search strategy

The studies included in this review were identified by searching Pubmed, Embase, Web of science, Cochrane, CINAHL, Academic Search Premier and Science Direct. The search was not limited, and thus all languages and publication types (e.g. reviews or conference abstracts) were included. The search was most recently updated on April 17, 2015.

We created two search themes, which were combined in the search by AND. The first theme was created for AAAs by using all terms for abdominal aortic aneurysm, such as abdominal aneurysm or abdominal aorta aneurysms. The second term consisted of all terms for pharmacology, including specific drug group names, such as medical treatment or drugs or hydroxymethylglutaryl-coA reductase inhibitors. Details on the search strategy are available in the appendix I.

### Inclusion criteria

Only studies providing original clinical data on an effect of pharmaceutical therapy on AAA growth were included. Hence, we excluded all animal studies as well as studies that exclusively described an effect of pharmaceutical intervention on molecular processes in the aneurysm wall; all reviews (n=79) and commentaries.

Two authors (VK and JL) independently reviewed the results of the search strategy. A first selection was made on title; all articles potentially reporting on an effect of a pharmaceutical intervention on abdominal aortic aneurysm disease were included. A second selection was made by reading the abstract of articles that were selected on basis of the title. The final selection was made on basis of the full text.

Quality of the identified studies was scored using the STROBE scoring system<sup>9</sup>.

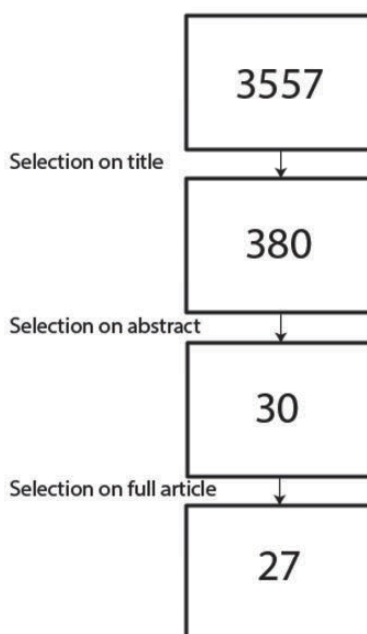
Statistical analysis was not performed because of the marked heterogeneity of the included studies.

## RESULTS

The search strategies identified 3557 articles. Selecting on title and abstract narrowed the amount of articles to 30 original studies. Two of the 30 original studies were excluded because of missing data on AAA growth rate<sup>10,11</sup>. Another article, written in Danish<sup>12</sup>, was excluded since it was also published separately in English<sup>13</sup>. As a result, a total of twenty-seven original articles were available for this review (Figure 1). Identified studies are summarized in Table 1 and their quality assessed (STROBE scoring system<sup>9</sup>, supplemental Table).

### Anti-hypertensive drugs

Beta-blockers and other anti-hypertensive drugs were the first agents to be evaluated for their potential to reduce AAA expansion rate. Beta-blockers are evaluated in two randomized controlled trials (RCTs)<sup>14,15</sup>, three case-control studies<sup>16-18</sup> and two cohort studies<sup>19,21</sup>. The two earliest, very small studies (n=38 and n=12 cases) suggest a borderline-significant effect of beta-blockers on the aneurysm expansion rate<sup>17,18</sup>. Later, cohort studies, however, find no effect of beta-blockers on the growth rate of AAAs<sup>16,18,21</sup>. Similar to this, the two RCTs do not show an effect of propranolol treatment on the AAA expansion<sup>14,15</sup>.



**Figure 1.** Systematic search strategy. First selection on title, second on abstract and last selection was made by reading the full article.

There are several reports on other classes of hypertensives. A retrospective study suggesting an effect of ACE inhibition on AAA stability<sup>20</sup> was followed by five studies investigating an effect of ACE inhibitors on AAA growth. Four of these studies, two small retrospective analysis within a prospective case-control study<sup>16;21</sup> and two larger retrospective studies (n=1231 and n=242 cases) find no effect of ACE inhibitors on the aneurysm expansion<sup>21;22</sup>. In contrast, a recent prospective cohort study of 1701 patients participating in the UK small aneurysm trial, unexpectedly indicated a significant increase in aneurysm growth rate in patients taking ACE inhibitors, implying that ACE inhibitors may adversely affect AAA growth<sup>23</sup>. Other anti-hypertensive drugs (i.e. diuretics and calcium channel blockers) are evaluated in two retrospective analyses by respectively Wilimink et al. and Bhak et al. Both studies did not find an association between these antihypertensive agents and AAA growth rate<sup>16,19</sup>.

### Statins

A potential effect of statins on AAA progression is evaluated in twelve studies. Six studies report a beneficial effect of statin use on the AAA growth<sup>24-29</sup>. In contrast, six other reports fail to show an effect of statins on AAA growth<sup>22;23;30-32</sup>. Eight out of the twelve studies had a prospective design, but none of them were randomized clinical trials<sup>19;22;24;26;27;29-31</sup>. Most studies do not specify the type of statin that is investigated. Simvastatin and Atorvastatin are the dominant statins in the four studies that specify the statin type<sup>28-31</sup>.

There is an apparent paradox in conclusions for an effect of statins with the earlier small studies reporting an association between the statin use and reduced AAA expansion<sup>27-29</sup>, but the more recent studies failing to confirm a relationship<sup>24;25;30</sup>. Moreover, none of the larger studies (viz. including more than 250 patients) found a difference in AAA expansion rate between statin users and non-statin users<sup>22;23;31</sup>.

### Macrolides

A presumed role for chlamydia in AAA growth led to studies testing an effect of macrolide treatment on the growth rate of small AAAs. Two RCTs evaluate the effect of roxithromycin on the expansion rate. The first, conducted in 2001, reports a significant lower expansion rate in the roxithromycin treated patients ( $p=0.02$ )<sup>13</sup>. The second study, also a small RCT, reports a borderline effect of a four-week treatment with roxithromycin on AAA progression ( $p=0.055$ )<sup>33</sup>. The effect of azithromycin, another member of the macrolide class, is investigated in a larger RCT conducted by Karlsson et al in 2009<sup>34</sup> (n= 247). This study does not observe a significant difference between the AAA expansion rate in the azithromycin-treated patients and controls.

### Tetracyclines

In 2001, a small RCT showed a pronounced effect of 3-months doxycycline treatment on AAA expansion for the 6 – 12 and 12-18 months follow-up periods<sup>35</sup>. Next, a phase II study by Baxter et al. open safety and feasibility study revealed significant a significant reduction in MMP9 levels after 6 months of doxycycline treatment. Nevertheless, no significant change was seen for the overall AAA expansion rate<sup>36</sup>. Results from an adequately powered multicenter RCT fail to show a beneficial

**Table 1.** Full survey of all articles included in this systematic review.

	Year	Intervention	Study Design	Participants (Cases/Controls)	Outcomes
PATI (13)	2002	Propranolol	RCT	Total: 548  (276/272)	AAA diameter growth (mm/y): Cases: 2.02  Controls: 2.60
Lindholt (14)	1999	Propranolol	RCT	Total: 54  (30/24)	Relative Risk of expansion:  Cases: 2.44 (0.88-6.77) Controls: 1.17 (0.74-1.85)
Wilmink (15)	2002	Anti-hypertensive drugs:  Calcium channel blockers  ACE inhibitors  Diuretics  Beta blockers	Prospective case- control study	Total: 5811  (48/284)  (24/308)  (54/278)  (77/255)	AAA diameter growth (mm/y):  Cases: 0.5 Controls: 0.8 Cases: 0.02 Controls: 0.8 Cases: 0.8 Controls: 0.7 Cases: 0.8 Controls: 0.7
Gadowski (16)	1994	Beta-blockers	Prospective case- control study	Total: 111  (38/83)	AAA diameter growth (mm/y) Cases: 3.0  Controls: 4.4
Leach (17)	1988	Beta-blockers	Retrospective case-control study	Total: 27  (12/15)	AAA diameter growth (mm/y) Cases: 1.7 Controls: 4.4
Bhak (18)	2015	Beta-blockers Cholesterol lowering Anti-hypertensive Aspirin	Prospective cohort study	Total: 534  unclear unclear unclear unclear	Adjusted difference in AAA diameter growth (mm/y) 0.009 -0.02 -0.001 -0.01
Kortekaas (20)	2014	ACE inhibitors	Prospective case- control study	Total: 286	Difference in growth rate: -0.24 mm/ year

Significance	Strobe score	Study qualities	Study limitations
NS		1. Study medication was randomly and double blinded assigned 2. Valid power calculation	1. Slow growing AAAs and patients already using beta blockers excluded 2. Low compliance, high drop-out rate: 26.8% and 42.4% of the patients in the placebo arm and the propranolol arm stopped their medication 3. Mislabeling of a batch of study medication 4. No correction for non random drop out
NS		1. Study medication was randomly and double blinded assigned	1. High drop out rate: 60% and 25% of the patients in the propranolol and placebo arm stopped their medication 2. Power calculation missing
	13.5/22	1. Large study size	1. Observational study, data derived from two separate screening populations with different baseline characteristics 2. Limited number of cases 3. Power calculation missing 4. No correction for non random drop out
NS			
NS			
NS			
NS			
NS	11.5/22	1. Longterm follow up	1. Observational study 2. Heterogeneous with respect to type and dose of beta blocker
NS	11.5/22		1. Observational study 2. Retrospective study 3. Limited number of cases
p=0.004	14.5/22	1. Large number of overall participants	1. Number of patients per group unclear 2. Observational study 3. Both CT and ultrasound measurements
0.51 0.18 0.78 0.48 p>0.05	16.5/22	1. One-observer measurements only	1. Observational study

Table 1. (continued).

Year	Intervention	Study Design	Participants (Cases/Controls)	Outcomes
			(82/286)	
Thompson (21)		Prospective cohort study	Total: 1231	Difference in AAA diameter growth between cases and controls (mm/y): ACE-inhibitors: -0.28
	ACE-inhibitors		294	
	Statins		383	Statins: -0.29
Sweeting (22)		Prospective cohort study	Total: 1701	AAA diameter growth (mm/y) Cases: 3.33 Controls: 2.77
	ACE-inhibitors		169	
	Calcium Channel Blockers		440	Cases: 2.76 Controls: 2.5
	Beta Blockers		255	Cases: 2.70 Controls: 2.85
	Statins		21	Cases: 2.07 Controls: 2.84
	Anti Platelet Therapy		501	Cases: 2.89 Controls: 2.80
Periard (23)	Statins	Retrospective case-control study	Total: 94 (50/44)	AAA diameter growth (mm/y) Cases: 2.93 Controls: 4.39
Karrowni (24)	Statins	Retrospective case-control study	Total: 211  (136/75)	AAA diameter growth (mm/y)  Cases: 0.9 Controls: 3.2
Karrlson (25)	Statins	Retrospective case-control study	Total: 213 (85/128)	AAA diameter growth (mm/y): Cases: 1.6

Significance	Strobe score	Study qualities	Study limitations
		1. Large study size	<ul style="list-style-type: none"> <li>2. Significant difference in baseline characteristics</li> <li>3. Power calculation missing</li> <li>1. Patients lost to follow (n=158) up had a significantly lower AAA growth rate</li> </ul>
NS			2. Time effect, patients identified between 1984 and 2007
NS		1. Large study size	<ul style="list-style-type: none"> <li>3. No correction for non random drop out</li> <li>4. Secondary analysis, study not powered for an evaluation of a ACE inhibitor or statin effect</li> <li>1. Observational study</li> </ul>
p=0.009		<ul style="list-style-type: none"> <li>2. Longterm follow-up</li> <li>3. Data partially-adjusted and fully-adjusted available</li> </ul>	2. Patients included between 1991-1995
NS			
NS			
NS			
NS			
p=0.01	17.5/22		<ul style="list-style-type: none"> <li>1. Observational study</li> <li>2. Retrospective study</li> <li>3. Uncommon definition of AAA (&gt;25 mm)</li> <li>4. Limited number of size measures</li> <li>5. A higher number of CT estimates (over estimates AAA size) in the non statin group</li> </ul>
p<0.001	15.5/22	1. AAA patients who at follow-up were found to have a change in statin therapy were excluded	<ul style="list-style-type: none"> <li>1. Observational study</li> <li>2. Retrospective study</li> <li>3. Mixed imaging modalities and absent definition of max. diameter</li> <li>4. Only 10% of the patients was imaged at 3 or more occasions</li> </ul>
p=0.008	9.5/22	1. Consistent aortic diameter measurements via ultrasound	<ul style="list-style-type: none"> <li>1. Observational study</li> <li>2. Retrospective study</li> </ul>

Table 1. (continued).

	Year	Intervention	Study Design	Participants (Cases/Controls)	Outcomes
					Controls: 2.5
Schlosser (26)	2008	Statins	Prospective case-control study	Total: 147  (63/84)	Adjusted estimated difference in growth rate for statin use: -1.2 mm/year
Schouten (27)	2006	Statins	Retrospective case-control study	Total: 150  (59/91)	Adjusted estimated difference in growth rate for statin use: -1.6 mm/year
Sukhija (28)	2006	Statins	Prospective case-control study	Total: 130  (75/55)	AAA size changes from baseline(mm) until endpoint: Cases: 4.6 to 4.5 Controls: 4.5 to 5.3
Meij, van der (29)	2013	Statins	Retrospective case-control study	Total: 142  (103/39)	No growth data available
Ferguson (30)	2010	Statins	Prospective cohort study	Total:652  (349/303)	Statins: OR 1.23 (95% CI 0.86-1.76)
		Aspirin		(363/289)	Aspirin: OR 1.10 (95% CI 0.78-1.56)
		Beta-blockers		(182/470)	Beta-blockers: OR 1.13 (95% CI 0.76-1.67)
		ACE inhibitors		(242/410)	ACE inhibitors: OR 0.91 (95% CI 0.64-1.31)

Significance	Strobe score	Study qualities	Study limitations
p=0.021	16.5/22	1. AAA expansion rates were adjusted for age, initial AAA diameter, and hyperlipidemia in the multivariate linear regression model	<ul style="list-style-type: none"> <li>3. Sub-analysis of a studying evaluating an effect of azithromycin</li> <li>4. Details regarding statin therapy missing</li> <li>1. Observational study</li> </ul>
p=0.006	16.5/22	<ul style="list-style-type: none"> <li>1. Patients with an inflammatory (n=12) and mycotic (n=1) AAA were excluded</li> <li>2. Different types of statins were recorded</li> </ul>	<ul style="list-style-type: none"> <li>2. Retrospective study</li> <li>3. Time effect, inclusion window 1996-2007</li> <li>1. Observational study</li> <li>2. Retrospective study</li> <li>3. Statin users also used more warfarin derivates and angiotensin II antagonists</li> <li>4. Amongst cases a wide range of different statin types was used</li> <li>5. Statins were not randomly assigned</li> <li>6. Power calculation missing</li> </ul>
p<0.001		1. Measurements were consistently made with CT-scan	<ul style="list-style-type: none"> <li>1. Observational study</li> <li>2. Power calculation missing</li> </ul>
NS		1. One-observer measurements only	<ul style="list-style-type: none"> <li>1. Significant differences in baseline characteristics and cardiovascular risk management between cases and controls</li> <li>2. Growth data missing</li> <li>3. Non-randomized</li> <li>4. Power calculation missing</li> </ul>
		1. Sample size calculations were made	1. Observational study
NS		2. Different types of statins were recorded	2. Growth data missing
NS			3. Significant differences in baseline characteristics
NS			
NS			

Table 1. (continued).

	Year	Intervention	Study Design	Participants (Cases/Controls)	Outcomes
Morosin (31)	2008	Statins	Retrospective case-control study	Total: 121 (34/87)	AAA diameter growth (mm/y) Cases: 1.9 Controls: 2.6
Vammen (11)	2001	Roxithromycin	RCT	Total: 58 (27/31)	AAA diameter growth (mm/y) Cases: 1.56 Controls: 2.75
Hogh (32)	2009	Roxithromycin	RCT	Total: 84 (42/42)	AAA diameter growth (mm/y) Cases: 1.61 Controls: 2.52
Karrlson (33)	2009	Azithromycin	RCT	Total: 213 (106/105)	AAA diameter growth (mm/y) Cases: 2.2 Controls: 2.2
		Aspirin	Retrospective case-control	(101 /100)	Controls: 2.2 Cases: 1.8
Morosin (34)	2001	Doxycycline	RCT	Total: 32 (17/15)	Controls: 2.6 AAA diameter growth (mm/y) Cases: 1.5 Controls: 3.0
Baxter (35)	2002	Doxycycline	Prospective cohort study	Total: 36	AAA diameter (mm) At baseline: 41.0 mm ± 0.9 mm At 6 months: 42 .7 mm ± 1.3 mm
Meijer (36)	2014	Doxycycline	RCT	Total: 286 (144/142)	AAA diameter growth (in 18 months) Cases: 4.1 mm [95% CI, 3.6 to 4.5 mm] Controls: 3.3 mm [CI, 2.8 to 3.7 mm]
Aorta Trial (37)	2014	Mast Cell Inhibitor (CD007)	RCT	Total: 326 10mg (80/84)	AAA diameter growth (mm/y) Cases (10mg): 2.58

Significance	Strobe score	Study qualities	Study limitations
NS			<ol style="list-style-type: none"> <li>1. No randomization</li> <li>2. Power calculation missing</li> </ol>
p=0.02		<ol style="list-style-type: none"> <li>1. Roxithromycin was randomly assigned</li> <li>2. Well defined exclusion criteria</li> </ol>	<ol style="list-style-type: none"> <li>1. Power calculation missing</li> </ol>
NS		<ol style="list-style-type: none"> <li>1. Roxithromycin was randomly assigned</li> <li>2. Single-observer measurements only</li> </ol>	<ol style="list-style-type: none"> <li>1. Power calculation missing</li> <li>2. Possible selection bias as only one third of the eligible AAAs was included</li> </ol>
NS		<ol style="list-style-type: none"> <li>1. Azithromycin was randomly assigned</li> <li>2. In addition to ultrasound, for each patient a volume calculation by CT scan was made</li> </ol>	<ol style="list-style-type: none"> <li>1. Power calculation missing</li> <li>2. Differences in baseline characteristics</li> </ol>
p=0.004			<ol style="list-style-type: none"> <li>1. Observational study</li> <li>2. Retrospective study</li> </ol>
NS		<ol style="list-style-type: none"> <li>1. One-observer measurements only</li> <li>2. Doxycycline was randomly assigned</li> </ol>	<ol style="list-style-type: none"> <li>1. Power calculation missing</li> <li>2. 3-month intervention</li> <li>3. Major differences in baseline AAA size between the groups</li> </ol>
NS			<ol style="list-style-type: none"> <li>1. Missing control group</li> <li>2. Treatment period 6 months</li> <li>3. Power calculation missing</li> </ol>
p=0.016		<ol style="list-style-type: none"> <li>1. One-observer measurements only</li> <li>2. Doxycycline or placebo were randomly assigned</li> <li>3. Long-term treatment with doxycycline</li> <li>4. Valid power calculation</li> </ol>	<ol style="list-style-type: none"> <li>1. High number of elective repairs</li> <li>2. Doxycycline dose of 100 mg was possibly too low or too high</li> <li>3. Drop-outs where not followed</li> </ol>
NS		<ol style="list-style-type: none"> <li>1. Mast Cell Inhibitor was randomly assigned</li> <li>2. AAA diameter was measured via 2D Ultrasound</li> </ol>	<ol style="list-style-type: none"> <li>1. No proof for an effect on the aneurysm wall</li> <li>2. Power calculation missing</li> </ol>

Table 1. (continued).

	Year	Intervention	Study Design	Participants (Cases/Controls)	Outcomes
				25mg (78/84)	Cases (25mg): 2.33
				40mg (84/84)	Cases (40mg): 2.70 Controls: 2.04
Lindholt (37)	2008	Aspirin	Prospective case-control study	Total: 148	AAA diameter growth (mm/y) AAA baseline <40 mm:  Cases: 2.52  Controls: 2.23 AAA baseline >40-50 mm:  Cases: 2.92 Controls: 5.18
		Aspirin			AAA diameter growth (mm/y) Cases: 1.8 Controls: 3.2
Franklin (39)	1999	NSAIDs	Unclear case-control study	Total: 78  (19/59)	AAA diameter growth (mm/y) Cases: 1.8 Controls: 3.2

NS = not significant

effect of 18 months doxycycline therapy on AAA progression. On the contrary, an acceleration in AAA growth rate is reported during 18 months follow up period<sup>37</sup>.

### Anti-Mast Cell therapy

Sillesen et al. investigated whether the mast cell inhibitor CRD007 (pemirolast) could halt growth of small AAA. However, no difference in AAA growth rate was found between placebo and the mast cell inhibitor treated patients<sup>38</sup>.

### Anti-platelet therapy

Five studies investigated the potential of anti-platelet therapy in stabilizing human AAA growth<sup>19;23;31;34;39</sup>. A first case-control study including 167 patients reports a decrease in AAA progression in those patients with a diameter between 40 and 49 mm. Patients with an AAA diameter smaller than 4.0 cm had similar expansion rate with or without using aspirin<sup>39</sup>. Significant reduced AAA progression in patients using aspirin was reported in a sub-analysis of a case-control data of a small RCT investigating the effect of azithromycin. Average growth rate of the 101 patients using aspirin was 1.8 mm/year compared to 2.6 mm/year in those not on antiplatelet therapy ( $p < 0.01$ )<sup>34</sup>. In contrast analyses performed on patients participating in the UK small aneurysm trial<sup>23</sup>, the ADAM study<sup>19</sup> and a cohort study incorporating 363 patients<sup>31</sup> failed to identify an effect of platelet therapy on aneurysm progression.

Significance	Strobe score	Study qualities	Study limitations
		3. Long-term treatment with the mast cell inhibitor	
AAA baseline <40 mm: NS			1. Overall growth data not available 2. Observational study 3. Contradictory conclusions for small and intermediate AAA 4. Power calculation missing 5. Self-reported aspirine use
AAA baseline >40-50 mm: p=0.017			
p=0.004		1. Matched cases and controls	1. Conference abstract only 2. Unclear study design

One small study (n=19) investigated the effect of non-steroidal anti-inflammatory drugs (NSAIDs) on AAA growth<sup>40</sup>. The median growth rate of the AAA diameter of 1.8 mm/year compares favorably to the 3.2 mm/year in patients not taking NSAIDs,  $p < 0.01$ .

## DISCUSSION

This systematic review shows that the number of studies evaluating a potential effect of pharmaceutical strategies to quench AAA growth in humans is limited. The majority of identified studies is of moderate quality, and initial promising reports are not confirmed by later larger studies. At this point no pharmaceutical therapy can be recommended for the stabilization of AAA.

The search strategies identified original 27 papers that evaluate the potential of pharmaceutical intervention for AAA stabilization. Identified interventions can be subdivided into strategies that are part of general cardiovascular risk management (anti-hypertensive agents, statins, anti-platelet therapy), and into “anti-inflammatory” strategies: macrolides, tetracyclins, and mast cell inhibition.

The majority of studies was of moderate quality as illustrated by a low to moderate score in the STROBE scoring system<sup>9</sup>. Most studies have a retrospective design, and have a small sample size<sup>41</sup>. Interpretation is hampered by poor matching, lack of standardized diameter measurements, and inappropriate statistical analyses. Studies on longitudinal data such as aneurysm progression are prone to non-random drop-out<sup>42</sup>. For example older patients are more likely to drop-out because of death, but are less likely to undergo repair due to different risk estimates. On the same

**Table 2.** Overview of ongoing clinical trials.

Name	Intervention	Estimated completion date	Clinical trial number
PISA	Anti-hypertensives	December 2013	NCT01425242
AARDVARK	ACE inhibitors	October 2014	NCT01118520
ACZ885	Canakinumab (anti IL1-beta)	December 2015	NCT02007252
TicAAA	Ticagrelor	December 2015	NCT02070653
TEDY	Telmisartan	August 2016	NCT01683084
BASE	ACE vs beta blockers	October 2016	NCT01904981
N-TA <sup>3</sup> CT	Doxycycline	June 2017	NCT01756833
ACA4	Cyclosporin A	September 2018	NCT02225756

token patients with larger aneurysms, or fast growing AAA are more likely to drop out prematurely because of repair. As such follow up studies in AAA patients require specific statistical approaches,<sup>43</sup> a prerequisite that is not met in most studies. Moreover, it was observed that initial promising studies from small cohorts were not confirmed by later larger studies, an observation hinting at the phenomenon of selective reporting<sup>44</sup>.

Most data is available for cardiovascular risk management (beta-blockers, ACE-inhibitors and statins). Trials with the beta-blocker propranolol experienced a high dropout rates because of poor tolerability<sup>14,15</sup>. Statins and ACE inhibitors are well tolerated, yet a recent meta-analysis on the available data concludes that these drug classes do not influence AAA progression<sup>45</sup>.

The second group of tested interventions was anti-inflammatory, with anti-inflammatory referring to an anti-microbial action, in the case of AAA because of a suspected causative role for Chlamydia infection in the disease, or alternatively anti-inflammatory in the context of chronic tissue inflammation that is thought to drive AAA progression (doxycycline, mast cell inhibition)<sup>46</sup>. Although aspirin has anti-inflammatory properties, it is unclear whether the dose used for anti-platelet therapy is sufficient to exert an anti-inflammatory effect on the aneurysm wall. Again, there was no evidence for a beneficial effect of anti-inflammatory strategies on AAA progression. On the contrary, evidence was found for growth acceleration in patients taking doxycycline<sup>37</sup>.

The above conclusions sharply contrast with the available preclinical evidence that shows that pharmaceutical interference with aspects of RAS system, cholesterol metabolism, vascular inflammation or protease activity alleviates aneurysm formation in rodent models of the disease<sup>4,5</sup>; an observation pointing to an impaired translatability of the available preclinical models<sup>46</sup>.

In conclusion, interpretation of the available data is hampered by the moderate quality of the available data. A role for beta blockers, doxycycline and the mast cell inhibitor pemirolast is ruled out in RCTs. Available observational data for ACE-inhibitors and statins is not consistent with a beneficial effect on aneurysm progression. A number of interventions are currently evaluated in clinical trials (Table 2). At this moment no therapy can be recommended although it cannot be excluded that AAA growth and rupture are disparate processes. Consequently although some interventions do not influence AAA progression, they may influence AAA rupture rate<sup>47</sup>, a notion that requires independent confirmation. Moreover, although cardiovascular risk management does not influence AAA progression, it is important to point out that risk management is indicated in AAA patients as this group is at an extremely high cardiovascular risk<sup>2</sup>.

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# SUPPLEMENTAL DATA

**Supplemental table 1.** Full STROBE score of all articles selected in this review.

STROBE score Full article	PATI (14)	Lindholt (15)	Wilmink (16)	Gadowski (17)	Leach (18)	Bhak (19)	Kortekaas (21)	Thompson (22)	Sweeting (23)	Periard (24)	Karrowni (25)	Karrlson (26)	Schlosser (27)
Titel & Abstract (study design and balanced summary)	1	1	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Background & Rationale	1	1	1	1	1	1	1	1	1	1	1	1	1
Objectives & Hypothesis	0.5	0.5	1	1	1	1	1	1	1	1	1	x	1
Study Design (early in paper)	x	1	1	0.5	0.5	1	1	1	1	1	1	0.5	1
Setting (locations, dates, periods recruitment, exposure, follow-up & data collection)	1	0.5	0.5	0.5	0.5	1	1	1	1	1	1	0.5	1
Participants (criteria, selection methods, follow-up)	1	0.5	x	1	1	0.5	1	1	1	0.5	1	1	0.5
Variables (outcomes, exposure, confounders, predictors)	0.5	0.5	0.5	x	1	x	1	0.5	x	1	0.5	x	1
Measurements (source of data, methods)	1	1	1	1	1	1	1	0.5	1	1	1	1	1
Bias (incl efforts to avoid)	0.5	0.5	0.5	1	1	0.5	1	x	x	1	0.5	x	1
Study size (explain how arrived)	1	x	x	x	1	x	x	x	x	x	x	x	0.5
Quantitative variables	1	1	1	1	x	1	x	1	1	x	1	1	1
Statistics (incl missing data)	0.5	0.5	1	1	x	0.5	1	0.5	1	1	1	1	0.5
Participants (report numbers in each stage, flow diagram)	1	1	0.5	0.5	0.5	x	1	1	x	1	x	x	0.5
Descriptive data (characteristics of study participants)	1	1	x	0.5	x	0.5	1	1	1	1	1	1	1
Outcome data (numbers in each exposure category)	1	1	0.5	1	0.5	x	x	1	0.5	1	0.5	0.5	1
Main results (unadjusted estimates and confounder adjusted)	1	1	x	x	x	1	0.5	1	1	1	1	x	1
Other analysis	1	x	x	x	x	1	x	x	x	1	1	x	x
Key results (summary with reference to objectives)	1	0.5	0.5	0.5	x	1	1	1	1	1	1	x	0.5
Limitations (incl direction and magnitude of potential bias)	1	0.5	1	x	0.5	1	1	0.5	0.5	1	1	x	1
Interpretation (overall considering objectives, limitations & results similar studies)	0.5	1	1	0.5	0.5	0.5	1	x	0.5	1	0.5	0.5	0.5
Generalisability (external validity)	x	0.5	1	0.5	1	0.5	0.5	0.5	x	0.5	x	x	x
Funding (give source)	x	1	1	x	x	1	1	x	x	x	x	1	1
Total	16.5	15.5	13.5	11.5	11.5	14.5	16.5	14.0	13.0	17.5	9.5	16.5	16.5

Schouten (28)	Sukhija (29)	Meij, van der (30)	Ferguson (31)	Morosin (32)	Vammen (13)	Hogh (33)	Karrlson (34)	Morosin (35)	Baxter (36)	Meijer (37)	Sillesen (38)	Lindholt (39)	Franklin (40)	STROBE score Abstract
0.5	0.5	0.5	0.5	0.5	1	0.5	1	1	1	1	0.5	0.5	0.5	Titel (study design)
1	1	1	1	1	1	1	1	1	1	1	1	1	x	Authors (contact details)
1	x	1	1	1	0.5	0.5	1	1	1	x	0.5	1	1	Study Design
1	0.5	1	1	1	x	0.5	1	x	1	1	1	1	x	Objective & Hypothesis
1	0.5	0.5	1	1	1	1	1	1	0.5	1	1	1	x	Setting (methods incl follow-up dates)
1	0.5	1	0.5	1	1	0.5	1	x	0.5	1	1	1	x	Participants (eligibility criteria & sources)
1	x	x	x	x	x	x	x	0.5	x	1	1	x	1	Variables (primary outcome)
0.5	0.5	1	1	0.5	1	0.5	1	1	0.5	1	1	1	x	Statistical Methods (incl confounding control)
1	1	x	x	x	x	x	x	0.5	x	0.5	0.5	x	0.5	Participants (begin & end)
x	x	x	1	x	0.5	1	0.5	x	x	1	1	1	0.5	Main results (incl measures of variability and uncertainty)
1	x	1	1	1	1	x	1	1	x	1	1	1	1	Conclusions
1	1	1	0.5	0.5	0.5	0.5	0.5	0.5	1	1	1	0.5		
x	x	x	x	1	1	1	1	1	1	1	x	1		
1	1	1	1	1	1	1	1	1	1	1	1	1		
1	0.5	1	0.5	1	1	1	1	1	1	1	1	1		
1	x	1	x	1	1	0.5	0.5	0.5	0.5	1	1	1		
x	x	x	x	1	x	x	1	x	x	1	x	x		
1	0.5	x	1	x	1	1	1	0.5	1	1	1	1		
1	x	1	1	1	1	1	1	x	1	1	0.5	1		
0.5	x	1	0.5	1	1	0.5	1	0.5	1	1	0.5	0.5		
x	0.5	0.5	0.5	x	x	x	0.5	x	0.5	0.5	x	x		
1	x	1	x	x	1	1	x	1	x	1	x	x		
16.5	8.0	14.5	13.0	13.0	15.5	13.0	17.0	14.5	13.5	20	15.5	15.5	4.5	



# Chapter

# 3

## AN ASSOCIATION BETWEEN CHRONIC OBSTRUCTIVE PULMONARY DISEASE AND ABDOMINAL AORTIC ANEURYSM BEYOND SMOKING RESULTS FROM A CASE-CONTROL STUDY

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# ABSTRACT

## Objectives

It is currently unclear whether the parallels between Abdominal Aortic Aneurysms (AAA) and chronic obstructive pulmonary disease (COPD), are explained by common risk factors alone, such as cigarette smoking, or by a predetermined cause. Given the persistent controversy with regard to the association between AAA and COPD we studied this association in depth.

## Methods

We conducted a case-control study comparing patients with a small abdominal aortic aneurysm (maximum infrarenal diameter 35-50 mm, n=221) with controls diagnosed with peripheral artery disease (PAD, n=87). The controls were matched to the cases for life-time cigarette smoking. Pulmonary function was measured by spirometry, and all subjects completed a questionnaire on medical history and smoking habits (current, former, and never smokers).

## Results

Aneurysm patients were similar to controls with respect to gender ( $p=0.71$ ), life-time cigarette smoking (39 vs 34 pack years,  $p=0.23$ ) and history of cardiovascular disease (45% vs. 55%,  $p=0.12$ ). Aneurysm patients had more airway obstruction (Forced Expiratory Volume in 1 second/Forced Vital Capacity (FEV1/FVC) ( $0.69 \pm 0.12$  vs.  $0.78 \pm 0.11$ ,  $p < 0.001$ )) which was most pronounced in never smokers ( $0.73 \pm 0.07$  vs  $0.86 \pm 0.07$ ,  $p < 0.001$ ). COPD was more prevalent in aneurysm patients (44%; 98/221) than in controls (20%; 17/87) (adj. OR 3.0; 95%CI 1.6 – 5.5,  $p < 0.001$ ). In particular, a major proportion of AAA patients was newly diagnosed with COPD; only 40 of 98 patients (41%) with COPD (mild, moderate or severe/very severe) were known before with obstructive pulmonary defects and received treatment.

## Conclusions

This study confirms an association between AAA and COPD and shows that this association is independent from smoking. Findings also demonstrate that COPD is under-diagnosed in AAA patients.

## WHAT THIS PAPER ADDS

The findings of this study suggest that the increased prevalence of chronic obstructive pulmonary disease (COPD) in patients with an abdominal aortic aneurysm (AAA) is independent from smoking. Along with other observed parallels between AAA and COPD, results of this study hint at a predetermined cause of these diseases, warranting further investigation of common genetic, inflammatory and remodeling pathways. Identification of common mechanistic pathways might be highly relevant for future AAA and COPD research and research collaboration initiatives. The burden of undetected COPD is relevant to those involved in the care of AAA patients.

## INTRODUCTION

Several parallels are observed between abdominal aortic aneurysms (AAA) and chronic obstructive pulmonary disease (COPD), both with regard to risk factors as well as to the underlying pathophysiology. Both diseases have a particularly strong relationship with smoking, and smoking cessation appears to be the principle disease-modifying intervention<sup>1,2,3</sup>. Moreover, their pathophysiologic bases are best described as a persistent pro-inflammatory response that is associated with proteolysis and excess matrix turn-over<sup>2,3,4,5</sup>.

A relation between AAA and COPD was first suggested by Cronenwett et al., who reported in 1985 that COPD was more common in patients with a ruptured aneurysm<sup>6</sup>. Several successive reports also indicated an association between AAA and COPD, although the by far largest study in aneurysm patients rejected an independent association (a summary of the reports is found in Table 1)<sup>7,8,9,10,11,12,13</sup>. Moreover, interpretation of these reports is complicated by considerable heterogeneity in outcome measures, definitions and control populations in these studies. In particular, a relation between AAA and COPD that is beyond that of smoking alone remains unclear.

We performed pulmonary testing in a cohort of patients with a small abdominal aneurysm and compared the results with a control group of patients with clinical peripheral artery disease (PAD). As there is a large overlap in risk factors, in particular the predominance of smoking, and patient characteristics between AAA and PAD populations, it was reasoned that this group constituted the most optimal control group<sup>3,14</sup>. With this study, we aim to test whether the suggested relationship between AAA and COPD extends beyond smoking alone.

## METHODS AND MATERIAL

### Design

The study enrolled patients with a small infrarenal AAA, who participated in a larger study, the Pharmaceutical Aneurysm Stabilization Trial (PHAST-trial <http://www.trialregister.nl/trialreg/admin/rctview.asp?TC=1345>).

This multicenter trial studied whether 18 months doxycycline treatment reduced aneurysm progression in 286 randomized Caucasian patients with small AAA (35-50 mm). The trial was conducted between January 2009 and June 2011 in 14 Dutch hospitals (manuscript submitted). Primary approval for the PHAST-trial and this sub-study was obtained from the Medical Ethical Review Board of the Leiden University Medical Center.

Presence of AAA and size of maximum aortic diameter was assessed by single observer ultrasound measurement of the anteroposterior diameter (Siemens P50, 1.67-4.0 MHz phased array transducer). Included were all patients with a confirmed AAA sized 35-50 mm. All measurements were performed at baseline, i.e. immediately before randomization.

The control population consisted of patients with established PAD referred to the outpatient department of vascular surgery at the Leiden University Medical Center and the Deventer Hospital. All patients underwent abdominal ultrasound to exclude an aortic aneurysm (i.e. infrarenal aortic diameter <25 mm). PAD was defined by a history of manifest intermittent claudication with an ankle-brachial index (ABI) <0.9, or by a history of lower extremity revascularization. Patients with a known history of lung carcinoma, lung surgery or aneurysmal diseases (i.e. an aneurysm on other locations;

**Table 1.** Review of previous reports on the relation between abdominal aortic aneurysm and chronic obstructive pulmonary disease. Cronenwett et al were the first to report on this relation, although they were rather focused on aneurysm rupture. Overtime, these studies show an increase in quality with respect to the used definitions, measures and analyses. However, considerable heterogeneity still exists, complicating translation to the general AAA population.

Study	Number AAA	Population	Method	Definition	Conclusion	Limitation
Cronenwett 1985	67	AAA patients selected for non-surgical management	spirometry	mild to moderate FEV1 > 50% pred., severe FEV1 < 50% pred.	Obstructive pulmonary disease predictive for aneurysm rupture	relation with aneurysm rupture, no conclusions on non-ruptured aneurysm
Bengtsson 1991	39	AAA screening and general population controls	spirometry	unknown	Smokers with affected lung function are at risk for AAA	unclear definitions of pulmonary function, general population as control
Smith 1993	219	AAA screening population, general population controls	unknown	unknown	a relation between AAA and COPD	unclear definition of pulmonary function, general population as control, no adjustment for smoking
Laarhoven 1993	36	COPD-patients	COPD by hospital record	mild or severe emphysema, FEV1/VC < 55%	high prevalence of AAA in COPD patients	no control population, no statistical analysis
Lindholt 1998	139	AAA screening population, AAA in COPD vs AAA in non-COPD	COPD by hospital record	As defined by World Health Organization	association caused by medication and cardiovascular history	COPD underestimation without spirometry testing. No adjustment for smoking
Lederle 2000	1917	AAA screening population	COPD as a questionnaire item	unknown	no association between AAA and COPD after adjustment for smoking	questionnaires insufficient to detect COPD
Sakamaki 2002	118	Japanese AA patients, with present AAA or TAA, without AA with CAD, without both	spirometry with reversibility testing	airway obstructive disease FEV1/FVC < 0.70	AAA risk factor for COPD	patients at high surgical risk, exclusion of currently smoking patients and patients treated for COPD
Fowkes 2006	89	AAA surgical waiting-list and general population controls	spirometry max of three measurements,	Modification GOLD classification	association, independent of cigaret smoking and cardiovascular history	significant differences between cases and controls on co-variables smoking and cardiovascular disease

e.g. the popliteal artery) were excluded. Because all patients in the AAA group were between the ages of 50 and 89 years, only controls older than 50 years were included.

Prior to measurement of respiratory functions, the controls were matched to the cases for life-time cigarette smoking (group matching). Matching was performed for four categories of smoked pack years (PY) (non-smokers (0 PY), 1-20 PY, 21-40 PY and more than 40 PY) based on the frequency distribution of the PHAST cohort.

### Measurements

Eligibility was based on the patients' medical records and all patients were interviewed about smoking habits, presence of a diagnosis of COPD and history of cardiovascular disease. Smoking status was defined by the three categories of current, former, and never smokers. Life-time cigarette consumption was analyzed by pack years (number of years smoked \* average number of daily smoked cigarettes / 20). Referral to or treatment by a physician for COPD was regarded as a previous diagnosis of COPD. Questions on cardiovascular history included myocardial infarction, cerebrovascular accident or transient ischemic attack, peripheral artery disease, medication for hypertension or diabetes mellitus. Body mass index (BMI) was calculated as  $\text{kg}/\text{m}^2$ .

Pulmonary function was measured by spirometry in all participants with standardized, portable equipment (MicroLab 3500, MicroMedical Ltd, California, US). Measures of respiratory functions consisted of forced expiratory volume in one second (FEV1), forced vital capacity (FVC), and FEV1/FVC ratio. Published prediction equations were used to calculate the percentage of predicted FEV1 for each participant (pFEV1%). The maximum of three repeated respiratory tests was used in the analysis. A modification of the Global Initiatives for Chronic Obstructive Lung Disease (GOLD) definitional criteria was used to classify subjects into the mutually exclusive categories of severe/very severe COPD (FEV1/FVC < 0.7 and FEV1 < 50% predicted), moderate COPD (FEV1/FVC < 0.7 and FEV1 50% to < 80% predicted), and mild COPD (FEV1/FVC < 0.7 and FEV1  $\geq$  80% predicted)<sup>15</sup>.

### Statistical analysis

Baseline data on respiratory functioning of 221 patients of the PHAST trial were available. Calculation of the sample size was based on the assumption that a 15% difference in mean FEV1 is clinically relevant<sup>12</sup>. To detect a difference with a level of 0.05 and 90% power, 90 control patients were required.

The analysis was carried out by using SPSS version 17 for Windows (SPSS Inc. Chicago, IL). Data are presented as means  $\pm$  standard deviation (SD). The patient characteristics including gender, age, smoking history, BMI and cardiovascular risk factors were systematically recorded and compared descriptively. Continuous variables were analyzed with the T-test and categorical variables with Chi-square test. We used linear regression analysis to estimate differences in outcome measures between the two groups. Multiple logistic regression analysis was performed to ascertain whether chronic obstructive airway disease was independently associated with the presence of an AAA, compared with gender, age, smoking status, presence of diabetes mellitus and a history of cardiovascular diseases. Differences in change of aneurysm growth parameters were assessed by repeated measurements analysis using linear mixed models with a random intercept and slope per patient. To assess whether growth rates were comparable between patients with and without

COPD, or within COPD disease stages, interaction terms between the disease stage and aneurysmal diameter were used. All test were two-tailed, P-values smaller than 0.05 were considered to be statistically significant.

## RESULTS

Our study population consisted of 221 patients with an AAA and 87 control patients with PAD without an AAA. The demographic and clinical characteristics of the two groups are outlined in Table 2.

Aneurysm and control groups were similar with respect to gender ( $p=0.71$ ), life-time cigarette smoking (39 vs 34 pack years,  $p=0.23$ ) and history of cardiovascular disease (45% vs 55%,  $p=0.12$ ). The percentage of current smokers was higher in the PAD patient group than in those with AAA (45% vs 30%,  $p=0.029$ ). AAA patients were slightly older (71 vs 69 year,  $p=0.03$ ) and heavier (BMI 27.4 vs 26.2,  $p=0.01$ ) and were less frequently diagnosed with diabetes mellitus (15% vs 30%,  $p=0.006$ ).

Significant differences in indices of airway obstruction were found (Table 3). Mean FEV1/FVC ratio was significantly reduced in aneurysm patients compared with controls (0.69 vs 0.78,  $p<0.001$ ). This reduced FEV1/FVC ratio in AAA patients was consistently found in all three smoking cohorts i.e. current (0.69 vs 0.75,  $p=0.013$ ), former (0.68 vs 0.79,  $p<0.001$ ) and most pronounced in never smokers (0.73 vs 0.86,  $p<0.001$ ). No significant differences were found in FEV1 and pFEV1% measures. In contrast, aneurysm patients had a significantly larger FVC (3.23 vs 3.46,  $p=0.024$ ). No relationship was found between annual aneurysm expansion rates in patients with COPD (2.3 mm/y) and those without (2.5 mm/y) ( $p=0.20$ ) (data not shown).

**Table 2.** Patient characteristics of cases with abdominal aortic aneurysm and controls.<sup>a</sup>

	PAOD n=87	AAA n=221	p
Age	69 ± 9	71 ± 7	<b>0.032</b>
Male gender	85%	87%	0.71
Body height (cm)	175 ± 8	175 ± 8	0.42
Body weight (kg)	80 ± 13	84 ± 14	<b>0.014</b>
BMI	26.2 ± 3.4	27.4 ± 3.7	<b>0.013</b>
Smoking			<b>0.029</b>
current	38 (45%)	66 (30%)	
ever	37 (44%)	132 (60%)	
never	10 (12%)	23 (10%)	
Pack years	34 ± 27	39 ± 31	0.23
DM	25 (29%)	32 (15%)	<b>0.006</b>
MI	23 (27%)	62 (28%)	0.89
Stroke	5 (6%)	11 (5%)	0.79
TIA	12 (14%)	20 (9%)	0.21
PAOD	87 (100%)	53 (24%)	<b>&lt;0.001</b>
All CVD (other than PAOD)	46 (55%)	98 (45%)	0.12

BMI; body mass index, DM; diabetes mellitus, MI; myocardial infarction, TIA; transient ischaemic attack, PAD; peripheral artery disease, CVD; cardiovascular disease.

<sup>a</sup> Data are presented as means SD except for percentages (%) and p-values are calculated using logistic regression. Bold p-values indicate a significant difference between AAA and PAD groups.

**Table 3.** Spirometry outcome measures in cases with abdominal aortic aneurysm and controls.<sup>a</sup>

	PAOD n=87	AAA n=221	p
FVC (L)	3.23 ± 0.78	3.46 ± 0.83	<b>0.024</b>
FEV1 (L)	2.50 ± 0.69	2.39 ± 0.71	0.21
FEV1 % predicted	84.0 ± 17.8	80.4 ± 19.0	0.13
FEV1/FVC ratio	0.78 ± 0.11	0.69 ± 0.12	<b>&lt;0.001</b>
current smokers	0.75 ± 0.11	0.69 ± 0.14	<b>0.013</b>
former smokers	0.79 ± 0.10	0.68 ± 0.12	<b>&lt;0.001</b>
never smokers	0.86 ± 0.07	0.73 ± 0.07	<b>&lt;0.001</b>

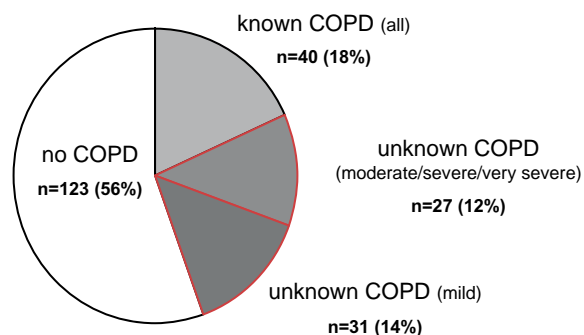
FVC; forced vital capacity, FEV1; forced expiratory volume in 1 s, L; litre. FVC is slightly increased in aneurysm patients. Mean FEV1/FVC ratio is significantly reduced in aneurysm patients compared with controls, most pronounced in never smokers.

<sup>a</sup>Data are presented as mean SD.

Classifying the results according to the GOLD criteria revealed that COPD is more prevalent in aneurysm patients (98/221; 44%) than in PAD patients (17/87; 20%). The unadjusted Odds Ratio of aneurysm patients for COPD is 3.3 (CI95% 1.8 – 5.9,  $p < 0.001$ ), and 3.0 (95%CI 1.6 – 5.5,  $p < 0.001$ ) when adjusted for age, gender, smoking, and presence of diabetes mellitus and a history of cardiovascular disease. In particular, a major proportion of aneurysm patients was newly diagnosed with COPD; only 40 of 98 patients (41%) with COPD (mild, moderate or severe/very severe) were known before with obstructive pulmonary defects and received treatment. (Fig. 1)

## DISCUSSION

This study demonstrates an increased prevalence of obstructive pulmonary disease in patients with an abdominal aortic aneurysm; a relationship that appears independent from smoking. Our findings clearly support the longstanding notion of an association between AAA and obstructive airway



**Figure 1.** Distribution of chronic obstructive pulmonary disease in AAA patients after spirometry. Of 221 patients with AAA, a total of 98 (44%) patients had COPD, as determined by spirometry and defined by the GOLD classification. 40 patients were previously diagnosed and treated for COPD (all stages of GOLD-classification). An additional 27 patients were newly identified with moderate or severe/very severe COPD and mild COPD was found by spirometry in 31 patients.

disease. Although a major proportion of COPD in aneurysm patients is most likely smoking-induced, our results suggest a relationship that is beyond that of smoking alone as clearly illustrated by spirometry results in never smokers. Findings from this study also show that obstructive pulmonary disease often remains unrecognized in aneurysm patients.

In 1985, Cronenwett et al. reported an association between rupture of abdominal aortic aneurysms and obstructive pulmonary disease<sup>6</sup>. Later reports extended this notion, and described a generally increased prevalence of COPD in AAA patients, and of aneurysmal disease in COPD patients<sup>7,8,9,10,11,12</sup>. Unfortunately, interpretation of these studies is hampered by methodological shortcomings such as identification of COPD on basis of medical records or questionnaires, which are known to underestimate the presence of pulmonary disease<sup>16,17</sup>. Moreover, the implication of the studies is complicated by considerable heterogeneity in outcome measures, definitions and control populations. Whether the presumed association is dominated by smoking as the primary risk factor for both diseases remains unclear. Additionally, findings from these studies are not supported by the largest study in AAA patients thus far (the Veterans Affairs Aneurysm Detection and Management (VA) study) where the association between COPD and AAA was lost after adjustment for smoking<sup>13</sup>. The reason for this apparent discrepancy is unclear, but may also relate to the use of questionnaires in the VA study to assess the presence of pulmonary disease, which has a positive predictive power of only 58% to recognize COPD<sup>16,17</sup>.

Given the contrasting findings and the persistent controversy with regard to the role of cigarette smoking in the association between AAA and COPD, we considered a re-evaluation relevant. The choice for PAD patients as the control population was based on the parallels in risk factors between AAA and PAD, which not only include dominance of smoking as the primary risk factor but also other common non-modifiable risk factors such as age and male gender<sup>14</sup>. As more than 85% of both populations were former or current smokers, with a similar burden of pack years, a considerable proportion of patients with pulmonary impairment was expected in both groups. However, significantly more airway obstruction was present in aneurysm patients, indicating that the high prevalence of COPD is not explained by smoking alone. This observation suggests that the relation between AAA and COPD reflects a common susceptibility, a notion that is supported by converging pathophysiologic pathways including metalloproteinase-9 and neutrophil elastase.<sup>18,19</sup> Such a common predisposition is suggested by the observation that the difference in FEV1/FVC ratio between AAA and PAD patients was most pronounced in never smokers. Identification of a common mechanisms or pathways might be highly relevant for future aneurysm and pulmonary research and research collaboration initiatives. In our opinion, further investigation of common genetic, inflammatory and remodeling pathways is warranted.

In line with two previous observations we found no relationship between the presence of obstructive pulmonary disease and expansion of aneurysm diameter<sup>9,20</sup>. Whether aneurysm growth is related to an actual decline in FEV1 values can however not be determined with this study. To that end, a long-term prospective study design would be required including a much larger sample of aneurysm patients, assessing pulmonary changes over time<sup>2</sup>.

This study has limitations. Firstly, we performed a case-control study designed to overcome restraints of previous reports. However, like all observational studies, our results are also subject to bias from confounding factors. We matched both cohorts to reduce imbalance with respect

to smoking as the most apparent confounding factor. Yet, small but significant differences with regard to potential confounders remained between the groups. We mathematically adjusted for these potential confounders. Results for this analysis showed that these adjustments only minimally affected the results. Secondly, we used a 2.5 to 1 ratio for cases and controls. This ratio was based on the power calculations for the study. Although the significant outcomes confirm the adequacy of the power calculation, a larger control group would have resulted in smaller confidence intervals.

Third, we used fixed ratios and cut off points to determine obstructive pulmonary defects for reasons of simplicity and comparability. While this is a common approach, this may result in over-diagnosis in elderly patients because of the variable nature of obstructive pulmonary diseases and the heterogeneity among patients. Although COPD is operationally defined by results on spirometry, an adequate diagnostic process should include post-bronchodilator spirometry, questionnaires and other clinical, physiological and radiologic measures. It is clear that FEV1 measures and indices alone have epidemiological value but they are not sufficient for clinical decision-making<sup>2</sup>.

Finally, we would consider an additional histopathological analysis of matched aneurysmal and pulmonary tissues from AAA patients highly relevant. Yet, such a study would rely on tissues obtained during post mortems. Given the extremely low number of post mortem nowadays such a study is extremely hard to conduct and one has to rely on the abundance of literature on inflammatory and proteolytic pathways in both conditions.<sup>3-5,18,19</sup>

### Implications

Data from our clinical cohort shows that 59% COPD cases were previously undetected; only 40 of the 98 detected AAA patients had a diagnosis of COPD and received proper treatment. Spirometry more than doubles the number of patients with a diagnosis of COPD, indicating that COPD is under-diagnosed in the AAA patients<sup>21</sup>. This can be partially explained by low physical activity in elderly patients, as symptoms of COPD such as shortness of breath may remain unnoticed. These findings suggest that it is clinically relevant to screen each AAA patient for obstructive pulmonary disease<sup>22</sup>. Early detection of COPD is useful as appropriate disease modification is available. For COPD, the only approach that has proven useful on modifying the course of the disease is smoking cessation. However, the usefulness of long-acting beta agonists or muscarinic antagonists in alleviation of the symptoms is also well documented, thus enhancing quality of life<sup>23</sup>. Moreover, COPD has been identified as a comorbidity associated with increased cardiovascular risk and poor outcome for any major vascular procedure<sup>24,25</sup>. Appropriate preoperative care, with a detailed respiratory assessment and careful attention to pulmonary function in the postoperative period, is necessary to favor the postoperative course of aneurysm patients<sup>26</sup>.

### Conclusion

The findings of this study show that the increased prevalence of chronic obstructive pulmonary disease (COPD) in patients with an abdominal aortic aneurysm (AAA) is independent from smoking. In addition, chronic obstructive pulmonary disease often remains unrecognized in aneurysm patients.

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# Chapter

# 4

## ACTIVATION OF THE VITAMIN D RECEPTOR SELECTIVELY INTERFERES WITH CALCINEURIN-MEDIATED INFLAMMATION: A CLINICAL EVALUATION IN THE ABDOMINAL AORTIC ANEURYSM

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## ABSTRACT

### Introduction

In-vitro and in-vivo studies attribute potent immune regulatory properties to the vitamin D receptor (VDR). Yet, it is unclear to what extent these observations translate to the clinical context of (vascular) inflammation. This clinical study evaluates the potential of a VDR agonist to quench vascular inflammation.

### Methods and Results

Patients scheduled for open abdominal aneurysm repair received paricalcitol 1 µg daily during 2-4 weeks prior to repair. Results were compared to matched controls. Evaluation in a parallel group showed that AAA patients are vitamin D insufficient (median plasma vitamin D: 43 [30-62 (IQR)] nmol/L). Aneurysm wall samples were collected during surgery, and the inflammatory footprint studied. The brief paricalcitol intervention resulted in a selective 73% reduction in CD4+ T-helper cell content ( $P < 0.024$ ) and a parallel 35% reduction in T-cell (CD3+) content ( $P < 0.032$ ). On the mRNA level, paricalcitol reduced expression of T-cell associated cytokines IL-2, -4 and 10 ( $P < 0.019$ ). No effect was found on other inflammatory mediators. On the protease level, selective effects were found for cathepsin K ( $P < 0.036$ ) and L ( $P < 0.005$ ). Collectively these effects converge at the level of calcineurin activity. An effect of the VDR agonist on calcineurin activity was confirmed in a mixed-lymphocyte reaction.

### Conclusions

A brief course of the VDR agonist Paricalcitol has profound effects on local inflammation via reduced T-cell activation. The anti-inflammatory potential of VDR activation in vitamin D insufficient patients is highly selective and appears to be mediated by an effect on calcineurin-mediated responses.

## INTRODUCTION

Inflammation plays a key role in the progression of the abdominal aortic aneurysm (AAA). The vitamin-D receptor (VDR) is a widely expressed nuclear receptor with an expression pattern that comprises most leucocytes, endothelial cells as well as vascular smooth muscle cells<sup>1,2</sup>. In-vitro studies show that activation of the VDR has strong immune-modulatory (anti-inflammatory) effects, and in the context of atherosclerotic disease it has been proposed that activation of the VDR may quench vascular inflammation. Although the validity of this concept has been well established in animal models, clinical studies consistently fail to show a benefit of VDR activation<sup>3,4</sup>. The basis for this discrepancy is unclear. Possible explanations include differences in inflammatory responses between preclinical disease models and the human situation<sup>5,6</sup>. Moreover, it has been pointed out that observations from situations of true vitamin D deficiency cannot simply be extrapolated to conditions characterized by a (sub) normal vitamin D status<sup>7</sup>. Considering the apparent gap between pre-clinical expectations and outcomes of clinical interventions we decided to explore the anti-inflammatory potential of VDR activation on the human vasculature.

The pathology of AAA is characterized by a broad and intense inflammation that comprises almost all aspects of the native and adaptive immune response<sup>8,9</sup>. In some patients, open surgical repair is indicated, thereby providing access to tissue. As such this procedure provides a unique opportunity to test the anti-inflammatory potential of pharmaceutical interventions.

In the present study, we investigated the anti-inflammatory potential of a 2-4 week treatment with the potent VDR agonist, paricalcitol, in patients scheduled for elective, open AAA repair. Results from this study show that the anti-inflammatory potential of the VDR-agonist paricalcitol in patients with subnormal vitamin D status is restricted to a selective effect on the calcineurin/NFAT axis; an observation that is supported by the ability of paricalcitol to effectively suppress lymphocyte proliferation in the mixed lymphocyte reaction.

## MATERIAL AND METHODS

### Patient populations

This open proof-of-concept study was approved by the Medical Ethical Committee of the Leiden University Medical Center. Written informed consent was obtained from all patients. Patients scheduled for open AAA repair were eligible for the study. Decision for open-repair was based on anatomical (e.g. neck, elongation), and patients characteristics (e.g. age) and preferences. Patients with impaired liver dysfunction (ALAT >3 times upper limit of the reference values, hypercalcemia and/or hyperphosphatemia, patients on digoxin as well as patients with inflammatory disease or (suspected) so-called inflammatory aortic aneurysms, were excluded from participation in the study. The study was started in November 2008 and the final patient was included in November 2010.

Patients received paricalcitol 1 µg once a day in the 2-4 weeks preceding their planned elective open repair. The final dose was taken in the evening before the surgery. Control AAA wall samples were obtained from the LUMC biobank, these samples were matched for sex, age, maximum AAA diameter and statin use. AAA wall tissue was taken from the anterior-lateral aneurysm wall at the level of the maximal diameter of the aneurysm. All wall samples (viz. samples both study

samples and biobank samples) were collected immediately after opening of the aneurysm sac. Adhering thrombus was carefully removed and wall samples were immediately halved. One half was snap-frozen in CO<sub>2</sub>-cooled iso-pentane or liquid nitrogen and stored at -80° C until use for mRNA (RT-PCR) and protein (ELISA) analysis. The other half was fixed in formaldehyde (24 hours), decalcified (Kristensens solution, 120 h), and paraffin embedded for histological analysis. All analyses were performed in an investigator-blind fashion.

Assessment of baseline vitamin D status was not foreseen in the study protocol. In the light of the study findings we considered information on the vitamin D status of AAA patients in retrospect relevant. To that end we measured vitamin D levels in available plasma samples from AAA patients who participated in the PHAST study.<sup>10</sup> Selected samples were all from patients from the same study center as the patients from whom aneurysm wall samples were available.

### Immunohistochemistry

Slides were incubated overnight with antibodies against myeloperoxidase (MPO; rabbit polyclonal, 1:4000 dilution, DakoCytomation, Heverlee, Belgium), CD3 (polyclonal rabbit, 1:400 dilution, Abcam, Cambridge, UK), CD4 (clone 4B12, 1:200 dilution, DakoCytomation), CD8 (clone C8/144B, 1:200 dilution, DakoCytomation), CD20 (clone L26, 1:1000 dilution, DakoCytomation), CD68 (clone KP6, 1:1200, DakoCytomation), and CD138 (clone B-B4, 1:1000 dilution, Serotec, Oxford, UK)<sup>9</sup>.

For each section 6 representative medium power fields (3 photographs for 'medial' and 3 for the 'adventitial layer) were photographed at a 20x magnifier, and the number of positive cells counted.

### Semi quantitative mRNA analysis

Total RNA extraction was performed using RNeasy (Qiagen, Venendaal, The Netherlands) and glass beads<sup>9</sup>. Copy-DNA was prepared using kit #A3500 (Promega, Leiden, The Netherlands) and quantitative real-time polymerase chain reaction (Taqman system) analysis was performed for human interleukin (IL)-1 $\beta$ , IL-2, IL-4, IL-6, IL-8, IL10, Tumor necrosis factor (TNF) - $\alpha$ , Interferon- $\gamma$ , MCP-1, Perforin, B-lymphocyte-induced maturation protein-1 (BLIMP-1), MMP (matrix metalloproteinase)2, -3, -9 and 12, the Cathepsins K, L and S, on the ABI-7500 Fast system (Life Biosciences, Nieuwerkerk aan den IJssel, The Netherlands) using established primer/probe sets (Assays on Demand, Life Biosciences) and Taqman Gene Expression Master Mix (Life Biosciences). Analyses were performed according to the manufacturer's instructions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as a reference and for normalization.

### Protein analysis

Aortic wall tissues were pulverized in liquid nitrogen and homogenized in two volumes lysis buffer (10 mM Tris pH 7.0, 0.1 mM CaCl<sub>2</sub>, 0.1 M NaCl, 0.25% (v/v) Triton X-100). Samples were subsequently centrifuged at 10,000g for 15 minutes at 4°C, and the supernatant protein extract was snap-frozen in liquid nitrogen and stored at -80°C until analysis. Protein content in thawed protein extracts homogenates was determined with a BCA protein assay kit (Pierce, Rockford, IL, USA). Cytokine/chemokine protein levels in these homogenates were measured by separate ELISAs for IL-6, IL-8

(PeliKane compact kit, Sanquin, Amsterdam, The Netherlands), and MCP-1 (Quantikine kit, R&D Systems, Abingdon, UK).

Relative tissue MMP2 (pro and activated form) content was assessed by Western blot using anti-MMP2 (sc-10736, Santa Cruz Biotechnology, Dallas USA ) and GAPDH (sc-25778, Santa Cruz Biotechnology) for normalization. Blots were visualized using supersignal West femto substrate kit (Life Technologies, Bleiswijk, The Netherlands) and chemiluminiscense visualized and quantified on a Chemidoc Touch Imaging system (Biorad Laboratories, Veenendaal, The Netherlands) .

### T-cell proliferation

Mixed Lymphocyte Reactions (MLRs) were set up with 50  $\mu$ l of  $1 \times 10^6$  donor PBMCs with 105 irradiated (30 Gy/3000 Rad) HLA-mismatched stimulator cells (antigen-specific stimulus) or donor PBMC were stimulated with the mitogen phytohemagglutinin (PHA, non-antigen specific stimulus) in triplicate in 96-well round-bottomed plates (Greiner Bio-one) in the presence of different concentrations paricalcitol or one the comparators (Tacrolimus or Mycophenolic acid). Proliferation was measured on day 5 by incorporation of 3H-thymidine, which was added during the last 16 h of culture. The results were expressed as the median counts per minute (cpm) for each triplicate culture.

### Statistical analysis

Statistical analyses were performed with SPSS 20.0 (SPSS Inc., Chicago, IL, USA). For the comparisons, a P-value  $<0.05$  was considered statistically significant. Differences between the groups were evaluated by ANOVA (normally distributed data) or the Kruskal Wallis test in case of non-normally distributed continuous data. As all the observations in the paper fit within a theoretical framework no correction for multiple testing was performed.

## RESULTS

### Patient population

Baseline characteristics of the pre-operative paricalcitol-intervention group (n=11) and the matched control group (n=11) used in the molecular analysis are shown in table 1. Paricalcitol was well tolerated and there were no drop-outs.

Plasma levels showed that all patients in the parallel AAA cohort are vitamin D insufficient by current standards<sup>11</sup> (median level 43 [30-62 (IQR) nmol/L]).

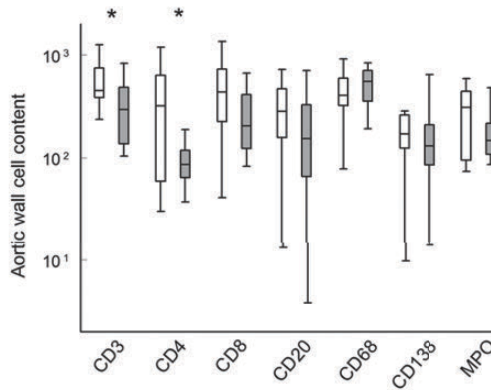
### Paricalcitol intervention

AAAs are characterized by a broad cellular inflammatory component consisting of macrophages, neutrophils, and T- and B-cells<sup>8,9</sup>. Immuno-histochemical analysis showed that paricalcitol reduced the aortic wall T-cell (CD3<sup>+</sup>) and T-helper cell (CD4<sup>+</sup>) content ( $P < 0.024$  and  $P < 0.032$  respectively), but did not influence the relative abundance of the other inflammatory cell types (i.e. monocytes/macrophages (CD68<sup>+</sup>), neutrophils (MPO<sup>+</sup>), cytotoxic T-cells (CD8<sup>+</sup>), B-cells (CD20<sup>+</sup>) and plasma cells (CD138<sup>+</sup>)) (Figure 1) or distribution over the media and adventitia (Figure 2).

Evaluation of an effect of paricalcitol treatment on the mRNA levels of inflammatory mediators showed a selective reduction in the Th1/Th2 cytokines IL2, -4 and -10. No effect was seen on

**Table 1.** Baseline patient characteristics of the paricalcitol intervention study. Median [inter quartile range]

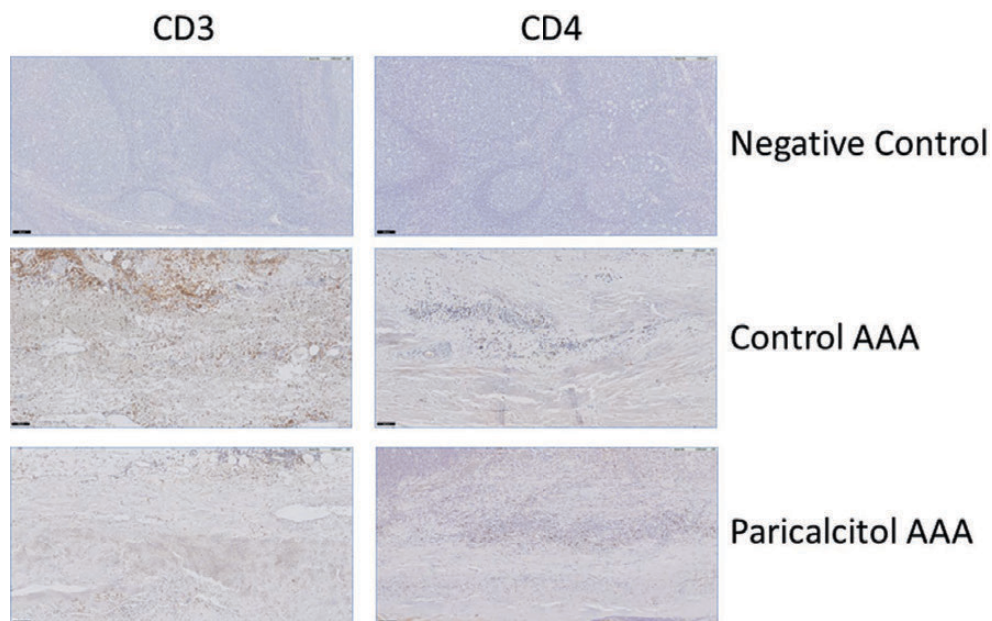
	Paricalcitol n=11	Controls n=11
Age (year)	72 [61-76]	72 [67-77]
AAA diameter (mm)	57 [52-62]	57 [51-67]
Male sex	11/12	11/12
(ex) smokers	11/12	10/12
Statin use	10/12	10/12
ACE inhibitor use	3/12	2/12



**Figure 1.** Effect of Paricalcitol on aneurysm wall leucocyte content. Semi-quantitative analysis of aortic wall T-cell (CD3), T-helper cell (CD4), and cytotoxic T-cells (CD8), B-cell (CD20) monocyte/macrophage (CD68), plasma cell (CD138), and neutrophil (myeloperoxidase (MPO) content. Cell counts are based on reflect the number of positive cells per 6 medium power fields. Cell content is expressed as the number of cells per mm<sup>2</sup>. Non-treated controls (white bars); Paricalcitol-treated patients (grey bars). Boxplots indicate the inter quartile ranges with the median. The vertical lines represent the range. \*P<0.03.

the mRNA and protein levels of general proinflammatory cytokines (Table 2, Figure 3). Paricalcitol did not influence the expression of the B-cell associated marker BLIMP-1 or the cytotoxic T-cell/NK cell marker perforin. On the protease level paricalcitol reduced the expression of the cathepsins K and L, and increased the expression of MMP2 (Table 2). No effect was found on the expression of other MMPs. The change in MMP2 mRNA expression was not followed by an increase aneurysm wall protein content. On the contrary, VDR activation reduced both pro and activated MMP2 content (relative expression: 1.27 (0.50) vs 0.74 (0.24) (proMMP2) and 0.31 (0.13) vs 0.18 (0.04) (activated MMP2) respectively, P<0.04).

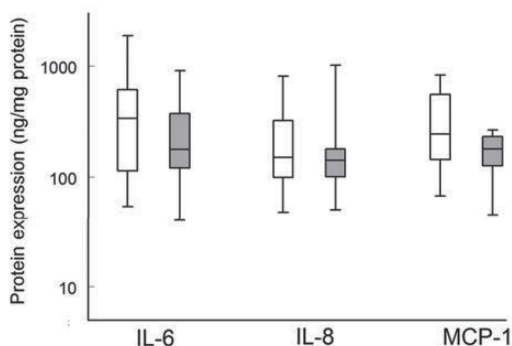
An apparent selective effect on CD4<sup>+</sup> T-helper cells, T-helper cell-associated cytokines and a selective effect on cathepsin K and L expression imply an effect of paricalcitol on the Calcineurin/NFAT signalling pathway. We explored such an effect<sup>12</sup> in a mixed lymphocyte reaction. Figure 4 shows that the effect size of paricalcitol mediated T-cell proliferation was almost equal to that of tacrolimus. Cytotoxicity assays showed that this effect is not explained by excess cell death (results not shown).



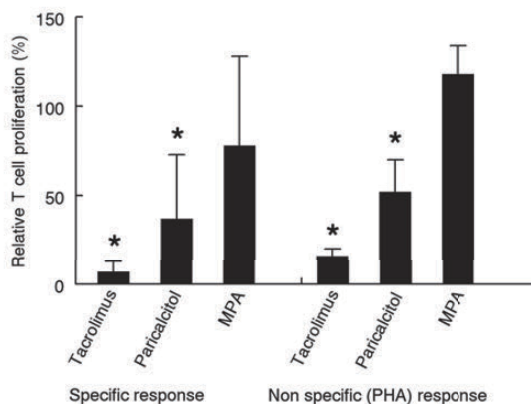
**Figure 2.** Immunohistological staining for CD3 and CD4 in AAA wall samples from control and paricalcitol-treated individuals. AAA, abdominal aortic aneurysm.

**Table 2.** Relative mRNA expression of selected inflammatory mediators, proteases, cytokines, and cell activation markers (log transcript level relative to GAPDH (GAPDH=0)). Frozen material was not available from 1 Paricalcitol patient.

	Control n=11	Paricalcitol n=10	p
IL1 $\beta$	-1.49 [-1.65 – -1.30]	-1.23 [-1.47 – -0.99]	0.122
IL-2	-1.42 [-1.62 – -1.26]	-1.98 [-2.30 – -1.90]	0.000056
IL-4	-2.76 [-3.17 – -2.51]	-3.49 [-3.61 – -3.01]	0.019
IL-6	-0.32 [-1.10 – 0.03]	-0.77 [-1.08 – -0.46]	0.595
IL8	-0.44 [-1.28 – -0.21]	-0.42 [-0.71 – -0.18]	0.295
IL-10	-1.77 [-2.06 – -1.53]	-2.13 [-2.28 – -2.00]	0.006
MCP-1	-0.05 [-0.75 – 0.44]	-0.36 [-0.55 – -0.15]	0.421
TNF $\alpha$	-1.32 [-2.17 – -0.87]	-1.01 [-1.21 – -0.90]	0.188
Interferon $\gamma$	-2.18 [-2.65 – -1.62]	-1.57 [-1.98 – -1.21]	0.119
MMP2	-0.41 [-0.99 – -0.11]	0.03 [-0.17 – 0.18]	0.011
MMP3	-1.61 [-1.89 – -1.09]	-1.51 [-2.06 – -1.09]	0.929
MMP9	-0.94 [-1.34 – -0.20]	-0.54 [-0.59 – -0.22]	0.105
MMP12	-1.02 [-1.76 – -0.44]	-0.66 [-0.88 – -0.50]	0.106
Cathepsin K	-1.16 [-1.41 – -0.94]	-0.90 [-1.07 – -0.83]	0.036
Cathepsin L	-0.14 [-0.26 – 0.10]	-0.43 [-0.56 – -0.19]	0.005
Cathepsin S	-0.74 [-1.03 – -0.31]	-0.41 [-0.56 – -0.32]	0.079
Perforin	-1.68 [-2.71 – -0.74]	-0.95 [-1.07 – -0.92]	0.057
BLIMP	-1.19 [-2.56 – 0.14]	-0.57 [-0.81 – -0.41]	0.125



**Figure 3.** Similar aneurysm wall protein interleukin-6, interleukin-8, and monocyte chemo-attractant protein-1 content in non-treated controls (white bars) and Paricalcitol-treated patients (grey bars). Boxplots indicate the inter quartile ranges with the median. The vertical lines represent the range. No differences were found between the two groups (ANOVA). IL, interleukin.



**Figure 4.** Relative effects of tacrolimus, paricalcitol and mycophenolic acid (MPA) on the specific and non-specific T-cell response in a mixed lymphocyte reaction (control condition = 100%). \* $P < 0.04$ . PHA=phytohemagglutinin.

## DISCUSSION

This clinical study shows that a brief 2-4 week intervention with the VDR agonist paricalcitol exerts clear effects on aspects of vascular inflammation in the AAA. The selective effect on T-helper cells, the proteases cathepsin K and L, and the results of the mixed lymphocyte response are consistent with a selective effect on the calcineurin/NFAT axis.

Reports on the anti-inflammatory potential of VDR agonists are multiple and diverse. Reported mechanisms include, among others quenching of NF $\kappa$ B<sup>13</sup> and MAPK<sup>14,15</sup> activation, as well as interference with macrophage activation<sup>16</sup> or the Ang-2-Tie-2-MLC kinase cascade<sup>17</sup>. Using the abdominal aortic aneurysm as a clinical example of comprehensive vascular inflammation<sup>8,9</sup> we show that the effects of VDR activation through the vitamin D analogue paricalcitol are restricted, and include an effect on the aneurysm wall T-helper (CD4+) cell content and its associated cytokines,

as well as an effect on the expression of the cysteine proteases cathepsins K and L. The approximate 75% reductions in both T-helper cell content and in the T-helper cell related cytokines IL-2, 4 and 10 suggests that the reduction in cytokine expression primarily reflects a reduction of aortic wall cell content rather than an effect on cell activation. For logistic reasons assessment of vitamin D status was not included in the study protocol. As of the remarkable findings we considered information on the vitamin D status of AAA patients relevant. To address this point we assessed vitamin D plasma levels in plasma samples from a group of AAA patients in this trials' study center who participated in the PHAST study. All these patients were vitamin D insufficient, viz. none of the AAA patients tested was vitamin D deficient or sufficient<sup>11</sup>.

The observed selective effects on T-cell activation and the (contrasting) effect on the cysteine proteases cathepsin K and L, converge at the level of the calcineurin/NFAT axis; such an effect that has been clearly demonstrated for vitamin D in in-vitro studies<sup>18,19</sup>. A NFAT-1c responsive element has been described for the Cathepsin L promoter region<sup>20</sup>, as such the reduced cathepsin L expression may reflect reduced promoter activity. An alternative (but non-exclusive) explanation is that the reduced cathepsin L expression mirrors a reduction in CD4 content. Increased cathepsin K expression seemingly contrasts with the findings for cathepsin L. Yet, cathepsin K expression is in part regulated by NFAT-2<sup>21</sup>, a factor that in contrast to NFAT-1c requires phosphorylation rather than de-phosphorylation (NFAT1c) for nuclear transfer. Hence, it has been pointed out that calcineurin inhibitors promote NFAT-2 activity<sup>22</sup>. No effect was found of VDR agonist on RANKL expression<sup>23</sup>, (results not shown). Opposed associations have been reported for a link between MMP2 expression and the calcineurin axis, as such it is unclear whether the increased MMP2 expression links to an effect on this axis or, alternatively reflects a separate effect of VDR activation or a statistical type I error<sup>24,25</sup>. This latter possibility is supported by reduced MMP2 protein content in paricalcitol treated patients.

In two previous studies, we have shown that brief 2-4 week pre-operative interventions with doxycycline and the ACE-inhibitor Ramipril profoundly reduced aortic wall inflammation through, respectively, effects on AP-1 (doxycycline)<sup>26</sup> and NFκB<sup>27</sup> pro-inflammatory pathways. Similarly, it was found that statins dose-dependently reduce NFκB-driven inflammation in the aneurysm wall<sup>28</sup>. Lacking clear effects on other inflammatory pathways, VDR activation appears highly selectively influencing NFAT mediated inflammation. Our observations not necessary exclude an effect of VDR activation on other pathways as reported in experimental studies. Yet, such effects may only be apparent when studying vitamin D deficient individuals, and are missed in the real for life situation with most patients having suboptimal (i.e. with plasma levels beyond 20 nmol/L as found in AAA patients) or normal vitamin D levels.

The observed selective effect of VDR activation on NFAT mediated inflammation implies a role for VDR agonists in pathologies with established benefit from NFAT inhibition inhibitors, either as a low-toxic, moderate potent mono therapy, or alternatively as an add-on therapy, allowing tempering of calcineurin inhibitor dose; thereby potentially limiting the negative side effects of this class of compounds.

A critical point is whether the observed effects of VDR activation are beneficial in the context of AAA disease or vascular disease in general (atherosclerosis). Although cathepsin K<sup>29</sup> and L<sup>30</sup> have both been implicated in the process of aneurysm formation<sup>31</sup>, an apparent dominant role in AAA

progression is challenged by the observation that reductions in cathepsin K and L expression during respectively statin<sup>28</sup> and ACE-inhibitor therapy<sup>27</sup> are not followed by an effect on aneurysms growth. It is unclear whether and if the effects of VDR activation on T-helper cell content will influence AAA disease. A role for T-helper cells in the context of AAA disease remains elusive<sup>32</sup> with reported observations from animal studies being inconclusive<sup>33,34</sup>, and clinically accelerated aneurysm progression during intense immune suppression<sup>35</sup>.

Along these lines the potential benefit of VDR activation in the context of atherosclerotic disease remains unclear with clear benefits in preclinical studies, and consistent epidemiological association between vitamin D (sub) deficiency and manifest atherosclerotic disease<sup>2</sup>, but very limited evidence for a benefit of vitamin D supplementation on manifestations of atherosclerotic disease<sup>3,4</sup>.

This interventional study has a number of limitations. The study is not placebo controlled and small. Yet given the progressive decline in open AAA repair procedures, interventional studies like this become more and more difficult to perform. We have chosen for an individual matching procedure of cases and control material from our tissue bank; this approach reduces clinical variation in the study and removes potential strong confounders, and is very suited to observe subtle differences in pathways. However, the matched design does not circumvent biases as in the double blinded randomized trials. The consistent data from the different platforms used in this study make a type I-error extremely unlikely. A larger sample size would obviously have resulted in smaller confidence intervals, but this is getting more and more complicated in an era of endovascular aneurysm repair. We cannot exclude that minor effects on other inflammatory pathways are missed due to a type II statistical error. Yet, if such effects exist, they presumably compare weakly to the effect exerted on the NFAT pathways, and to pleiotropic effects on NFκB and AP-1 signalling exerted by respectively statins<sup>28</sup> and ACE-inhibitors<sup>27</sup>, and doxycycline<sup>26</sup>. A further limitation of the study is that evaluation of baseline vitamin D status was not included in the study protocol. We therefore assessed vitamin D levels in plasma from AAA patients participating PHAST study who were included in same centre as the patients in this study<sup>10</sup>. Without any exception all patients in this AAA group had insufficient vitamin D plasma levels; as such it is unclear whether and how the observations from this study translate to vitamin D deficient and sufficient individuals.

In conclusion the anti-inflammatory potential of VDR activation in vitamin D insufficient individuals is highly selective and appears mediated by an effect on the NFAT/Calcineurin axis.

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# Chapter

# 5

## INHIBITION OF CYSTEINE PROTEASE ACTIVITY REDUCES ANEURYSM EXPANSION THROUGH DECREASED MATRIX DEGRADATION

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# ABSTRACT

## Aim

Abdominal aortic aneurysm (AAA) is a common vascular dilatation disorder in elderly people that may become lethal after aortic rupture. Current treatment exclusively relies on preventive surgical repair of larger AAA. Pharmaceutical intervention decreasing aneurysm progression to reduce the need for surgical repair is currently missing. The pathology of AAA is best described as a chronic inflammatory condition of the vessel wall accompanied by enhanced protease activity. Cysteine proteases are associated with matrix remodeling and are abundantly expressed in aneurysms. To test whether cysteine proteases constitute a target for pharmaceutical AAA stabilization, we evaluated the efficacy of the broad-spectrum cysteine protease inhibitor E64.

## Methods and Results

Human AAA tissue shows enhanced cysteine protease expression and a strong increase (10-fold) in protease activity as illustrated by the increased presence of cysteine protease-mediated collagen (CTX)-fragments compared to control age-matched aortic tissue. In the angiotensin-II (AngII) and elastase AAA mouse model, we showed that E64 treatment reduced aneurysm severity of the ascending and descending aorta or aneurysm formation, respectively. We revealed inhibited breakdown of the elastic laminae and prevented loss of collagen in the vessel wall, promoting integrity of the aorta. In the elastase model, in which the aorta is locally treated with elastase, E64 was superior to doxycycline ( $p=0.04$ ) in inhibition of aortic dilatation (ultrasound,  $p<0.001$ ).

## Conclusion

This study showed that the broad-spectrum cysteine protease inhibitor, E64, decreased aneurysm formation in two established mouse models of the disease, indicating that cysteine proteases are suitable potential targets for pharmaceutical AAA stabilization in humans.

## INTRODUCTION

An abdominal aortic aneurysm (AAA) is a dilatation of the terminal aortic segment. Small AAAs are generally clinically silent, yet larger aneurysms can rupture, causing an often fatal bleeding. The current approach towards AAAs is surveillance of smaller aneurysms, and preventive surgical elimination of larger aneurysms once the diameter has become larger than 55 mm. Accordingly, pharmaceutical intervention inhibiting AAA progression may have major advances, both from patients' and from a socio-economical perspective<sup>1</sup>.

Although elastin degradation is considered the hallmark of AAA-disease, collagen degradation is held responsible for the actual aneurysmal dilatation and ultimate rupture of the aneurysm<sup>2</sup>. The vascular type collagens type-I and -III are highly resistant towards proteolytic degradation, and specific collagenolytic enzymes are required for initiation of collagen degradation. Others and we previously identified the cysteine proteases, cathepsin K, -L and -S as prominent collagenolytic enzymes in human AAA samples<sup>3-8</sup>. Moreover, deficiency of cystatin C, the primary endogenous inhibitor of cathepsins, has been associated with human aneurysm formation<sup>8,9</sup>. Genetic mouse models with cathepsin deficiency underline their importance in this pathology. Mice do not develop aneurysms spontaneously and the two most established murine AAA models are the angiotensin II- (AngII) induced and the elastase-induced aneurysm model<sup>10-12</sup>. Mice lacking the protease cathepsin K, -L, or -S are resistant to AAA formation<sup>13-15</sup>. Significantly, cystatin C deficiency in mice recapitulates many features of human aortic aneurysms<sup>9</sup>. Together these observations pose the cysteine collagenase network as an important pharmaceutical target to stabilize AAAs. To test the validity of this approach, we here employ the broad-spectrum cysteine protease inhibitor E64<sup>16</sup>, in two established murine AAA models.

## METHODS

### Patients

The investigation conforms the principles outlined in the Declaration of Helsinki (59<sup>th</sup>, October 2008). Human AAA tissue was obtained during surgery for asymptomatic AAA. Peri-renal aortic patches of age-matched organ donors were used as controls. Sample collection was performed in accordance with the guidelines of the medical ethical committee of the Leiden University Medical Center.

### Collagen Degradation Assay

Homogenates of aortic wall tissues (ICTP assay; N=3 control and N=8 AAA, CTX assay; N=12 both groups) were normalized for protein content. Collagen degradation was measured by CTX-assay (Serum Cross lags; Nordic Biosciences, Milsbeek, The Netherlands) and ICTP assay (Uniq ICTP RIA, Orion Diagnostica Oy, Espoo, Finland). The CTX epitope reflects cathepsin K-mediated collagen turn-over and the ICTP assay the matrix metalloproteinase-mediated collagen degradation<sup>17</sup>.

### Murine Aneurysm Models

All murine investigations were performed conform the Directive 2010/63/EU of the European Parliament or conform the *Guide for the Care and Use of Laboratory Animals* published by the US

National Institutes of Health (NIH Publication No. 85-23, revised 1996). Local approval was obtained from the institutional animal welfare committees.

### Angiotensin II – induced model of AAA

This AAA-model relies on angiotensin II (AngII) infusion (1.44 mg/kg/day) via an osmotic minipump for 4 weeks<sup>10</sup>. ApolipoproteinE deficient (ApoE<sup>-/-</sup>) male mice of approximately 8 months old were used for the aneurysm formation experiment. The mice treated with the cysteine protease inhibitor E64 (C<sub>15</sub>H<sub>27</sub>N<sub>5</sub>O<sub>5</sub>; N-(trans-epoxysuccinyl)-L-Leucine-4-guanidinobutylamide; Sigma Aldrich Zwijndrecht, The Netherlands)<sup>6</sup>, received 0.24 mg/kg/day E64 for 4 weeks via the minipump that also contained AngII. Mice were primed through a bolus injection of E64 (0.48 mg/mouse i.p.) on day one. As an extra control group for aortic dilatation, five ApoE<sup>-/-</sup> mice were given a minipump containing saline, which did not lead to aneurysm formation. After 4 weeks the mice were harvested and their aortas analyzed.

Aneurysms were classified according to Daugherty et al<sup>10</sup>, measuring the maximal abdominal aortic diameter macroscopically and taking into account thrombus formation in an aneurysm or if multiple aneurysms/dissections were present per aorta. In addition, the circumference (to calculate diameter) of the ascending aorta was measured where the aorta leaves the heart.

### Cytokine levels in serum

Murine monocyte chemoattractant protein 1 (JE), murine interleukin-8 (KC), interleukin-6 (IL-6), interferon gamma (IFN- $\gamma$ ) and tumor necrosis factor alpha (TNF- $\alpha$ ) were determined in serum of the control and E64-treated mice by specific ELISAs.

### Elastase-induced model of AAA

In male (8-12 weeks old) wild-type (WT; C57BL/6) mice aneurysms were generated via porcine pancreatic elastase (PPE) infusion as previously described<sup>12</sup>. In short, an isolated aorta segment was perfused with elastase solution for 5 minutes, after which the flow was restored. The experimental E64-treated mice (n=13: 0.48mg E64/kg/day) received E64 via an osmotic minipump placed one day prior to elastase infusion. A control group received a saline containing minipump (n=17), and served as positive control, where maximal aortic expansion could be determined. A second group was treated with doxycycline (in drinking water; n=12), an established inhibitor of aneurysm growth in mice and decreased vascular inflammation in human aneurysm vessel wall<sup>18-21</sup>. Ultrasound measurements were made at baseline and at day 7 and 14 after the infusion by means of the Vevo 770 Imaging system using an RMV 704 microvisualization scan head (Visualsonics, Ontario, CA).

### (Immuno)Histochemistry

Upon harvest, human or murine aortic segments were fixed, embedded in paraffin and sectioned for histological analysis. To detect the different cysteine proteases in human AAA tissue, sections were incubated with primary antibodies against cathepsin K, -L, or -S (generous gift from Dr. Weber, Institute of Biochemistry, Martin-Luther University of Halle-Wittenberg, Halle, Germany (cathepsin K and S), or R&D systems Abbingdon, UK (Cathepsin L)).

To visualize elastic laminae and quantify breaks, Lawson-staining was performed. To detect collagen, sections were stained with Picro Sirius Red solution. Three sections per mouse were blindly evaluated and mean values for elastic laminae breaks or collagen area was calculated for each animal. Collagen area was measured using Leica QWin software.

### Statistical analysis

The data are expressed as mean  $\pm$  the standard error of the mean (SEM). The non-parametric Mann-Whitney U-test was used to compare the human or murine aneurysm data.

A 2-tailed Student t test was used to compare elastic lamina breaks, collagen content, and cytokine data from the two groups of mice. The level of significance was set at  $p < 0.05$ .

## RESULTS

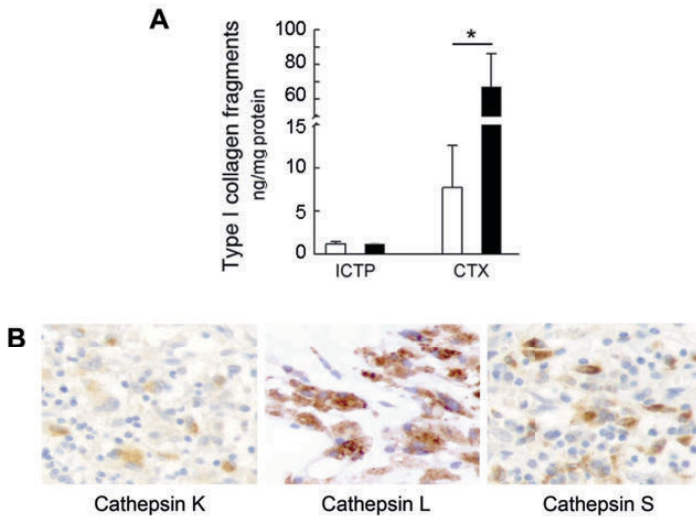
### Cysteine protease activity in human AAA

Although the main focus in AAA research has been on the role of matrix metalloproteinases (MMPs), cysteine proteases are now recognized as key factors in vessel wall destabilization<sup>20</sup>. We previously determined that expression of members of both classes are increased in aneurysm tissue, compared to aortic (atherosclerosis) tissue from age-matched control individuals<sup>21</sup>. To establish to what extent MMPs and cysteine proteases are involved in AAA, we now determined their activity, by measuring their specific collagen degradation products. The ICTP assay (MMP-activity)<sup>17</sup>, showed minimal levels of MMP-specific fragments in control and AAA tissue (Fig.1A). However, we observe abundant CTX fragments (cathepsin K activity) in AAA tissue compared with control aortic wall (10-fold increase;  $p < 0.001$ )(Fig.1A). Cathepsin K, L and S are each abundantly expressed in human AAA tissue, predominantly by macrophages (Fig.1B), and are probably all involved in generation of CTX fragments. In conclusion, macrophage-derived cysteine proteases contribute significantly to matrix degradation in human AAA.

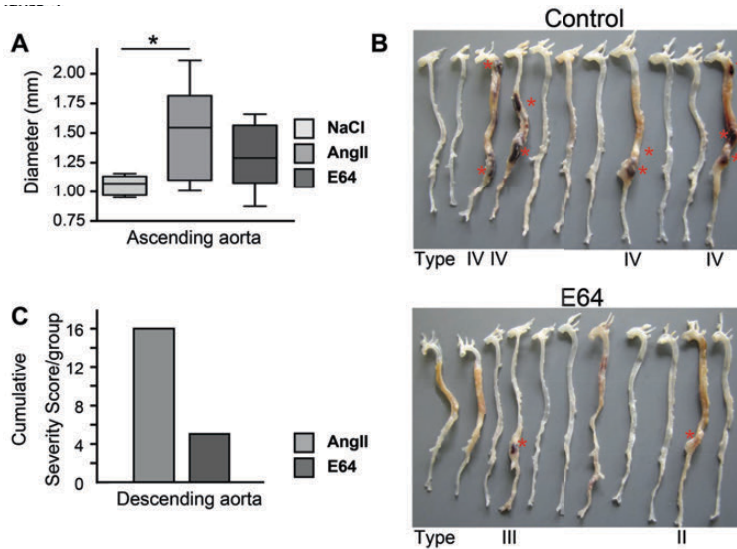
### E64 in the AngII-model decreased AAA severity

The importance of cysteine proteases in aneurysm formation was first investigated in the AngII-induced AAA model by blocking their activity via cysteine protease inhibitor E64<sup>22, 23</sup>. Mice were treated with AngII without or with E64, and after four weeks the aorta was analyzed for aortic dilatation. Dilatation of the ascending aorta is a typical feature in the AngII model<sup>24</sup>. To assess the normal aortic diameter of ApoE<sup>-/-</sup> mice, we incorporated the analysis of mice that received saline (N=5) instead of AngII for 4 weeks. The normal wideness of the ascending aorta is  $1.1 \pm 0.1$  mm (Fig. 2A). However, in response to AngII infusion (N=11) the ascending aorta diameter expands significantly to a diameter of  $1.6 \pm 0.4$  mm ( $p < 0.03$ ). E64 treatment (N=11) reduced the aortic diameter to  $1.3 \pm 0.3$  mm (saline vs E64 is not different;  $p = 0.08$ ).

In addition, aneurysms in the descending aorta were typed according to the scoring system as described before<sup>10</sup>. We observed 4 severely affected aortas with multiple aneurysms per aorta (scored as type IV; Fig. 2B) in the control group, compared with 2 milder phenotype type II (dilated aorta) and type III (dilated with thrombus) aneurysms in the E64 treated group (Fig. 2B). Cross sections of these aneurysms were stained to visualize collagen in red or elastic laminae in purple,



**Figure 1.** Collagen degradation and cathepsin expression in human aneurysm. Collagen degradation was measured in human aortic tissue from control (white) and AAA specimens (black) (A). The levels of collagen-I fragments derived by MMP-mediated (ICTP) or cysteine proteases (CTX) degradation were determined (\* $p < 0.001$ ) (B).



**Figure 2.** E64 reduces aneurysm formation in the AngII-model. Increased aortic dilatation is observed in the ascending aorta upon AngII-treatment when comparing with saline-treated mice ( $p < 0.03$ ) (A). In addition, 4 (out of 11) mice showed severe aorta pathology (type IV), which is reduced to 2 (out of 11) mice with minor aneurysm formation in the E64-treated group (type II and III) (B). The cumulative aneurysm severity score for the descending aorta is shown (C).

and revealed extensive medial degradation (Supplemental Fig. 1). The cumulative aneurysm score of 16 in control mice and 5 in the E64-treated group revealed protection by E64 (Fig. 2C). Taken together, the E64-treated mice showed decreased aneurysm pathology.

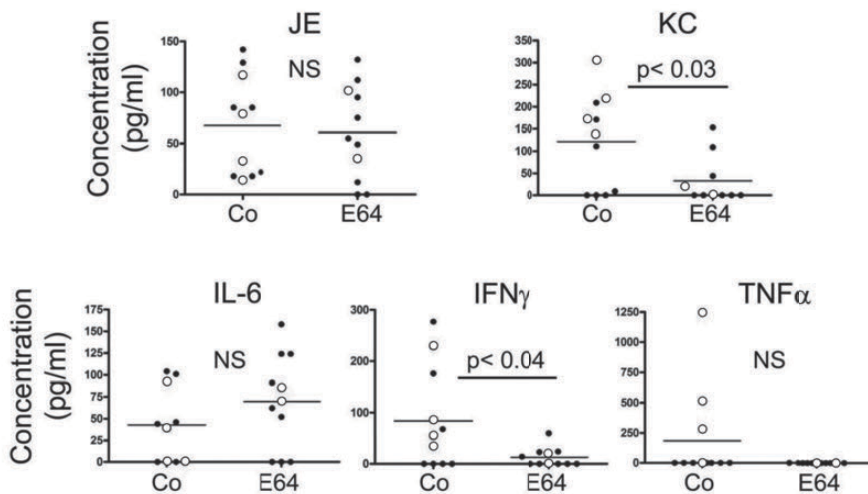
### E64 reduced several specific circulating pro-inflammatory factors

AngII infusion has a pro-inflammatory effect, which is causative in induction of aneurysms<sup>10</sup>. Therefore, we determined the effect of E64 on systemic inflammatory responses by measuring a panel of hallmark cytokines/chemokines in the circulation of the mice. E64 treatment reduced circulating KC ( $p < 0.03$ ) and IFN $\gamma$  ( $p < 0.04$ ) levels, whereas no effect was found on JE, IL-6 and TNF $\alpha$  serum levels (Fig. 3).

### E64 protects against extracellular matrix degradation

In the AngII-model the vessel wall was severely damaged at the site of the macroscopically visible aneurysms (Supplemental Fig. 1). Consequently, we decided to measure elastic lamina breaks only in macroscopically *un*-affected aortas at the location where AAA normally form (N=7 control and N=9 E64), to analyze aortic damage in an early stage of the disease. Elastic lamina degradation was observed in the aortas of all AngII-treated mice, yet the number of breaks per aorta was significantly decreased in the E64-treated group (Fig. 4A;  $p = 0.05$ ). The analysis also revealed that the elastic lamina breaks often occur at sites where atherosclerotic lesions have developed (Fig. 4B).

Quantification of the abundance of the collagen matrix in the media of the vessel wall showed that aortas from E64-treated mice contained 50% more collagen compared to untreated mice (Fig. 4C;  $p < 0.02$ ), indicating that collagen had disappeared from the media to a higher extent in



**Figure 3.** E64 reduces several specific cytokines. The expression level of the chemokines JE and KC and the cytokines IL-6, IFN $\gamma$  and TNF $\alpha$  was determined in serum of control (Co) and E64-treated mice. White dots indicate mice with an aneurysm.

control mice (reduced red staining in media) (Fig. 4D). Thus, both the elastin and collagen matrix components are more intact in response to E64 treatment.

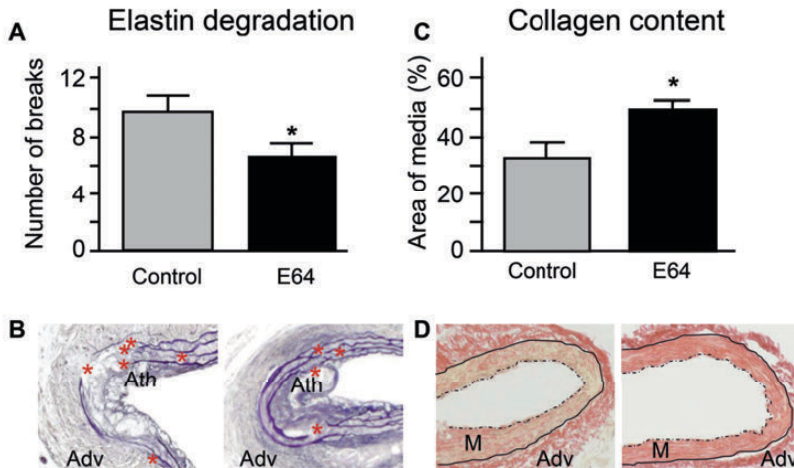
### E64 in the Elastase model prevents AAA formation

To validate the seemingly protective effect of E64, in yet another AAA model, we performed aortic elastase infusion experiments. Mice that received additional saline developed an aneurysm (as defined by  $\geq 50\%$  increase in aortic diameter) within 14 days ( $85\% \pm 22\%$  increased diameter) (Fig.5A). Mice that received E64-treatment, however, were protected from aortic dilatation ( $p < 0.001$ ). E64 was even more effective ( $32\% \pm 16\%$  increased diameter) than the established inhibitor of aneurysm formation in mice, doxycycline ( $51\% \pm 21\%$ ), that we incorporated in our study as a control for treatment effectiveness ( $p < 0.05$ ). Representative ultrasound photographs depict the lumen of the abdominal aorta in the different groups, revealing the preserved vessel diameter in the E64- and doxycyclin-treated mice (Fig.5B).

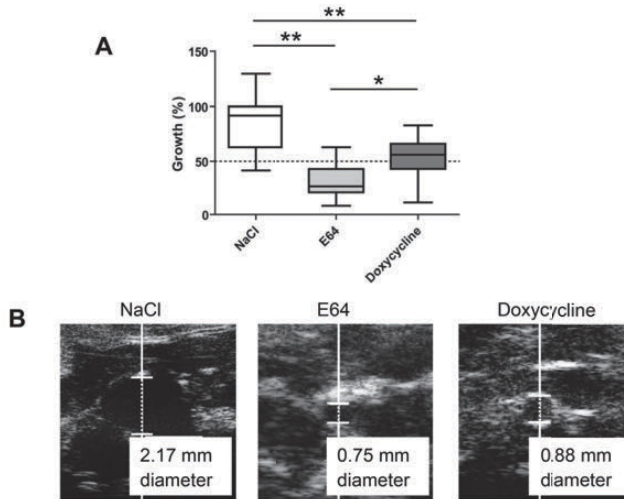
## DISCUSSION

This study shows that cysteine protease-mediated matrix degradation is prominent in human AAA, and that comprehensive cysteine protease inhibition prevents extensive degradation of the vessel wall and aortic dilatation in two established mouse models.

AAA pathology comprehends chronic inflammation accompanied by proteolytic imbalance, with ultimate aortic rupture due to loss of collagen fibers<sup>25</sup>. Cathepsins K, -L and -S exhibit collagenolytic enzyme activity, yet for a long time this was assigned to the MMP subfamily of collagenases<sup>3</sup>. To



**Figure 4.** Reduced elastin breaks and enhanced collagen content after E64 treatment. Elastic lamina breaks were counted as a measure for medial integrity of the abdominal aorta (A; \* $p = 0.05$ ). Examples of elastic lamina disruptions (red star) in the media in the presence or absence of E64 are shown (B). Collagen content of the vessel wall was analyzed as a measure for vessel wall stability (C; \* $p < 0.02$ ). Collagen (red) degradation was most prominently observed in the media (D). Dotted line indicates internal elastic lamina and black line indicates outer elastic lamina. Adv, adventitia; Ath, atherosclerosis; M, media.



**Figure 5.** E64 prevents aortic dilatation in the elastase model. Upon elastase infusion more than 50% increase in aortic diameter is observed after 2 weeks, which is inhibited by E64 or doxycycline (\*\* $p < 0.001$ )(A). The E64-treated mice revealed less diameter growth than the doxycycline group (\* $p < 0.05$ ). Representative ultrasound photographs are shown (B).

to assess the relative contribution of cathepsins and MMPs in collagen degradation, we measured CTX and ICTP fragments and observed minimal levels of the MMP-mediated ICTP fragments, whereas CTX levels were 10-fold higher in aneurysm compared with normal aortic tissues. This may reflect a minor role for the MMPs in aneurysm formation, however, it should be noted that ICTP fragments are larger than CTX fragments and contain multiple cathepsin cleavage sites. As such, cysteine proteases degrade ICTP fragments, which may explain the relatively low levels of ICTP fragments<sup>17</sup>.

Others and we previously demonstrated increased expression of cathepsin K, -L and -S in human aneurysm tissue, at both mRNA and protein level<sup>3-8</sup>. While the expression level of the proteases was increased, a reduction in the expression of its endogenous inhibitor cystatin C was correlated with aneurysm pathology in human and mouse<sup>8,9</sup>. We now investigated the effect of cysteine protease inhibitor E64 in two murine AAA models. E64 prevented AAA formation and severity in both models, yet more potently in the elastase AAA model. This may be due to differences between the AAA models, with the AngII model having a more systemic effect compared to the local focus in the elastase model<sup>26</sup>, or more practical that the concentration used in the elastase model was more efficient.

AngII is a strong pro-inflammatory factor, mediating signaling via the AngII-receptor-1. We observed reduced levels of KC and IFN $\gamma$  upon E64 treatment. We have previously shown in human AAA tissue that the monocyte/neutrophil chemo-attractant interleukin-8 (IL-8) is increased in the vessel wall<sup>27</sup>. In the current study, we demonstrated that KC, the murine equivalent of IL-8, is high in the serum of AngII-infused mice and decreased upon E64 treatment and correlates with a decrease in vessel wall damage. The role of IFN $\gamma$  in development of AAA is controversial<sup>28,29</sup>. In our study, increased IFN $\gamma$  is associated with increased aorta pathology, which is in line with the findings of Xiong *et al*, who demonstrated that IFN $\gamma$ -deficient mice do not develop AAA<sup>29</sup> and with Zhou

*et al*, who revealed IFN $\gamma$  production promotes AAA formation<sup>30</sup>. The reduced cytokine levels may reflect decreased aorta wall damage upon E64, because elastic lamina breaks and collagen degradation products in itself are a pro-inflammatory stimulus and matrix degradation was reduced in E64-treated mice.

Our study, showing that E64 inhibits AAA development, is in line with observations that cathepsin K or -L deficiency results in decreased aneurysm formation in mice in the elastase model<sup>13,14</sup>, suggesting that cathepsin K and L are prominent cathepsins in this model. In the AngII-mediated AAA model cathepsin S deficiency causes decreased AAA development<sup>15</sup> yet, Bai *et al* reported that cathepsin K gene disruption did not affect aneurysm formation<sup>31</sup>, indicating that possibly redundancy by the other cathepsins compensates for the loss of cathepsin K in this model.

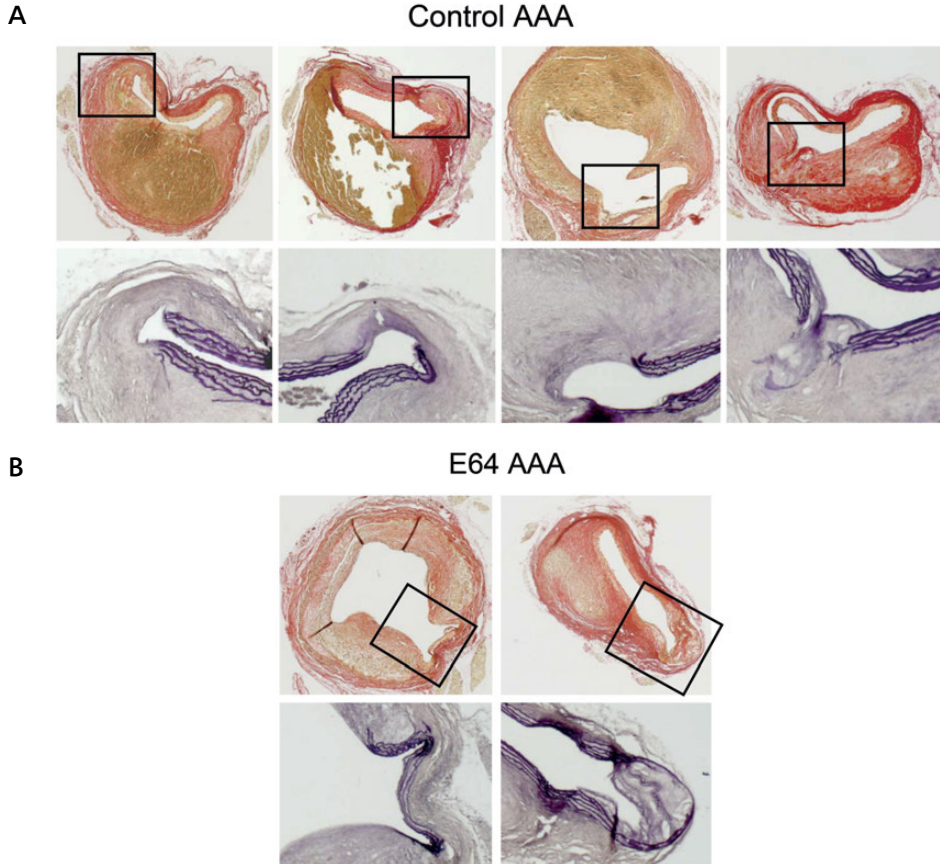
Our data suggests that the broad-spectrum cysteine protease inhibitor E64 is effective to reduce aneurysm formation through inhibition of matrix degradation. Inhibition of cysteine proteases has potential therapeutic value to treat AAA. At present, we do not propose the use of E64 in humans because of its limited cathepsin specificity, however, highly specific cathepsin K inhibitors have been developed to treat human osteoporosis and are currently tested in clinical trials<sup>32</sup>. Cathepsin S inhibitors have been generated, but need further validation in patients with autoimmune diseases, for whom they have been developed<sup>33</sup>. The application of such novel inhibitors in aneurysm patients holds promise for the future. In conclusion, cysteine protease inhibition results in decreased aneurysm formation, offering an attractive therapeutic approach to prevent AAA progression.

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## SUPPLEMENTAL DATA



**Supplemental Figure 1.** Aneurysms acquired in the AngII-AAA model. Collagen staining (red) reveals the collagen content and distribution throughout the aneurysm in control (A) and E64-treated (B) mice. The medial disruption is shown in more detail (box upper panel enlarged in lower panel) by staining of the elastic laminae (purple). Clearly, there is extensive aortic damage at the location of the aneurysm.



# Chapter

# 6

## CXCL8 HYPERSIGNALING IN THE AORTIC ABDOMINAL ANEURYSM: THE ORAL CXCR2 ANTAGONIST DF2156A FULLY ABROGATES EXPERIMENTAL ANEURYSM FORMATION

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# ABSTRACT

## Background

The chemokine CXCL8 is concurrently involved in neutrophil-mediated inflammation and angiogenesis, two prominent pathological characteristics of abdominal aneurysm disease (AAA). Previous work identified CXCL8 hyperexpression as a discriminative feature of AAA disease. In the absence of molecular targets for pharmaceutical aneurysm stabilization we considered an evaluation of the CXCL8 signaling cascades in human AAA and the potential of interfering with CXCL8 signaling relevant.

## Methods

ELISA's, Western blot analysis, real time PCR and array analysis were used to explore CXCL8 expression and signaling in aneurysm wall samples from patients undergoing elective AAA repair. A role for CXCL8 in AAA disease was tested through the oral CXCR1/2 antagonist DF2156A in the elastase model of AAA disease.

## Results

There is an extreme disparity in aortic wall CXCL8 content between AAA and atherosclerotic disease (median [IQR] aortic wall CXCL8 content: 425 [141 - 1261] (AAA) vs. 23 [2.8 - 89]  $\mu\text{g/g}$  protein (atherosclerotic aorta), ( $P < 1.5 \cdot 10^{-15}$ )) and abundant expression of the CXCR1 and 2 receptors in AAA. Array analysis followed by pathway analysis showed that CXCL8 hyper-expression in AAA is followed increased by IL-8 signaling ( $p < 0.000039$ ).

Interference with CXCL8 signaling through DF2156A fully abrogated AAA formation in the murine elastase model of AAA disease ( $p < 0.001$ ).

## Conclusion

Activation of the CXCL8-signaling pathway is a prominent and distinctive feature of AAA. Interference with the pathway constitutes a promising target for medical stabilization of AAA.

## INTRODUCTION

An Abdominal Aortic Aneurysm (AAA) is a common pathology and a major cause of death due to rupture<sup>1</sup>. Most AAAs are asymptomatic and remain undetected until rupture<sup>1</sup>. Hence, some countries instigated nationwide screening programs for the identification of AAA. These programs resulted in a major increase in patients with an identified AAA, most of them small in size.

In accordance to prevailing guidelines these patients with smaller AAAs are kept under surveillance until the AAA reach the threshold for repair at 55 mm. It is estimated that up to 70% of the patients in the watch and follow up program will eventually reach the 55 mm intervention threshold<sup>2</sup>. Accordingly, it has been pointed out that pharmaceutical intervention reducing or inhibiting progression of small AAA, and thus reducing the need for surgical repair could have major advantages; both from a patients' as from a socio-economical perspective<sup>3</sup>. Despite clear preclinical successes, no pharmaceutical intervention has been proven to be effective so far<sup>4</sup>.

The pathology of growing AAAs is thought to be a localized chronic inflammatory response that is accompanied and perpetuated by exaggerated angiogenesis and a proteolytic imbalance; the latter is being held responsible for a progressive weakening of the aortic wall<sup>5</sup>. The actual molecular basis has not been identified.

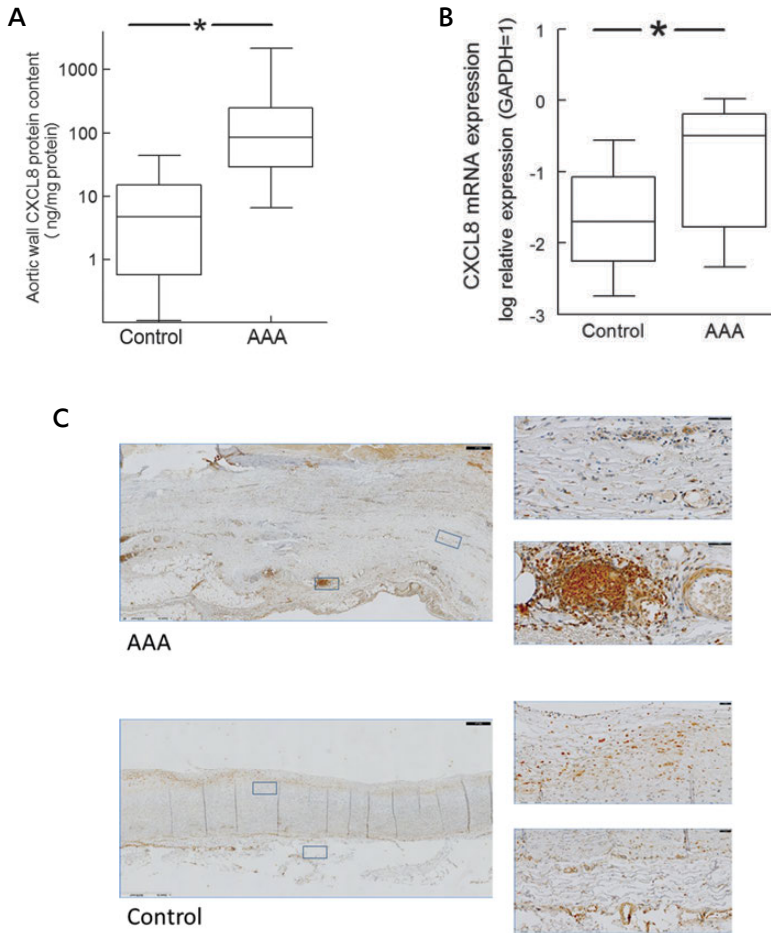
We previously documented CXCL8 hyper-expression as a clear distinctive and unique feature of AAA with 300-fold higher CXCL8 protein levels in the aneurysm wall than in advanced aortic atherosclerotic wall samples<sup>5,6</sup>. CXCL8 has comprehensive chemotactic effects on a wide-variety of immune cells, in particular *but* not-exclusively on neutrophils; a cell type that is explicitly implicated in AAA disease<sup>5,7,8</sup>. Moreover, CXCL8 stimulates protease expression and inflammation<sup>9</sup>, and exerts strong pro-angiogenic effects by promoting chemotaxis and proliferation of endothelial cells<sup>10-12</sup>.

In this context, we considered further examination of a putative role for CXCL8 signaling as a potential therapeutic target in AAA disease relevant. The present study confirms the CXCL8 hyper-expression and exaggerated activation of the CXCL8 downstream pathways in human aneurysms, and shows that interference with CXCL8 signaling through the oral CXCR1/2 antagonist DF2156A fully abrogates aneurysm formation in an accepted model of AAA disease (the murine elastase model).

## RESULTS

### CXCL8 expression in human abdominal aortic aneurysms

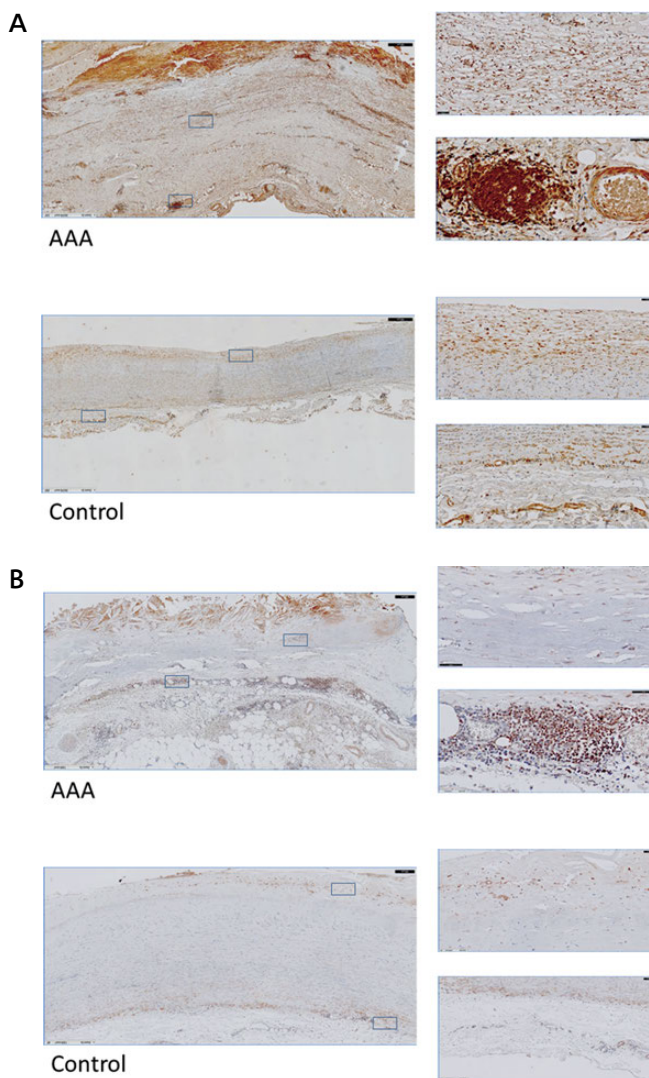
We first performed a validation of our previous observations of CXCL8 hyper-expression in 238 AAA wall samples and control aorta samples from the Aneurysm-Express Biobank. Results confirmed previous observations and showed a several hundred-fold increase CXCL8 protein content in aneurysm wall samples ( $P < 1.5 \cdot 10^{-15}$ , Figure 1A) and an approximately 16-fold higher CXCL8 mRNA expression ( $P < 0.01$ , Figure 1B). Immunohistological staining for CXCL-8 in the aneurysm wall shows comprehensively expression in macrophages, neutrophils, and smooth muscle cells; as well as in a subpopulation of lymphocytes, and occasional endothelial cells (Figure 1C). Expression in advanced aortic atherosclerotic disease on the other hand is essentially confined to foam cells, macrophages, and occasional smooth muscle cells the intimal layer and intimal border zone of the medial layer of the aortic wall, and incidental lymphocytes (Figure 1C).



**Figure 1.** CXCL expression in human AAA and controls (atherosclerotic aorta). A) Extreme disparity in aortic wall CXCL8 protein content. AAA vs control:  $P < 1.5 \cdot 10^{-15}$ . B) Aortic wall CXCL8 mRNA content. AAA vs Control  $P < 0.01$ . C) Representative samples showing aortic wall CXCL-8 distribution in AAA and control aorta. Overview 5X, detail 40x.

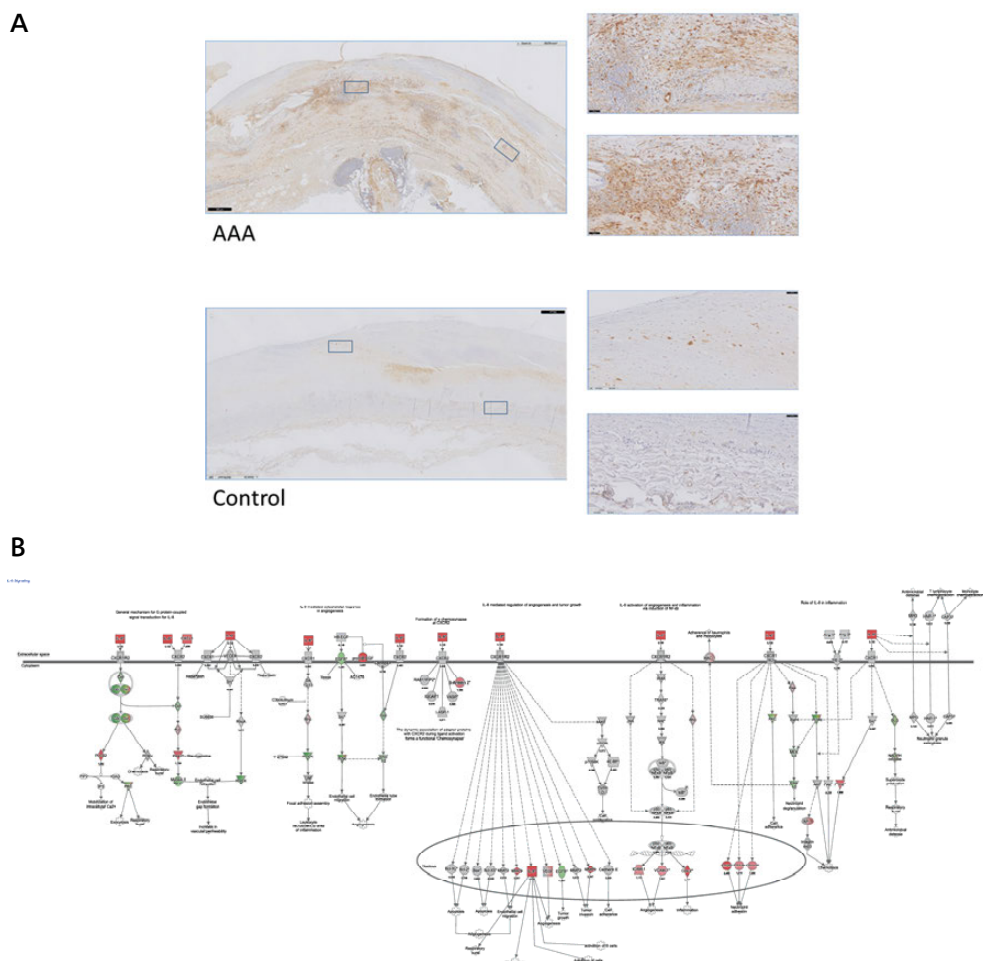
### Abundant presence and activation of the CXCL8- pathway in human abdominal aortic aneurysms

CXCL8 signaling in humans is through its receptors: CXCR1 and 2. CXCL8 binding to these receptors results in activation of multiple effector pathways such as phosphorylation of ERK (extracellular signal regulated protein kinase) 1/2. Immunohistochemical analysis shows abundant expression of both the CXCR1 and CXCR2 receptors (Figure 2) and enhanced ERK phosphorylation in AAA compared to aortic atherosclerotic disease. CXCL8 signaling was further explored through Ingenuity-based transcriptomics analysis. This analysis identified CXCL8 (IL-8) signaling pathway and Agranulocyte Adhesion and Diapedesis among the top upregulated pathways in AAA disease ( $p < 0.000039$  and  $< 0.00000049$  respectively).



**Figure 2.** CXCR1 and CXCR2 expression in human aortic wall. A) Representative samples showing aortic wall CXCR1 distribution in AAA and control aorta. Overview 5X, detail 40X. B) Representative samples showing aortic wall CXCR2 distribution in AAA and control aorta. Overview 5X, detail 40X.

CXCL8 has particularly strong effects on neutrophil chemotaxis, stabilization and activation, and is described to be the dominant promoter of CXC chemokine-mediated angiogenesis. Histologic evaluation shows abundant and dispersed neutrophils (MPO staining) in human aneurysms, while neutrophils are absent in control atherosclerotic samples (Figure 4). Occasional neutrophils in the vaso vasora confirmed the validity of the staining. This characterizes neutrophils abundance as a clearly distinctive feature of human AAA.

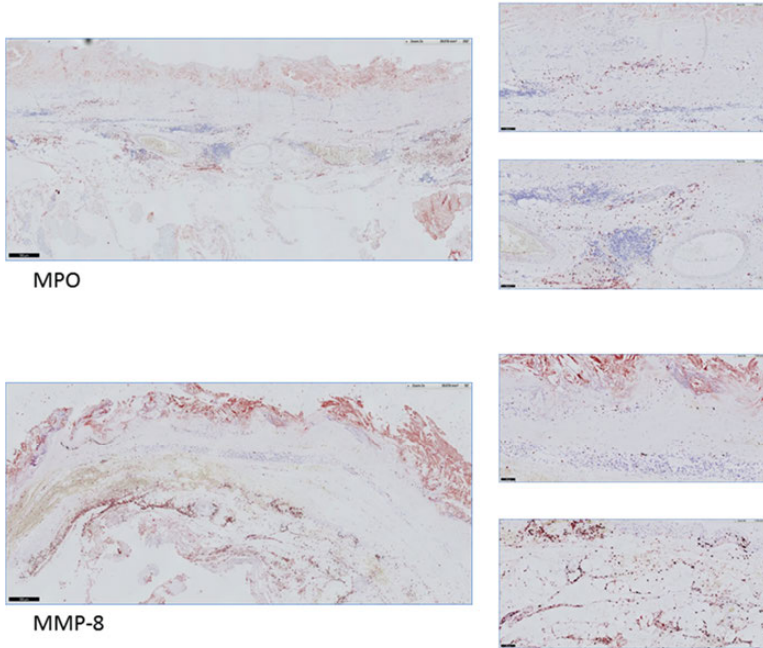


**Figure 3.** CXCL-8 signaling in human aorta. A) Representative samples showing aortic wall pERK1/2 distribution in AAA and control aorta. Overview 5X, detail 40x. B) CXCL-8 signaling in AAA (Ingenuity Pathway Analysis).

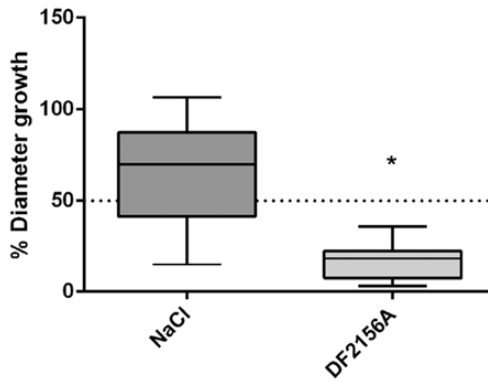
### CXCR1/2 inhibition abrogates aneurysm formation

To evaluate possible involvement of the CXCL8 in aneurysm formation, we tested whether the oral CXCR1/CXCR2 inhibitor (DF2156A) influences aneurysm formation in the established murine elastase model of the disease. Mice ( $n=10$ ) received DF2156A during two weeks via daily oral gavage starting from the day before elastase perfusion. Control animals ( $n=10$ ) received daily oral gavage with saline. At the day before elastase perfusion, 7 days and 14 days after perfusion the aortic diameter was measured via ultrasound. Aortic dilatation at day 7 was similar in both groups (12.7% SD  $\pm$ 8.5% (DF2156A) and 21.5 % SD  $\pm$ 14.9% (vehicle),  $p=0.161$ ). A daily gavage completely abolished aneurysm formation, measured at day 14, in all animals (17.7% dilatation SD  $\pm$ 9.6%, (DF2156A) and 71.9% SD  $\pm$  26.7% (vehicle)  $p<0.001$ ) (Figure 5).

DF2156A treatment almost completely quenched vascular inflammation and preserved the integrity of the vessel wall as shown by an increased collagen content and less elastin breaks

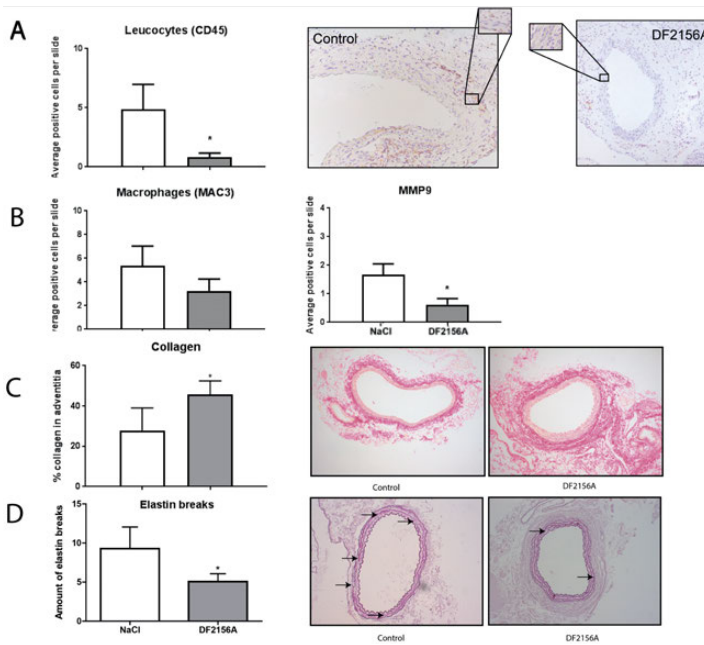


**Figure 4.** Neutrophil abundance in AAA.



**Figure 5.** The CXCR1/2 antagonist DF2156A fully abrogates AAA formation. The oral CXCR1/2 antagonist DF2156A (reperitaxim) fully abrogates aneurysm development in the elastase model of AAA disease. Percentage given is the increase in aorta diameter from baseline at day 14.  $P < 0.001$ .

(Figure 6d). Furthermore, treatment with the CXCR1/CXCR2 inhibitor resulted in significantly less leucocytes ( $p < 0.05$ ) and limited the MMP9 expression ( $p < 0.05$ ) compared to the controls (Figure 6a and b). While all mice revealed similar macrophage counts ( $p = 0.98$ ) (Figure 6b), which reveals that the effect on vascular inflammation is highly selective.



**Figure 6.** The CXCR1/2 antagonist DF2156A quenches vascular inflammation and preserves adventitial collagen, and medial elastin. The oral CXCR1/2 antagonist DF2156A reduces aortic wall leucocyte content (CD45 staining) and MMP-9 expression (both  $P < 0.05$ ) but does not influence aortic wall macrophage content (MAC3) staining, adventitial collagen and reduces medial elastin breaks. All data shown is for day-14.

## DISCUSSION

This study confirms CXCL8 hyper-expression and enhanced activation of the CXCL8 axis as a distinctive feature of AAA. Interference with the oral CXCL8 antagonist fully abrogated AAA formation, characterizing this axis as a potential pharmaceutical target for AAA.

Our previous work identified CXCL8 hyperexpression as a unique feature of human AAA.<sup>13</sup> These observations were validated in this study in an independent and large patient cohort (Aneurysm Express) using a different analysis platform. Immunohistochemistry for CXCL8 showed comprehensive expression in both leucocytes as well as mesenchymal cells. This pattern was clearly distinct from advanced atherosclerotic disease in which expression was predominantly confined to the intima, in particular to foam cells.

In humans CXCL8 signals through the CXCR1 and -2 receptors. These receptors have different affinities, suggesting distinct responses at varying CXCL8 levels. The activities are thought to be partially overlapping activities, but they also appear to mediate distinct aspects of CXCL-8 mediated inflammation<sup>22</sup>. The expression pattern is broad, and includes a wide variety of leucocytes, mesenchymal cells (smooth muscle cells, fibroblasts) and endothelial cells. Immunohistochemical staining for CXCR1 and 2 followed by morphological analysis showed abundant receptor expression in leucocytes as well as smooth muscle cells and (myo)fibroblasts of the aneurysm wall. Observations above not only identify CXCL8 hyperexpression as a clear distinctive feature between AAA and

atherosclerotic disease, but also show that the transcriptional machinery required for CXCL8 signaling is present in AAA. Prominent ERK1/2 phosphorylation, and Ingenuity based pathway analysis confirmed exaggerated CXCL8 signaling as a distinctive feature of AAA disease.

CXCL8 classically associates with neutrophil influx and neutrophil-mediated inflammation. CXCL8 not only acts as a strong chemo-attractant for neutrophils, but it also increases neutrophil half-life by preventing apoptosis, thereby further contributing to neutrophil abundance in AAA disease.<sup>23</sup> These neutrophils may contribute to the proteolytic imbalance through release of multiple proteases such as the serine protease neutrophil elastase and metalloproteinases MMP8 (neutrophil collagenase) and MMP9 (neutrophil gelatinase), but also through protease mediated degradation of protease inhibitors such as TIMPs (through the action of neutrophil elastase) and cystatin C (through MMP9 and neutrophil elastase)<sup>5</sup>. A crucial role for neutrophils in AAA disease is emphasized by several animal studies in which interference with either neutrophil activation or infiltration alleviates AAA formation and/or progression<sup>24,25</sup>. Apart from its effects on neutrophils, CXCL8 also has potent pro-angiogenic effects and influences other leucocytes (in particular M1 macrophages<sup>26</sup>) thereby contributing to a proinflammatory environment<sup>27</sup>. Angiogenesis is a characteristic feature that has been linked to vascular inflammation and AAA rupture, and as such has been brought forward as a therapeutic target for pharmaceutical AAA stabilization<sup>28</sup>.

The CXCL8-pathway has long been identified as potential pharmacological target for several acute and chronic inflammatory conditions<sup>21;29</sup>. Combined CXCR1/2<sup>30;31;33</sup> and selective CXCR2 inhibitors are currently under clinical evaluation<sup>32;33</sup>.

We tested the ability of the combined CXCR1/2 antagonist DF2156A to inhibit AAA formation in an established murine model of the disease. A single daily dose strongly reduced AAA formation; in fact the minimal dilatation observed presumably reflects the effect of pressure-perfusion and/or the loss of elastic recoil by the elastase treatment, and not the influx of any inflammatory cells. The effects exerted by DF2156A in the model by far exceed the effects reported for other established anti-inflammatory agents, such as doxycycline<sup>34</sup>, indomethacin<sup>35</sup> or cyclosporin<sup>36</sup>.

Considering the apparent failure of medical stabilization of small AAA so far, the potency of CXCR1/2 inhibition *in vivo* is remarkable and merits clinical evaluation. As mice only express CXCR2, we could not test the contribution of each receptor to the process of clinical AAA formation. Consequently it is unclear whether clinical trial with selective CXCR2 inhibitors would be equally effective as a combined CXCR1/2 antagonist.

In conclusion, to our knowledge, this study is the first to demonstrate full abrogation of aneurysm formation in the murine elastase model, emphasizing the critical role of the CXCR2-axis in aneurysm formation in the model. This and along with the clinical data, identifies activation of the CXCL8-pathway as a distinctive feature of AAA and characterizes this pathway as a promising (possibly the most promising) target for the medical stabilization of growing AAA.

## METHODS

### Human Samples

The investigation conforms the principles outlined in the Declaration of Helsinki (2013). Sample collection and handling was performed in accordance with the guidelines of the medical ethical

committee of the Leiden University Medical Center. We obtained tissue from anterior-lateral aneurysm wall during elective surgery for asymptomatic AAA (>5.5 cm or larger). Aortic tissue samples removed along with the renal artery during kidney ex-plantation from brain-dead, heart-beating, adult organ donor, were used as control samples. Aortic wall samples were divided in two parts. One half was immediately snap-frozen in CO<sub>2</sub>-cooled isopentane or liquid N<sub>2</sub> and stored at -80°C for later analysis. The other half was fixed in 4% formaline for 12 hours and decalcified. The latter segments were paraffin embedded and 4 µm sections were processed into slices. For immunohistochemistry, sections (n=10 AAA, n=10 control atherosclerotic aortic wall samples) were deparaffinized, treated for 10 minutes with H<sub>2</sub>O<sub>2</sub> to block endogenous peroxidase activity, and incubated overnight at room temperature with the primary antibody diluted in PBS-1% albumin. The following primary antibodies were used: human myeloperoxidase (DAKO), CXCL8 (bs-078012, Bioss, Huissen, The Netherlands), CXCR1 (ab124344, Abcam, Cambridge UK), CXCR2 (bs-1629R, Bioss), pERK1/2 (1481-1 Epitomics, Leiden, The Netherlands). CXCL8 mRNA expression was quantified by semi quantitative RT, to that end a total RNA extraction was performed according to manufacturer's instructions. cDNA was prepared by using a Promega kit (Proega, Leiden, the Netherlands) for RT-PCR. For the determination of mRNA expression we used an established CXCL8 primer/probe set (Thermo Fisher Scientific, Bleiswijk, The Netherlands), the mastermix (Eurogentec, Maastricht, the Netherlands) and the ABI-7700 system (Thermo Fisher Scientific) as previously described<sup>13</sup>. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Thermo Fisher Scientific) was used for normalization. Aortic wall CXCL8 protein content was determined using the Aneurysm-Express Biobank<sup>14</sup> (n=238 AAA samples and n=26 control atherosclerotic samples) via ELISA, employing Luminex multianalyte profiling technology<sup>15;16</sup>, using a bio-plex system (Bio-Rad, Veenendaal, the Netherlands). Total protein concentration of every sample was quantified via a BCA protein measurement method (Thermo Fisher Scientific). All measured concentrations were related to the protein concentration of every sample. Inter-assay coefficient of variation was <10%. Microarrays: RNA extraction was performed from full thickness aortic wall samples from 31 AAA patients (mean age 69.5 yrs. mean diameter 62.3±12.1 mm) collected during elective aneurysm repair and 9 control samples (infra renal aorta obtained during kidney procurement for donation).

RNA from aneurysm wall was labeled and hybridized to Illumina HumanHT-12 v4 BeadChips (Illumina, Eindhoven, the Netherlands). Arrays were scanned with an Illumina iScan microarray scanner. Bead level data preprocessing was done in Illumina GenomeStudio.

Analysis of array data: Quantile normalization and background reduction were performed according to standard procedures in the Illumina GenomeStudio software.

Association of genome-wide expression data with AAA phenotype revealed 11486 transcripts with P<0.05. These differentially expressed transcripts were used as an input for pathway analysis through Ingenuity Pathway Analysis suite (<http://www.ingenuity.com>, accessed 2016). Levels of significance were determined using Fisher's exact tests implemented in the software.

### Elastase model

All murine investigations were approved by the Leiden University Medical Center animal welfare committee and were in compliance with the Dutch government guidelines. Eight-to-ten weeks old, male, C57BL/6 mice were obtained from Charles River, France. The aneurysms were created

via porcine pancreatic elastase (PPE) infusion as previously described<sup>17-20</sup>. After the elastase infusion 0.05-0.1 mg/kg/12hrs buprenorfine was given and the mice recovered with free access to food and water. The oral CXCR1/2 antagonist DF2156A (6 mg/kg), a generous gift from Dompé Pharma, Milan, Italy<sup>21</sup> was given (n=10) daily via oral gavage in 100 microliter of 0.25% carboxymethylcellulose diluted in PBS. Treatment was started the day before the elastase infusion and the mice were sacrificed 14 days after the infusion. Control animals (n=10) received daily oral gavage of 100 microliter of 0.25% carboxymethylcellulose diluted in PBS for 15 days. To compare the aortic growth rates of the different groups we measured the maximum axial diameter of the aorta by means of ultrasound one day prior to elastase infusion, after one week and two weeks after infusion by means of the Vevo 770 Imaging system using RMV 704 microvisualization scan head (Visualsonics, CA). At day 14 after the elastase infusion, the mice were sacrificed and the aorta was removed and embedded in paraffin for later analysis. Immunohistochemical sections were deparaffinized and incubated overnight at room temperature with the primary antibody diluted in PBS -1% albumin. The sections were incubated with CD45 (BD Pharmingen, Breda, The Netherlands), MAC3 (BD Pharmingen), MMP9 (Santa Cruz Biotechnology) and MPO (Abcam). Additional sections were stained with Sirius Red for collagen and Weigert's elastin stain to visualize elastic laminae. Six slides per animal were used per staining for analysis and only moderate or strongly reactive cells were counted as positive. The slides were blindly evaluated. The mean value for positive staining cells on six slices was calculated for each animal.

### Statistical Analysis

All values are shown as mean (SD) and probability values of  $P < 0.05$  were considered statistically significant. After performing an ANOVA test to explore the difference between human AAA and human atherosclerotic samples, an unpaired t-test was performed. The Mann-Whitney U test was used to detect significant difference in aortic diameter and in cell count between the two groups of mice. All analysis were performed using SPSS 23.0 (SPSS Inc. Chicago).

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# Chapter

# 7

## COLCHICINE INHIBITS AORTIC ANEURYSM FORMATION IN A RODENT MODEL

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## ABSTRACT

Abdominal aortic aneurysms (AAA) are characterized by chronic trans mural inflammation. Neutrophils, in particular, have been described to be pivotal in this process. Colchicine is known to diminish neutrophil chemotaxis and therefore might be beneficial for stabilizing AAA growth. The aim of this study is to test the effect of Colchicine in a murine model of AAA disease and to explore the mechanisms by which Colchicine might engage in the chronic inflammation seen in the human AAA wall.

We identified the effect of Colchicine on AAA formation in an established murine model of AAA disease. Male 8-10 week old, wild type C57/BL6 mice underwent aortic perfusion with porcine pancreas elastase. The mice were treated either with Colchicine or as a control group with saline. At baseline, day 7 and day 14 the aortic diameter was measured by ultrasound. 14 days after the elastase perfusion aortas were harvested for immunohistochemical analysis.

Experimentally treated animals had significantly less growth in aortic diameter ( $31.71\pm 14.97\%$ ) compared to the control animals ( $79.25\pm 34.88\%$ ) ( $p < 0.01$ ). Quantitative immunohistochemical staining indicated that the number of tissue leukocytes were significantly decreased in Colchicine treated animals (15 vs 44 cells per aorta,  $p < 0.05$ ).

We propose two mechanisms for Colchicine to engage in human AAA growth. The first mechanism that is proposed states that Colchicine might directly reduce neutrophil chemotaxis in the AAA wall. The second mechanism describes that Colchicine might indirectly interfere with neutrophils via the NALP3 inflammasome. Here we find the NALP3 inflammasome and its effector interleukins IL1 and IL18 prominently present in the human AAA wall.

This, and the finding that Colchicine reduces murine AAA formation after elastase perfusion indicate that Colchicine might be a promising means to stabilize human AAAs.

## INTRODUCTION

Excess protease activity caused by chronic inflammation is thought to be a critical factor in abdominal aortic aneurysm (AAA) growth by destructing structural proteins as elastin and collagen, which ultimately leads to AAA rupture<sup>32</sup>. Currently, pharmaceutical therapies that interfere with chronic inflammation are widely investigated in order to reduce the growth of small AAAs and thereby to reduce the need for surgical repair<sup>3,13</sup>.

To date however, no pharmaceutical intervention has been shown to effectively interfere with AAA progression<sup>31</sup>. Therefore further investigation of the mechanisms of chronic inflammation, observed during AAA progression, is needed. Recently, it has been described that neutrophils are one of the key elements of chronic inflammation. They are responsible for orchestrating an ongoing inflammatory immune response in the human AAA wall<sup>5</sup>. Additionally, neutrophils appear to be an important source of cytokines found in the aneurysm wall, e.g. CXCL8<sup>20</sup>. Previous research including our own has indicated that interfering with neutrophil chemotaxis, by inhibiting of the neutrophil receptors or by the using of L-selectin knockout mice reduces or even abrogates aneurysm formation<sup>15</sup>.

The immune modulatory drug Colchicine interferes with several aspects of neutrophil activation.

Besides the direct effect of Colchicine on neutrophils, an indirect effect of Colchicine on neutrophils has been described via the NALP3 inflammasome<sup>26</sup>. It has been described that the NALP3-inflammasome via caspase-1 activates IL1 $\beta$  and IL18. This induces endothelial cell and fibroblast IL6 and CXCL8 production thereby causing neutrophil chemotaxis and preservation<sup>11; 26; 29</sup>.

The aim of this study is to test the effect of Colchicine in a murine model of AAA disease and to explore the mechanisms by which Colchicine might engage in the chronic inflammation seen in the human AAA wall.

## METHODS

### Elastase model

All murine investigations were approved by the Leiden University Medical Center animal welfare committee and were in compliance with the Dutch government guidelines. Eight-to-ten week old, male, C57/BL6 mice were obtained from Charles River, France. The aneurysms were created via porcine pancreatic elastase (PPE) infusion as previously described<sup>1</sup>. After the elastase infusion 0.05-0.1 mg/kg/12hrs buprenorfine was given and the mice recovered with free access to food and water. Colchicine (1mg/L) (Sigma-Aldrich, St Louis, MO, USA), was daily provided by drinking water. Treatment was started the day before the elastase infusion and the mice were sacrificed 14 days after the infusion. Control animals received plain drinking water daily for 15 days. To compare the aortic growth rates of the different groups one observer blindly measured the maximum axial diameter of the aorta by means of ultrasound one day prior to elastase infusion, after one week and two weeks after infusion by means of the Vevo 770 Imaging system using RMV 704 microvisualization scan head (Visualsonics, Ontario, CA). At day 14 after the elastase infusion, the mice were sacrificed and the aorta was removed and embedded in paraffin for later analysis. Immunohistochemical sections were deparaffinized and incubated overnight at room temperature with the primary antibody diluted in 1% PBSA. The sections were incubated with CD45 (clone 30-F11,

BD Pharmingen, USA), MAC3 (clone M3/84, BD Pharmingen, USA) and MMP9 (C-20, Santa Cruz Biotechnology, USA). Additional sections were stained with Weigert's elastin stain to visualize elastic laminae. Staining with haematoxyline-phloxine and saffron (HPS) staining was performed to provide an overview of the murine aortic wall. Eight slides per animal were used per staining for analysis and only moderate or strongly reactive cells were counted as positive. The slides were blindly evaluated. The mean value for positive staining cells on eight slides was calculated for each animal.

### Human Samples

The investigation conforms to the principles outlined in the Declaration of Helsinki (59<sup>th</sup>, October 2008). Sample collection and handling was performed in accordance with the guidelines of the medical ethical committee of the Leiden University Medical Center. For the control samples; all human aortic arterial wall samples were provided by the Vascular Tissue bank (Department of Vascular Surgery, Leiden, The Netherlands). None of the patients in the study had a history of diabetic or chronic inflammatory disease. The primary cause of death in the control group was fatal brain injury due to a major head trauma or subarachnoidal bleeding.

The abdominal aortic aneurysm samples were obtained from the anterior-lateral aneurysm wall during elective surgery for asymptomatic AAA (>5.5 cm or larger).

Both control and aneurysm samples were cut in half. One half was immediately snap frozen in liquid nitrogen and stored at -80°C for mRNA (real time PCR analysis) and protein (multiplex analysis). The snap-frozen samples were partly used for total RNA extraction, which was performed according to manufacturer's instructions. Subsequently, cDNA was prepared and the RT-PCR for IL1 $\beta$  and IL18 (Life technologies, Paisley, UK) was conducted as previously described<sup>21</sup>. Another part of the snap-frozen human tissue samples was used for multiplex assay using a Bio-Plex 17 panel for multiple cytokines (Bio-Rad Laboratories, Hercules, CA, USA) as previously described<sup>20</sup>.

The other half of the control and aneurysm samples was fixed in 4% formalin for 12 hours and decalcified. Afterwards the fixed segments were paraffin embedded and 4  $\mu$ m sections were processed into slices. Immunohistochemical sections were deparaffinized, treated for 10 minutes with H<sub>2</sub>O<sub>2</sub> to block endogenous peroxidase activity and incubated overnight at room temperature with the primary antibody diluted in PBS-1% albumin. The following primary antibody was used: NALP3 (Abcam, Cambridge, UK). Envision mouse (Dako, Glostrup, Denmark) was used as secondary antibody. Sections were stained with DAB (Dako, Glostrup, Denmark).

### Statistical Analysis

All values are shown as mean (SD) and probability values of P<0.05 were considered statistically significant. The Mann-Whitney U test was used to detect significant difference in aortic diameter and in cell count between the two groups of mice. All analysis were performed using SPSS 20.0 (SPSS Inc. Chicago).

## RESULTS

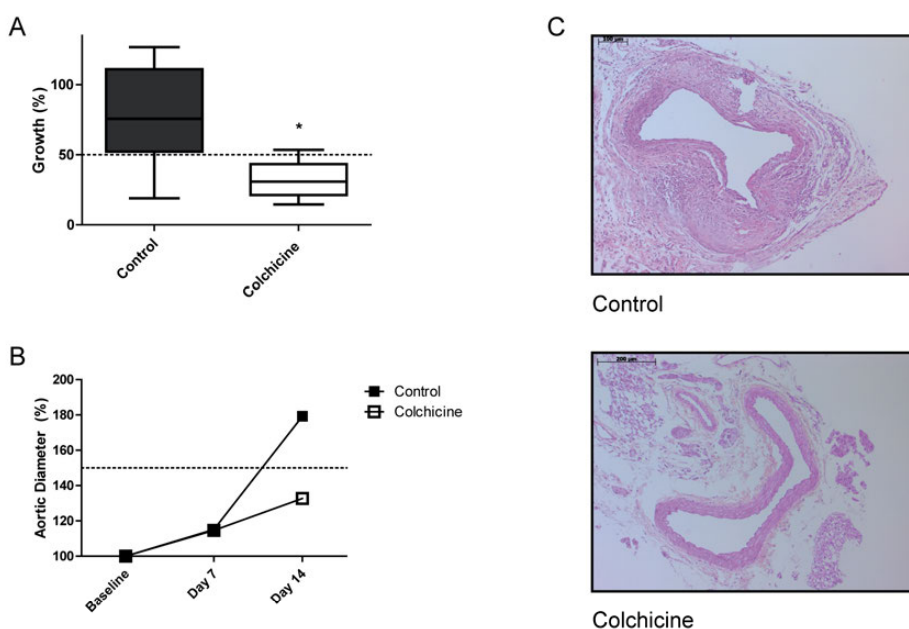
### Aortic dilatation after Colchicine treatment in the murine elastase AAA model

Aneurysm formation in control mice 14 days after elastase perfusion was found in 8 out of 9 mice (mean diameter increase  $86.76\% \pm 28.39\%$ ) and 1 revealed an increase in diameter of 19.93%. Colchicine treatment had profound effects an aneurysm was growing in only 1 of the 10 animals (mean diameter increase of  $31.71\% \pm 14.97\%$ ,  $p < 0.01$ ) (Fig. 1AB). Clear morphological differences between both groups were visualized with an overview HPS staining, the control animals revealed thickened, fibrotic aortic wall as the Colchicine treated animals had a preserved aortic structure (Fig. 1C).

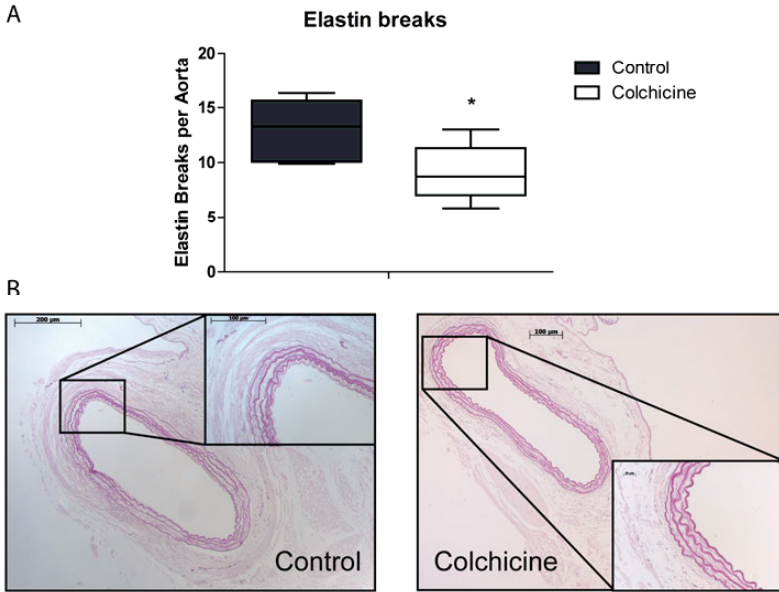
### Histological and immunohistochemical analysis of the murine aortas

Colchicine treatment preserved the elastin lamellae as the control aneurysmal aortas revealed loss of normal arterial architecture, with an increase in elastic lamellae breaks (Fig. 2).

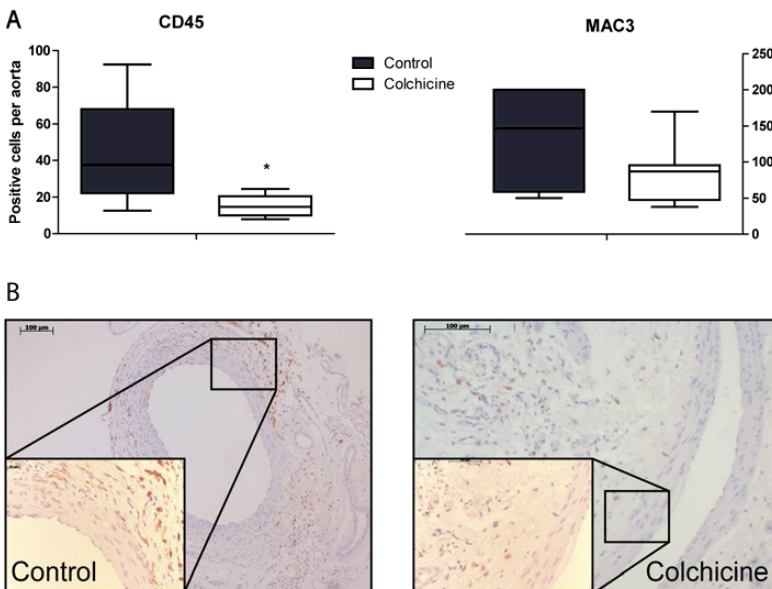
Quantitative immunohistochemical analysis showed that tissue leukocytes 14 days after elastase perfusion were significantly decreased in the Colchicine treated group as compared with the controls, 15 vs. 44 cells per aorta ( $p = 0.01$ ) (Fig. 3). The cells did localize mostly within the adventitia of the aortic wall. Colchicine did not significantly reduce the macrophage content



**Figure 1.** (A) Percentage aortic diameter growth measured by ultrasound in colchicine treated animals (colchicine) and in animals treated with saline (control) at day 14 compared with baseline. The data is presented as median range  $\pm$  minimal and maximum values. (B) Abdominal aortic development in colchicine treated animals and controls at the day before elastase perfusion (baseline), day 7 and day 14 after elastase perfusion. (C) Haematoxyline-phloxine and saffron (HPS) staining in saline treated animals (control) and colchicine treated animals (colchicine) 14 days after elastase perfusion.



**Figure 2.** (A) Elastin breaks per aorta measured in saline treated (control) and colchicine treated animals at day 14 post-elastase perfusion ( $p=0.01$ ) The data is presented as median range  $\pm$  minimal and maximum values. (B) Control and colchicine treated animals day 14 after elastase perfusion stained with Weigert's elastin stain to visualize elastic laminae.



**Figure 3.** (A) Leucocytes stained by CD45 in colchicine treated animals vs controls ( $p<0.01$ ) and macrophages visualized with MAC3 staining were similarly expressed in colchicine treated and control animals ( $p>0.05$ ). The data is presented as median range  $\pm$  minimal and maximum values. (B) Control and colchicine treated animals day 14 after elastase perfusion stained with CD45 stain to visualize leucocytes.

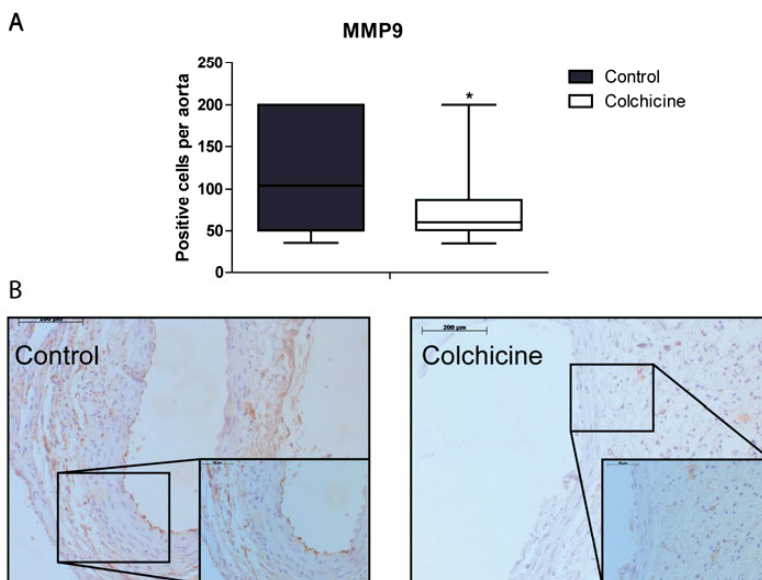
(Fig. 3), yet we found a significant decrease in neutrophil and neutrophil elastase (MMP9) expression in the Colchicine treated animals ( $p < 0.01$ ) (Fig. 4).

### Potential targets of Colchicine treatment in human aneurysms

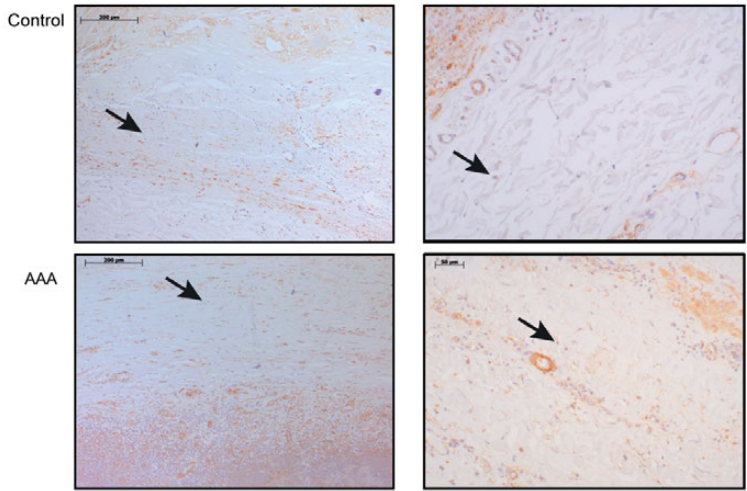
To evaluate the potential NALP3-route of neutrophil activation in human AAAs, we performed immunohistochemical staining for the NALP3 inflammasome on human AAA samples. By means of this staining, we revealed prominent expression of the NALP3-inflammasome in human abdominal aortic aneurysm tissue samples (Fig. 5). IL1 $\beta$  and IL18 were also observed to be strongly expressed in the AAA tissue. Compared with the control aortic tissue, the IL18 mRNA level was significantly higher in AAA ( $-1.75 \pm 0.50$  in control aorta,  $-1.03 \pm 0.32$  in AAA,  $p < 0.01$ ), IL1 $\beta$  expression was borderline significant ( $p = 0.09$ ). Yet, IL1 $\beta$  aortic protein content was significantly higher in the AAAs ( $0.67 \pm 0.72$  in the controls and  $5.27 \pm 4.80$  in the AAAs) (Fig. 6). These findings confirm presence of different Colchicine targets in AAA disease.

## DISCUSSION

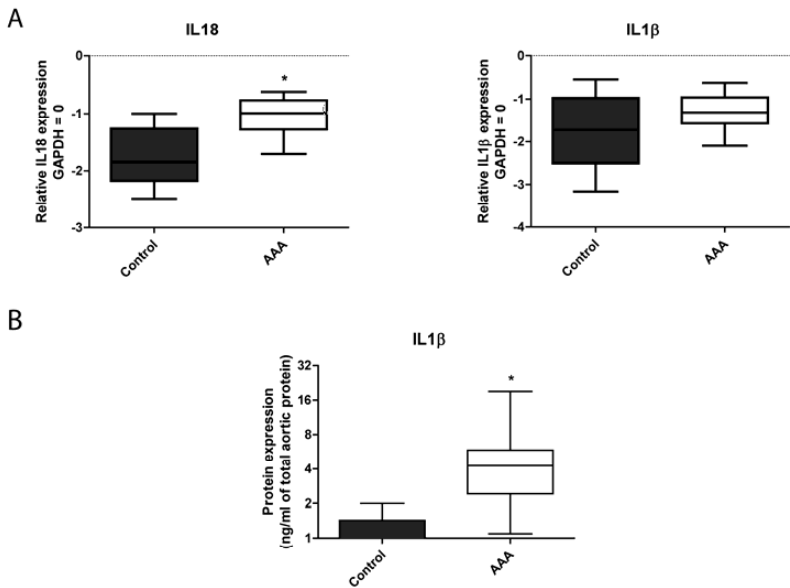
Neutrophils are thought to play a critical role in AAA disease. The immune modulatory compound Colchicine quenches neutrophil involvement through multiple and distinct mechanisms. In this study we show that Colchicine prevents AAA formation in an established murine model of elastase induced aneurysm formation. In this model, Colchicine protected the vascular wall from loss of elastin and preserved the structure of the aortic wall. Moreover, there were fewer leucocytes and neutrophils in the murine aortic wall in Colchicine treated mice compared with the controls.



**Figure 4.** (A) Matrix metallo protease 9 (MMP9) was significantly lower expressed in the colchicine treated animals ( $p < 0.01$ ) The data is presented as median range  $\pm$  minimal and maximum values. (B) Control and colchicine treated animals day 14 after elastase perfusion stained with MMP9.



**Figure 5.** NALP3-inflammasome stained in human abdominal aortic aneurysm and aortic atherosclerotic tissue (left 10x and right 20x magnification).



**Figure 6.** (A) mRNA expression of IL1 $\beta$  ( $-1.76 \pm 0.84$  in control aorta,  $-1.2 \pm 0.41$  in AAA,  $p=0.09$ ) and IL18 ( $p<0.01$ ) was analyzed via RT-PCR. (B) Differences in the protein expression level of IL1 $\beta$  was determined via a Bio-plex assay ( $p<0.01$ ).

AAAs are characterized by localized infiltration of inflammatory cells. The large amount of interleukins, TNF $\alpha$  and other pro-inflammatory cytokines released by this infiltration leads to an increase in expression of matrix metalloproteases and cathepsins, which in their turn digest

the extracellular matrix of the aortic wall and eventually thereby cause aneurysm rupture<sup>33</sup>. The activation of neutrophils, in particular, has been described to be pivotal in the process of AAA formation<sup>5</sup>. Interference with neutrophil influx or activation in the aortic wall in the elastase model has been found to completely abrogate the aneurysm formation<sup>10; 28</sup>. Also the activation of neutrophils and the abundant release of its enzymes throughout the vascular wall cause an ongoing inflammatory immune response, which leads to the chronic inflammatory state of human AAAs<sup>24</sup>. Previously we and others revealed abundant expression of CXCL8 in human AAA wall<sup>20</sup>. CXCL8 is known to be a strong chemo-attractant for neutrophils and it also prevents apoptosis, which prolongs the neutrophil survival and thereby further contributes to neutrophil abundance in the aneurysm wall<sup>6</sup>.

Colchicine directly interferes with neutrophil chemotaxis and thus provides a promising drug to alter the chronic inflammatory state of AAAs. There are two mechanisms that explain the effect of Colchicine on neutrophils. Firstly, Colchicine inhibits the priming effect of tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) on neutrophils by reducing the synthesis of TNF $\alpha$  and by down regulating its receptors on macrophages and endothelial cells. Secondly, in low concentrations, Colchicine reduces adhesion of the neutrophils to endothelium<sup>7; 26</sup>.

It is now apparent that Colchicine may also influence NALP3-inflammasome mediated inflammation<sup>26</sup>. This inflammasome is known to activate the NF $\kappa$ B-cascade and inducing indirectly neutrophil activation<sup>2</sup>. If and how the NALP3-inflammasome is involved in human aneurysm disease is unknown. Yet, recently it has been described that a part of the gene encoding for the NALP3-inflammasome, the NLRP3 gene, alters patients' susceptibility to AAA<sup>30</sup>. We here show prominent expression of the NALP3 inflammasome in human AAA samples, as well as up regulation of interleukins IL1 $\beta$  and IL18, which are activated by this inflammasome. Therefore, the NALP3-inflammasome may be involved in AAA disease.

There are several mechanisms probable to contribute to the prominent involvement of this inflammasome in human aneurysms. One of the proposed mechanisms is, as within gout, activation of the via uric acid, which is significantly increased in the wall of aortic aneurysms<sup>29</sup>. Current studies reveal that in emphysema, a disease with several pathophysiological parallels with AAA, uric acid from dying cells can activate the inflammasome<sup>6; 8</sup>. Another mechanism for activation of this inflammasome is smoking. It is known that mitochondrial reactive oxygen species (ROS) induced by cigarette smoking can activate the NALP3 inflammasome<sup>9</sup>. Smoking is one of the key risk factors for aneurysm development and when the incidence of smoking is reduced, the prevalence of AAA drops equally<sup>19</sup>. Therefore, ROS induced by cigarette smoking might be a likely explanation for inflammasome activation in human AAA walls.

A further possible explanation for the activation of the inflammasome might be induction via cholesterol crystals. High cholesterol is a well-recognized risk factor for AAA disease and cholesterol crystals have been frequently reported in the AAA wall. In atherosclerotic disease they were found to trigger the inflammasome by entering macrophages and rupturing lysosomes<sup>14; 27</sup>. Additionally, a recent study suggests activation of the inflammasome contributes to mechanical stretch-induced lung inflammation, a factor we know is important in AAA formation<sup>34</sup>. Although there are ongoing concerns about the narrow therapeutic margin between the gastrointestinal side-effects of Colchicine and its therapeutic efficacy, Colchicine has been recently shown to be

safe and efficacious in preventing the post-pericardiotomy syndrome after cardiac surgery<sup>17</sup> and in the prevention of recurrent pericarditis<sup>22</sup>. No differences in side effects were reported between placebo and Colchicine. Moreover, a recent study, in which the patients received Colchicine in a low dose for a minimum of two years, concluded that Colchicine is an attractive therapy for secondary prevention of cardiovascular events<sup>25</sup>. This, and our findings that Colchicine in the murine AAA model reduces aneurysm growth, indicate that Colchicine might be a cost-effective option for prevention of aneurysm growth and rupture in clinical practice.

Before the drug is considered for prevention of AAA growth in aneurysm patients, however, more research has to be done to investigate the effect of Colchicine on human abdominal aortic aneurysms.

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# Chapter

# 8

## IL-6: A JANUS-LIKE FACTOR IN ABDOMINAL AORTIC ANEURYSM DISEASE

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# ABSTRACT

## Background and aims

An abdominal aortic aneurysm (AAA) is part of the atherosclerotic spectrum of diseases. The disease is hallmarked by a comprehensive localized inflammatory response with striking IL-6 hyperexpression. IL-6 is a multifaceted cytokine that, depending on the context acts as a pro- or anti-inflammatory factor. In this study we explore a putative role for IL-6 in AAA disease.

## Methods

ELISA's, Western blot analysis, real time PCR and array analysis were used to explore IL-6 expression and signaling in aneurysm wall samples from patients undergoing elective AAA repair. A role for IL-6 in AAA disease was tested through IL-6 neutralization experiments (neutralizing antibody) in the elastase model of AAA disease.

## Results

We confirmed an extreme disparity in aortic wall IL-6 content between AAA and atherosclerotic disease (Median [5<sup>th</sup>-95<sup>th</sup> percentile] aortic wall IL-6 content: 281.6 [ 0.0 – 1820.8] (AAA) vs. 1.9 [0.0-37.8] µg/g protein (atherosclerotic aorta), (p<0.001). Array analysis followed by pathway analysis showed that IL-6 hyper-expression is followed by increased IL-6 signaling (p<0.000039), an observation confirmed by higher aneurysm wall pSTAT3 levels, and SOCS1 and SOCS3 mRNA expression, (p<0.018).

Remarkably, preventive IL-6 neutralization i.e. treatment started one day prior to the elastase-induction resulted in 40% 7-day mortality due to aortic rupture. In contrast, delayed IL-6 neutralization (i.e. neutralization started at day 4 after elastase induction) did not result in ruptures, and quenched AAA growth (P<0.021).

## Conclusions

AAA disease is characterized by increased IL-6 signaling. In the context of elastase model of AAA disease IL-6 appears a multi-faceted factor that appears protective upon acute injury, but that is negatively involved in the perpetuation of the disease process.

## Non-standard Abbreviations and Acronyms

GAPDH: Glyceraldehyde-3-phosphate Dehydrogenase

IL-6: Interleukin 6

PBS: Phosphate Buffered Saline

pSTAT3: phosphorylated Signal Transducer and Activator of Transcription 3

SOCS1: Suppressors of Cytokine Signaling 1

SOCS3: Suppressors of Cytokine Signaling 3



## INTRODUCTION

An abdominal aortic aneurysm (AAA) is common pathology that is considered part of the atherosclerotic spectrum of diseases<sup>1</sup>. Current clinical management fully relies on surgical repair of larger (viz. maximum diameter 55 mm or more) AAA. Yet, while traditional open repair comes with significant perioperative morbidity and mortality, endovascular therapy requires life-long follow up and the cost-effectiveness of endovascular repair is being challenged<sup>2</sup>. It has thus been pointed out that pharmaceutical therapy quenching or halting aneurysm growth holds many promises, both from the patient's point of view as from the socio-economical point of view<sup>3</sup>.

It is generally assumed that AAA growth is driven by a comprehensive inflammatory response, and consequently that interference with the inflammatory response will alleviate aneurysm progression. The validity of this approach has been well shown in numerous animal studies, but thus far clinical interventions failed<sup>4-6</sup>; a conclusion pointing to incomplete understanding of the inflammatory aspects of AAA disease.

The inflammatory footprint of AAA is complex and includes both components of an acute inflammatory response such as neutrophils and NK cells as well as aspects of a chronic inflammation (plasma cells, tertiary follicles)<sup>3</sup>. On the molecular level the disease is best defined as a general pro-inflammatory response with comprehensive and intense upregulation of pro-inflammatory cytokines and chemokines, including notably high IL-6 levels<sup>7</sup>. In fact, it has been reported that aortic wall IL-6 protein levels in AAA exceed those in advanced atherosclerotic disease by several 100-fold, implicating IL-6 as a potential critical inflammatory factor in AAA disease<sup>7</sup>. This notion is supported by robust genetic evidence linking a polymorphism in the IL-6 gene promoter (IL-6-572G>C) to AAA disease (reported odds ratio: 6.00)<sup>8</sup>.

IL-6 has long been considered a pro-inflammatory cytokine and, through its pro-inflammatory activities, a culprit in the development and complications of atherosclerotic disease<sup>9,10</sup>. Yet, it is now becoming apparent that IL-6 is versatile multifunctional (Janus-like) cytokine with functions that extend beyond that of a pro-inflammatory cytokine; and include coordination of immune and acute responses, regulation of hematopoiesis<sup>9</sup>, as well as roles in tissue protection and regeneration<sup>11, 12</sup>. Consequently, the biology of IL-6 is complex and, depending on the context IL-6 may exert protective activities as well as detrimental actions<sup>13</sup>.

In light of these observations we considered an evaluation of a putative role for IL-6 in AAA relevant. To that end, we evaluated the IL-6 signaling pathways in human AAA disease, and tested a role for IL-6 in the initiation and progression of AAA in the murine elastase model, an established model of AAA disease.

## PATIENTS AND METHODS

### Human Samples

The investigation conforms the principles outlined in the Declaration of Helsinki (2013). Sample collection and handling was performed in accordance with the guidelines of the medical ethical committee of the Leiden University Medical Center and University Medical Center Utrecht. Control infra renal aorta was selected from a tissue bank of aortic wall patches that were obtained during kidney procurement for organ donation. Characteristics of these samples have been

described elsewhere<sup>14</sup>. Controls were age matched and only samples displaying atherosclerotic lesions were included.

Aneurysm wall samples (anterior-lateral wall) were collected during elective surgery for asymptomatic AAA (55 mm or larger). Wall samples were divided in two parts. One half was immediately snap-frozen in CO<sub>2</sub>-cooled iso-pentane or liquid N<sub>2</sub> and stored at -80°C for later analysis. The other half was fixed in 4% formalin for 24 hours followed by decalcification (Kristenssen solution). Next, these segments were paraffin embedded and 4 µm sections were processed into slices.

A further analysis of the effects of doxycycline on SOCS-1 and 3 signaling was performed on cDNA samples from an earlier doxycycline intervention trial<sup>15</sup>.

### Elastase model

All animal experiments were approved by the Leiden University Medical Center animal welfare committee, and performed in compliance with the Dutch governmental guidelines.

IL-6 activity was quenched through repeated doses (4 mg/kg) of an IL-6 neutralizing antibody (MAB406, R&D systems, Abingdon, UK). This antibody and dosing scheme was earlier shown to fully quench IL-1-induced hepatic CRP expression (a process mediated by IL-6 and STAT3 signaling) in a pilot study using human CRP transgenic mice.<sup>16</sup>

Eight-to-ten weeks old, male, wild-type (WT; C57BL/6) mice were obtained from Charles River (Chatillon-sur-Chalaronne, France). Animals were housed in a temperature and humidity-controlled room on a 12:12-h light–dark cycle with ad libitum access to water and normal chow diet.

Infra renal aneurysms were created via incubation of the isolated terminal aorta segment with type I porcine pancreatic elastase (4.5 U/mL; Sigma-Aldrich, Zwijndrecht, the Netherlands) as previously described<sup>17</sup>. Briefly, the aorta was exposed and a catheter positioned at the iliac bifurcation. The catheter was removed after elastase infusion, and the abdomen closed after hemostatic control. Mice were given 0.1 mg/kg/12hrs buprenorphine, allowed to recover with free access to food and water.

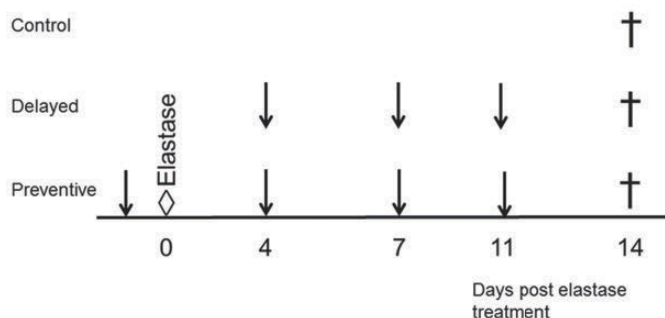
A role for IL-6 in AAA initiation and progression was assessed through repeated anti-IL-6 injections (IP) starting at the day before elastase infusion (day -1), followed by injections on day 3, day 7 and day 11 (n=9) (Figure 1). Control animals (n=9) received parallel IP injections with PBS.

There were a high number of unexpected deaths in the 24 hours following aneurysm induction in the treatment arm. To that end we set up a second experiment (n=8) in which IL-6 neutralization was delayed, i.e. therapy was initiated on day 3 after elastin treatment the infusion.

Aneurysm formation and growth in all groups was assessed by ultrasound (Vevo 770 Imaging system using RMV 704 micro-visualization scan head (Visualsonics, CA)). The maximum axial diameter of the aorta was measured at day -1 (one day prior to elastase infusion), day 7 and 14. Mice were sacrificed after the final aneurysm reading and their aorta was removed, formalin fixed and paraffin embedded.

### Aneurysm wall IL-6 content

Aorta wall IL-6 protein levels were determined in 238 AAA samples from the Aneurysm-Express Biobank.<sup>18</sup> Twenty six atherosclerotic aortic wall samples served as control. IL-6 content was



**Figure 1.** Study scheme of the elastase model. Arrows (↓) depict anti IL-6 injections. Mouse were sacrificed at day 14 (†) and the aorta harvested.

measured through Luminex multi-analyte profiling technology<sup>19,20</sup>, using a bio-plex system (Bio-Rad, USA), and the content normalized on basis of total protein content (BCA protein measurement method (Pierce Biotechnology, USA)). Inter-assay coefficient of variation was <10%.

### Immunohistochemistry

Human (n=10 AAA samples and n=10 control atherosclerotic aorta samples) and murine tissue sections were deparaffinized and incubated overnight at room temperature with the primary antibody diluted in PBS, 1% BSA, using the following primary antibodies for the human studies: IL-6 (Santa Cruz Biotechnology, USA), IL-6R or CD126 (Abcam, UK) and pSTAT3 (Abcam, UK). Envision mouse or Envision Rabbit (Dako, Denmark) were used as secondary antibody.<sup>15</sup>

Murine sections were incubated with CD45 (BD Pharmingen, USA), MAC3 (BD Pharmingen, USA), MMP9 (Santa Cruz Biotechnology, USA) and Smooth Muscle Alpha Actin (DAKO, Denmark). Further sections were stained with Sirius Red for collagen and Weigert's elastin stain to visualize elastic lamina. Eight slides per animal were used per staining for analysis and only moderate or strongly reactive cells were counted as positive. The slides were blindly evaluated. A mean value for positive staining cells in the eight sections was calculated for each animal.

### Microarrays

RNA extraction was performed from full thickness aortic wall samples from 31 AAA patients (mean age 69.5 yrs. mean diameter 62.3±12.1 mm) and 9 control samples (infra renal aorta obtained during kidney procurement for donation).

RNA from aneurysm wall was labeled and hybridized to Illumina HumanHT-12 v4 BeadChips. Arrays were scanned with an Illumina iScan microarray scanner. Bead level data preprocessing was done in Illumina GenomeStudio.

### Analysis of array data

Quantile normalization and background reduction were performed according to standard procedures in the Illumina GenomeStudio software.



Association of genome-wide expression data with AAA phenotype revealed 11486 transcripts with  $P < 0.05$ . These differentially expressed transcripts were used as an input for pathway analysis through Ingenuity Pathway Analysis suite (<http://www.ingenuity.com>, accessed 2016). Levels of significance were determined using Fisher's exact tests implemented in the software.

### Western blot analysis

Samples for Western blot were homogenized and lysed in the following buffer: 150mM NaCl, 50 mM Tris (pH 8.0), 1.0% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 10mM orthovanadate and Protease inhibitor cocktail (Roche, The Netherlands), and Western blot was performed as earlier described,<sup>14</sup> using the following antibodies: pSTAT3 (GeneTex, The Netherlands), STAT3 (Santa Cruz Biotechnology, USA) and alpha actin (Abcam, Cambridge, UK) for normalization. Donkey anti-rabbit IgG-HRP (Santa Cruz Biotechnology, USA) was used as the secondary antibody. Spots were visualized and quantified using the Super Signal West Dura Extended Duration Substrate (Pierce Biotechnology, Germany), and the luminescent image workstation (Roche Diagnostics) with labworks 4.6 software.<sup>15</sup>

### Semi quantitative RealTime PCR analysis

*IL-6*, *IL-6R* (CD126), *SOCS1* and *SOCS3* mRNA expression was quantified by semi quantitative RT, according to manufacturer's instructions in  $n=12$  AAA samples and  $n=16$  control atherosclerotic aorta samples. cDNA was prepared by using a Promega kit for RT-PCR. Semi quantitative RNA analysis was performed using the Taqman system for the determination of mRNA expression we used the established primer/probe sets (Life technologies, USA; *IL-6*: Hs00985641\_m1; *IL6r*: Hs01075666\_m1; *SOCS1*: Hs00705164\_s1; *SOCS3*: Hs02330328\_s1 and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase)), the mastermix (Eurogentec, Belgium) and the ABI-7700 system (Life Technologies, USA) as previously described. *GAPDH* (Life Technologies, USA) was used for normalization.<sup>15</sup>

### Statistical Analysis

An ANOVA test was performed to explore the difference between human normal aortic and the aneurysm samples. Subsequently an unpaired t-test was performed. Non-normally distributed data were log-transformed.

To evaluate the statistical significant difference between the mice treated for 15 days with anti-IL-6, mice treated for 10 days and the controls were evaluated with a Kruskal-Wallis ANOVA test. All parameters considered statistically significant ( $p < 0.05$ ) were hereafter evaluated pair-wise with the Mann-Whitney U test. Aneurysm formation in the elastase models was evaluated by the Chi square test.

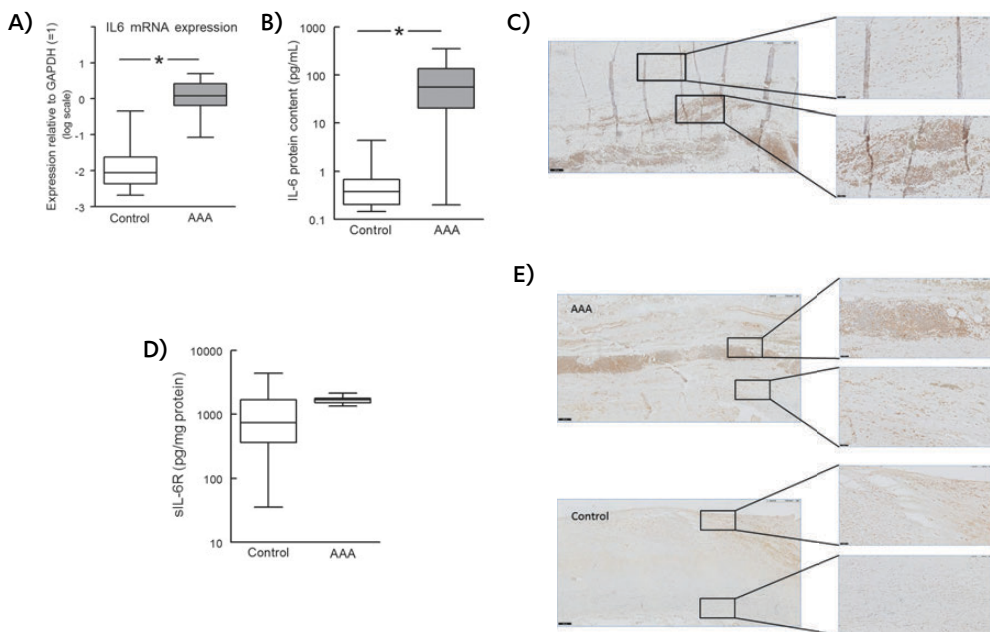
All values are shown as mean (SD) or as median [5<sup>th</sup> -95<sup>th</sup> percentiles]. Probability values of  $p < 0.05$  were considered statistically significant. The analyses were performed using SPSS 23.0 (IBM, Amsterdam, the Netherlands).

## RESULTS

### Elevated IL-6 expression and pathway activation in aortic aneurysm walls

The majority of studies on AAA wall cytokine profiles indicate abundant IL-6 protein expression, yet the current literature is not fully consistent. We therefore first validated previous reports<sup>7, 21</sup> of prominent aortic wall *IL-6* mRNA expression and elevated IL-6 protein levels in samples from the Aneurysm-Express Biobank. This analysis confirmed ample *IL-6* mRNA expression ( $p < 0.001$ , Figure 2A), and sharply increased IL-6 protein levels in AAA wall samples (aortic wall IL-6 content in AAA: 281.6 [ 0.0 – 1820.8]  $\mu\text{g/g}$  protein vs. 1.9 [0.0 – 37.8]  $\mu\text{g/g}$  protein median [5<sup>th</sup>–95<sup>th</sup> percentile] in atherosclerotic controls;  $p < 0.001$ , Figure 2B).

IL-6 signaling is thought to occur via two distinct pathways. Firstly, through the classic signaling route involving the IL-6 receptor (CD126)/GP-130 (CD130) receptor complex, and secondly via a trans-signaling route that involves signaling through the GP-130 (CD130)/soluble-IL-6 receptor (sIL-6r)<sup>9, 12</sup> complex. Signaling via this later route is thought to activate the non-classical signaling route<sup>21</sup>. While GP-130 is ubiquitously expressed, expression of the IL-6 receptor is thought to be more restricted. ELISA showed abundant sIL-6r in AAA and control wall samples (Figure 2D). Semi-quantitative RealTime

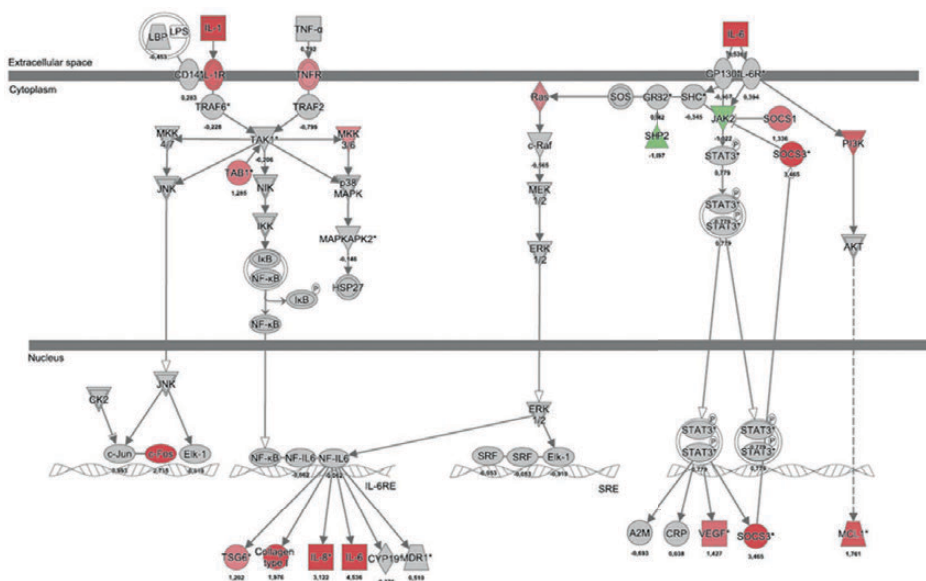


**Figure 2.** Aortic wall IL-6 expression. **(A.)** Aortic wall IL-6 mRNA expression relative to the house keeping gene GAPDH, in atherosclerotic controls and AAA,  $p < 0.001$ . **(B.)** Normalized aortic wall IL-6 protein content (ng/ml protein),  $p < 0.001$ . **(C.)** IL-6 localization in the human aneurysm wall. IL-6 is broadly expressed in lymphocytes, macrophages as well as mesenchymal cells. Overview 40X, details 200 X. **(D.)** Similar aortic wall soluble IL-6 receptor protein content (ng/mg protein) in AAA and atherosclerotic controls.  $p = ns$ . **(E.)** IL-6 receptor (CD126) expression in AAA and atherosclerotic controls. CD126 expression in control aorta is limited to the intimal layer, whereas diffuse but selective expression (subpopulations of lymphocytes, macrophages and mesenchymal cells) was observed in AAA tissue. Overview 40X, details 200X).

PCR for *IL-6r* mRNA showed increased expression in AAA versus atherosclerotic aortic controls ( $p < 0.01$ ). Immunohistochemical evaluation for the aneurysm wall showed diffuse expression IL-6r expression in mesenchymal cells and subpopulations of lymphocytes in the adventitial infiltrates (conclusions based on cellular morphology). IL-6r expression in the atherosclerotic controls was limited to the intimal layer (Figure 2E).

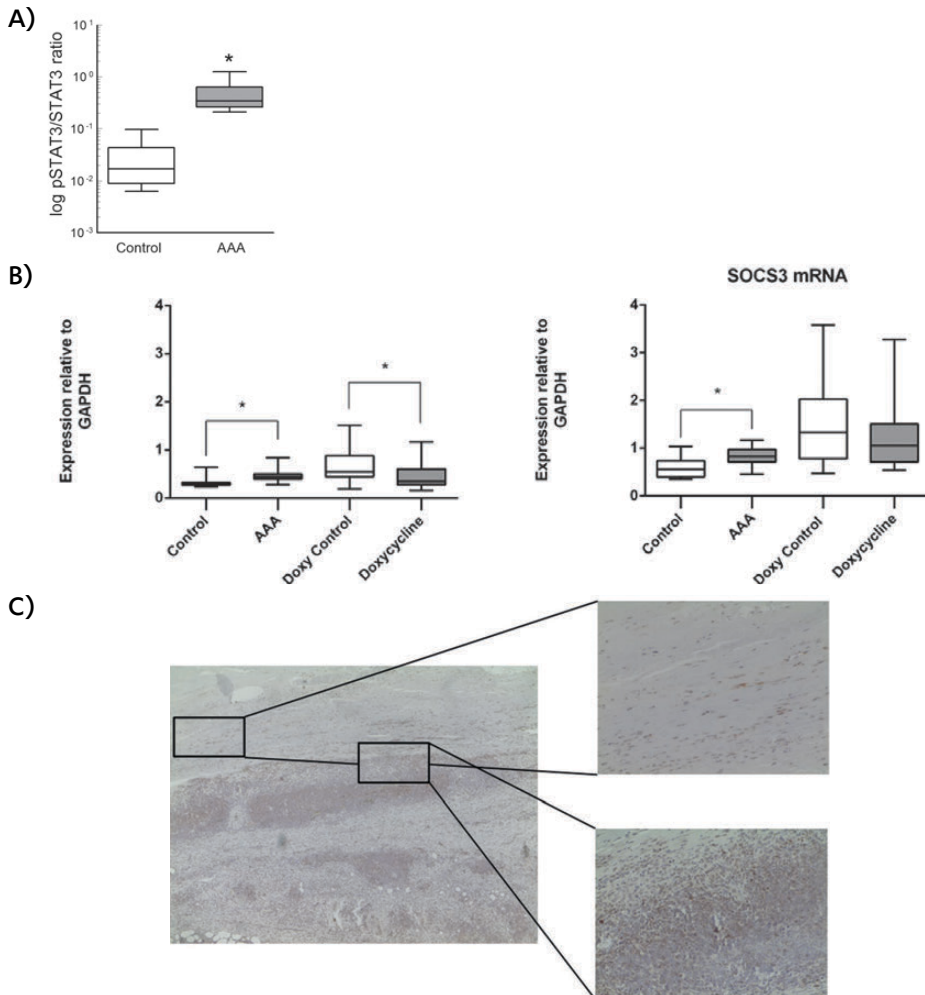
Signaling through both the classical, and non-classical route involves phosphorylation of the STAT-3 transcription factor, and induction of suppressor of cytokine signaling (SOCS) -1 and 3 expression. Ingenuity-based transcriptomics analysis shows upregulation of IL-6 signaling pathway in AAA disease (canonical IL-6 pathway:  $p < 0.000039$ ). More detailed analysis of the signaling networks indicated selective activation of the JAK-STAT signaling cascade but not of the RAS mediated ERK1/2 route (Figure 3). Western blot analysis confirmed the array data and showed increased STAT3 phosphorylation in AAA (Figure 4A,  $p < 0.018$ ). *SOCS1* and *3* mRNA expression was higher in AAA wall samples than in controls ( $p < 0.003$ , Figure 4B). Immunohistochemistry for phosphor-STAT3 indicates that IL-6 signaling in AAA and atherosclerotic controls mainly occurs in macrophages and smooth muscle cells (Figure 4C).

The tetracycline analogue doxycycline has broad anti-inflammatory properties that include quenching of aneurysm wall IL-6<sup>15</sup>. Paradoxically, a clinical trial concluded that doxycycline accelerates aneurysm growth<sup>5</sup>. We hypothesized that this effect may (partially) relate to suppression of SOCS expression. Comparison of *SOCS-1* and *3* mRNA expression in AAA wall samples from patients that did and did not receive doxycycline treatment showed that doxycycline treatment reduces AAA wall *SOCS-1* mRNA expression (Figure 4B,  $p < 0.037$ ).



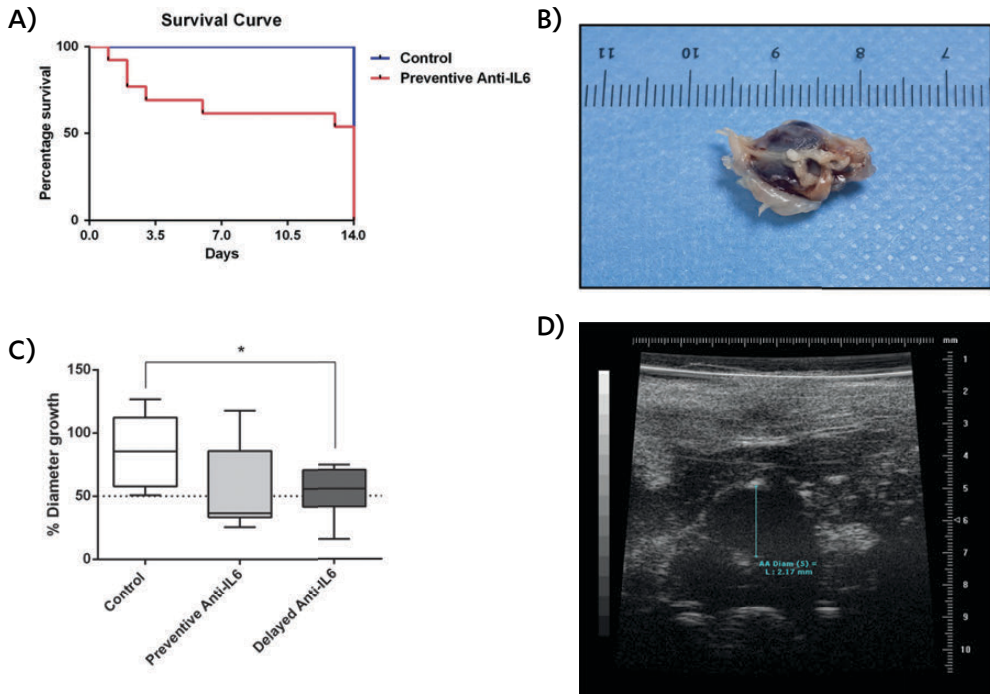
**Figure 3.** IL-6 signaling pathways in AAA disease (Ingenuity Pathway Analysis). Specific upregulation of STAT3 mediated IL6 signaling but not of the RAS-ERK1/2 axis in AAA disease (Ingenuity Pathway Analysis).





**Figure 4.** Enhanced IL-6 signaling in AAA disease. **(A.)** Similar aortic wall STAT3 but increased phosphorylated STAT3 (pSTAT3) content (Western blot analysis) in AAA tissue. Levels normalized on  $\alpha$ -actin content. (STAT3 ( $p = ns$ ) and pSTAT3 ( $p < 0.030$ )). **(B.)** Left bars: increased SOCS-1 and -3 mRNA expression in AAA tissue, ( $p$  respectively  $< 0.0008$  and  $0.0035$ ). Right bars: reduced aortic wall SOCS-1 mRNA expression after doxycycline therapy. **(C.)** phosphor-STAT3 distribution in AAA tissue, showing IL-6 signaling in macrophages and mesenchymal cells.

A possible role for IL-6 in AAA disease was investigated in the murine elastase model of AAA disease through quenching IL-6 by means of a validated IL-6 neutralizing antibody (Figure 1). Unexpectedly, preventive IL-6 neutralization (i.e. therapy started at the day prior to the elastase infusion) resulted in a 40% 7-day mortality (Figure 5A). Further evaluation showed that this mortality was due to aortic rupture (Figure 5A and B). No deaths or ruptures were observed in the control group. In the surviving mice, preventive anti-IL-6 treatment did not influence the number of aneurysms formed ( $>50\%$  increase in aorta baseline diameter,  $p > 0.05$ ) (Figure 5C).

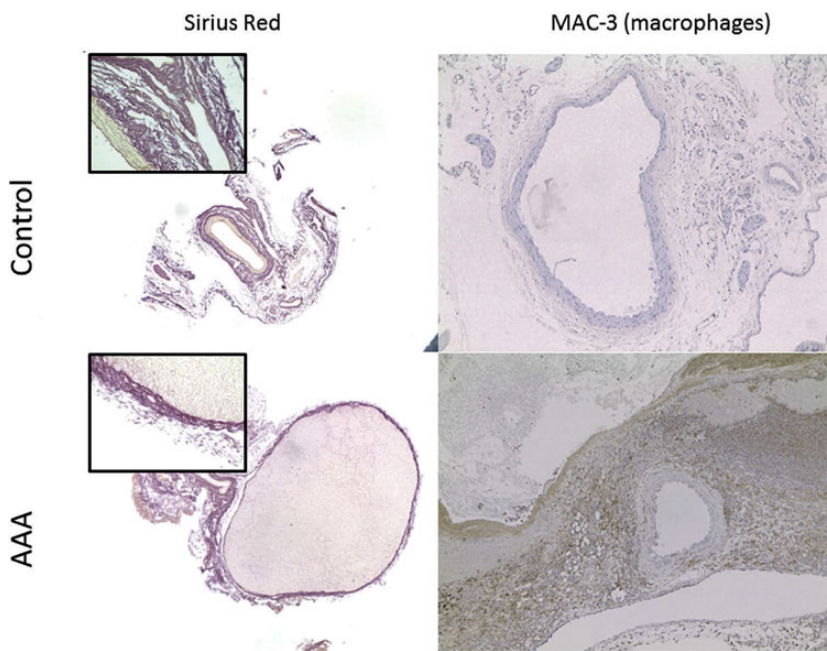


**Figure 5.** IL-6 neutralization in the elastase model of AAA disease. **(A.)** Survival curves of mice receiving preventive anti-IL-6 (i.e. anti-IL-6 was administered before the elastase perfusion), and vehicle treated control mice. Day 0 is the day of the elastin infusion. Mice were all sacrificed on day 14. **(B.)** Explanted aorta of one of the deceased mice that received preventive anti-IL-6 treatment showing a confined rupture (the array indicates the lumen of the aorta), and (right) an ultrasound showing a murine aneurysm. **(C.)** Delayed anti-IL6 (therapy initiated at day 4 after elastase infusion) quenches AAA formation ( $p < 0.03$ ). No effect was seen in the mice that survived whereas the preventive treatment resulted in similar aortic growth as the controls ( $p > 0.05$ ). **(D.)** Ultrasound image showing an aortic aneurysm in the elastase model.

The observed detrimental effects of IL-6 neutralization may relate to a possible protective role for IL-6 in the context of acute injury<sup>13</sup>. We therefore performed a second series of experiments in which IL-6 neutralization was delayed until day 4 (antibody treatment was started on the fourth day following elastase treatment). Delayed IL-6 neutralization did not result in rupture, moreover, the IL-6 neutralizing antibody reduced the AAA progression (Figure 5C, anti-IL-6 (delayed),  $p < 0.03$ ). Histological evaluation on day 14 (Figure 6) showed that IL-6 neutralization did not influence aortic wall leukocyte, macrophages or MMP9 content nor the number of elastin breaks and collagen content compared to control animals (Data not shown).

## DISCUSSION

The pathology of AAA is complex and poorly understood. On the molecular level, the disease is best described as an intense, localized comprehensive inflammatory response<sup>23, 24</sup>. At this point, it is still unclear which aspects of this inflammatory response are causatively involved in the disease



**Figure 6.** Histological examples of the elastase model. Histological staining for collagen (sirius red, left) and macrophages (MAC3, right) for a control and aneurysmal aorta .

process, and which factors are found by association<sup>25</sup>. Remarkably, while there is genetic evidence for an association between IL-6 signaling<sup>8</sup> and AAA disease, and while IL-6 hyperexpression has been reported as a discriminative feature of the disease<sup>7,21,26</sup>, no studies to date addressed a possible role for IL-6 in the disease. Results from this study confirm ample IL-6 expression and increased IL-6 signaling in human AAA tissue. IL-6 neutralization experiments in a mouse model of AAA disease suggest that the role of IL-6 could be protective in the context of aneurysm formation, but disadvantageous in the context of AAA progression.

Genetic and observational associations imply a link between IL-6 and AAA disease. A report by Smallwood and colleagues identifies a polymorphism in the IL-6 gene promoter as a rare independent risk factor for AAA<sup>8</sup>. Moreover, a Mendelian randomization approach found robust evidence for a causal link between the IL-6 receptor signaling pathway and AAA disease<sup>27</sup>. On the (aneurysm) tissue level most, the majority, but not all studies report increased IL-6 levels<sup>23,28, 29</sup>, and the aneurysm wall has been identified as a relevant source of plasma IL-6<sup>30</sup>.

Given the lack of full consistency with regard to IL-6 hyperexpression<sup>23</sup>, we first performed a validation experiment for our previous findings in a patient cohort<sup>18</sup> that is distinct from our earlier studies<sup>7</sup>. Results of this evaluation again showed an extreme disparity in aortic wall IL-6 content in AAA and atherosclerotic aorta wall samples with median AAA IL-6 content being more than 100-fold higher than in atherosclerotic controls.

Although IL-6 is generally considered a pro-inflammatory cytokine and a key-regulator of the acute phase response, it is now clear that the roles of IL-6 extend beyond its traditional function



in inflammation/infection, and also include regulation of hematopoiesis, metabolic control and roles in tissue protection/regeneration<sup>9,12</sup>. These disparate roles are thought to reflect different signaling routes: the *classical* IL-6 receptor route being responsible for the anti-inflammatory/tissue regenerative activities<sup>13</sup>, and the non-classical, *trans-signaling* route via the soluble IL-6 receptor being responsible for the pro-inflammatory activities. These observations characterize IL-6 as a Janus-like factor with the net effect depending on the activities of the two distinct signaling routes, and the cross-talk with other inflammatory mediators<sup>9,12,13</sup>.

IL-6 receptor (CD126), responsible for the classical signaling route was diffusely expressed in the mesenchymal cell population within the aneurysm wall as well as in subpopulation of leucocytes within the tertiary follicles. This pattern was clear distinct from aortic atherosclerotic disease in which the receptor was essentially expressed in the atherosclerotic region in the intima. At this point both signaling routes are thought to converge on STAT3 transcription factor effector pathway, and on SOCS-1 and 3 expression<sup>31-33</sup>. As such, elevated STAT3 phosphorylation, and increased SOCS-1 and 3 expression in AAA show that the increased IL-6 levels in AAA disease are followed by augmented IL-6 signaling. Yet, this data doesn't allow for distinction between signaling via the tissue protective (classical) route or via the pro-inflammatory (non-classical) signaling route.

We therefore decided for an experimental approach and tested whether quenching of IL-6 via a neutralizing antibody influenced aneurysm formation and progression in the elastase model of AAA disease. Unexpectedly we experienced early ruptures in almost 50% of the anti IL-6 treated animals, a notable phenomenon since, to the best of our knowledge ruptures have not been described for the elastase model. The aggravation of acute tissue damage during IL-6 neutralization proofs the efficacy of the antibody used, but also fits in other reports showing that IL-6 is protective in the acute phases of ischemia reperfusion injury and ischemia<sup>34-37</sup>. Yet, it is obviously unclear how whether and how these experimental findings from an acute model translate to the process of AAA formation in man i.e. whether IL-6 has a role in protecting against AAA formation.

We therefore performed a second series of experiments with delayed anti-IL-6 therapy started at day 4 after the elastase treatment. Delayed IL-6 neutralization did not result in ruptures, suggesting that the deleterious effects of IL-6 inhibition relate to processes involved in the initiation process of AAA formation in the elastase model. Delayed therapy did result in a significant reduction of aneurysm progression.

A critical question is whether these mouse observations translate to the human context. Although the acute aortic ruptures observed in this study may not appear relevant for human AAA, diverticular perforations have been observed during treatment with Tocilizumab, a monoclonal antibody targeting the IL-6 receptor<sup>11</sup>. As such it cannot be excluded that IL-6 neutralization may negatively affect clinical AAA disease. Data from the experiments with delayed therapy hint at a beneficial effect of IL-6 neutralization on AAA progression. A critical question is whether quenching IL-6 has beneficial effect in the clinical context. Our earlier studies show that statins, ACE-inhibitors and doxycycline therapy all profoundly reduce aneurysm wall IL-6 levels<sup>5,38,39</sup>, yet this is not followed by attenuation of aneurysm progression<sup>4</sup>. Although one could argue that halving IL-6 hyperexpression in AAA disease is not sufficient to interfere with AAA progression, it remains to be established whether a further suppression is clinically feasible. In fact, the observed accelerated aneurysm growth in patients receiving doxycycline-treatment may (partially) relate to a reduction in SOCS-1 expression in patients receiving doxycycline and/or a role of IL-6 in tissue remodeling<sup>40</sup>.

In conclusion, this study confirms exaggerated IL-6 levels and IL-6 signaling in AAA disease, and although indications were found for a beneficial effect of AAA neutralization in the elastase model of AAA disease, we also found indications for potential detrimental effects. Moreover, indirect interference with aneurysm wall IL-6 content in clinical studies is not followed by a reduction in AAA progression. As such this study does not identify IL-6 as a key-driver of AAA progression, yet the data does not rule out a role for IL-6 in AAA formation.



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# Chapter

# 9

## ADVENTITIAL ADIPOGENIC DEGENERATION IS AN UNIDENTIFIED CONTRIBUTOR TO AORTIC WALL WEAKENING IN THE ABDOMINAL AORTIC ANEURYSM

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# ABSTRACT

## Objective

The processes driving human abdominal aortic aneurysm progression are not fully understood. While anti-inflammatory and proteolytic strategies effectively quench aneurysm progression in preclinical models, so far all clinical interventions failed. These observations hint at incomplete understanding of processes involved in abdominal aortic aneurysm progression and rupture. Interestingly, strong clinical and molecular associations exist between popliteal artery aneurysms and abdominal aortic aneurysms however, popliteal artery aneurysms have an extremely low propensity to rupture. We thus reasoned differences between these aneurysms may provide clues towards (auxiliary) processes involved in abdominal aortic aneurysm-related wall debilitation. A better understanding of the pathophysiologic processes driving abdominal aortic aneurysm growth can contribute to pharmaceutical treatments in the future.

## Methods

Aneurysmal wall samples were collected during open elective and emergency repair. Control perirenal aorta was obtained during kidney transplantation, and reference popliteal tissue obtained from the anatomy department. This study incorporates various techniques including (immuno) histochemistry, Western Blot, quantitative polymerase-chain-reaction, microarray and cell culture.

## Results

Histological evaluation of abdominal aortic aneurysms, popliteal artery aneurysms and control aorta shows extensive medial (popliteal artery aneurysm) and transmural fibrosis (abdominal aortic aneurysm), and reveals abundant adventitial adipocytes aggregates as an exclusive phenomenon of abdominal aortic aneurysms ( $P < .001$ ). qPCR, IHC, Western blotting and microarray analysis showed enrichment of adipogenic mediators (C/EBP family  $P = .027$ ; KLF5  $P < .000$ ; and PPAR-  $P = .032$ ) in abdominal aortic aneurysm tissue. *In vitro* differentiation tests indicated a sharply increased adipogenic potential of abdominal aortic aneurysm adventitial mesenchymal cells ( $P < .000$ ). Observed enrichment of adipocyte related genes and pathways in ruptured abdominal aortic aneurysm ( $P < .000$ ) supports an association between the extent of fatty degeneration and rupture.

## Conclusion

This translational study identifies extensive adventitial fatty degeneration as an ignored and distinctive feature of abdominal aortic aneurysm disease. Enrichment of adipocyte-genesis and adipocyte-related genes in ruptured AAA point to an association between the extent of fatty degeneration and rupture. This observation may (partly) explain the failure of medical therapy and could provide a lead for pharmaceutical alleviation of abdominal aortic aneurysm progression.

## INTRODUCTION

An abdominal aortic aneurysm (AAA) is a localized, progressive dilatation of the terminal aortic segment. If left untreated, AAA's will rupture and a ruptured AAA constitutes a prominent cause of sudden death in elderly males.<sup>1</sup>

At this point the pathophysiology of AAA growth and ultimately rupture remains an enigma.<sup>2</sup> The prevailing concept is that aneurysm progression is essentially driven by a localized pro-inflammatory response and accompanying proteolytic imbalance; the latter being held responsible for a progressive, and ultimately fatal weakening of the vessel wall.<sup>3,4</sup>

Remarkably, while interference with inflammatory and/or proteolytic cascades proves highly effective in animal models of the disease, all clinical studies so far fail to show a benefit.<sup>5-10</sup> In fact, against all expectations interference with aspects of inflammation in the clinical setting may even accelerate disease progression.<sup>11,12</sup> Altogether these clinical observations suggest that factors beyond proteases and inflammation are involved in progression (and rupture) of human AAA.

Remarkable strong clinical and molecular associations exist between AAA and popliteal artery aneurysms (PAA).<sup>3</sup> Yet, while the natural history of AAA is that of rupture, the primary concern in PAA's is thrombosis and rupture is rare in the context of PAA.<sup>13</sup> Hence, we reasoned that inherent differences between these two forms of aneurysms may provide clues towards auxiliary processes contributing to AAA wall rupture. On this basis we considered a patho-histological (re-)examination of AAA and PAA wall samples relevant.

Results of this evaluation identify extensive adventitial adipocyte accumulation as a thus far unrecognized feature that distinguishes growing AAA from PAA. In this paper we explore fatty degeneration as an novel additional pathophysiologic mechanism in AAA, and test a possible association between the process and terminal aortic wall weakening (rupture) in the disease.

## MATERIALS AND METHODS

### Tissue samples

All tissue samples were obtained from the Vascular Tissue Bank at the Department of Vascular Surgery, Leiden, the Netherlands. AAA and PAA samples were collected during elective and emergency aneurysm repair. Reference (non-aneurysmal) infra renal abdominal aortic wall samples were obtained during clinical organ transplantation from unused donor aortic tissue.<sup>14</sup> Considering the ample presence of atherosclerotic lesions in AAA wall samples, we specifically selected donor specimens with advanced atherosclerotic disease (*viz.* all samples selected had earlier<sup>14</sup> been classified as late fibroadenoma or higher (Modified AHA classification according to Virmani))<sup>15</sup> as reference (control) tissue.

Reference popliteal artery samples were kindly donated by the department of human anatomy. Movat staining of these popliteal samples did not indicate intermediate or advanced atherosclerotic lesions. Sample collection and handling was performed in accordance with the guidelines of the Medical and Ethical committee in Leiden, The Netherlands and the code of conduct of the Dutch Federation of Biomedical Scientific Societies (<https://www.federa.org/codes-conduct>).

Handling of the aneurysmal tissues was as follows: after obtaining tissue specimens, the adhering thrombus was carefully removed and tissue divided in three sections. One section was fixed in

formaldehyde (4%) for 24h, and decalcified in Kristensen's solution for 120h followed by embedding in paraffin. A second section was snap frozen in liquid nitrogen and stored at -80°C to be used for mRNA and protein analysis. The third section was kept in NaCl 0.9% and adventitial mesenchymal cells were isolated within 24h. For this purpose tissue was split along the medial-adventitial border and the adventitial segment used for cell isolation.

### **Histology and Immunohistochemistry**

Histochemistry and immunohistochemistry (IHC) was performed on 4µm thick tissue sections. Slides were deparaffinized using xylene followed by rehydration. Histologic evaluation of vessel wall architecture was performed using Movat's pentachrome and Sirius Red staining according to local protocols. IHC was performed using heat induced epitope retrieval (HIER) combined with overnight incubation of primary antibodies. Antibodies against KLF-5 (AF3758, R&D Systems, Abingdon, United Kingdom) and PPAR- (AHP1461, AbdSerotec, Puchheim, Germany) were diluted to 1:200 and 1:1000 in 1% BSA respectively. Envision detection system (Dako, Amsterdam, The Netherlands) was used as secondary antibody. Samples were stained with DAB (Dako, Amsterdam, The Netherlands), and counterstained with Mayer's hematoxylin (Merck Millipore, The Netherlands). For quantification purposes one full length section (typically 10-15 mm) per patient was quantified. Stained slides were scanned at 400x magnification using Philips' IntelliSite Ultra Fast Scanner (Philips, Eindhoven, the Netherlands) and representative sections are shown.

### **Quantitative Polymerase Chain Reaction**

For quantitative polymerase chain reaction (qPCR) total RNA extraction was performed using RNeasy mini kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's instructions. Copy DNA was prepared (kit #A3500; Promega, Leiden, The Netherlands) and qPCR for C/EBP, PPAR-, KLF-5, C7orf68, ANGPTL4, ADAMTS9, SLC39A14, SRPX2 performed on the ABI-7700 system (Applied Biosystems by Thermo Fisher, Landsmeer, The Netherlands) using established primer/probe sets (Assays on Demand; Applied Biosystems by Thermo Fisher, Landsmeer, The Netherlands) and Mastermix (Eurogentec, Seraing, Belgium). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as for reference and normalization.

### **Microarray**

RNA extraction was performed from full thickness aortic wall samples from 31 elective and 17 ruptured AAA patients (mean age: 69.5±7.2 and 73.5±11.3 years; mean diameter 62.3±12.1 and 77.0±14.9 mm respectively) and 9 control samples (infra renal aorta obtained during kidney procurement for donation, mean age: 68.8±9 years; mean diameter 19.6±2.6mm). RNA from aneurysm wall was labeled and hybridized to Illumina HumanHT-12 v4 BeadChips. Arrays were scanned with an Illumina iScan microarray scanner. Bead level data preprocessing was done in Illumina GenomeStudio.

### **Analysis of array data**

Quantile normalization and background reduction were performed according to standard procedures in the Illumina GenomeStudio software. Differential expression was calculated using empirical Bayes statistics included in the limma package and transcripts with an absolute log 2

fold-change  $\geq 1$  were considered as differentially expressed. Differentially expressed transcripts were analysed using the Ingenuity Pathway Analysis (<http://www.ingenuity.com>) and levels of significance were determined using Fisher's exact tests implemented in the software.

### Isolation of adventitial cells

Upon medio-adventitial splitting, the adventitial matrix was digested using collagenase II solution (Worthington Biochemical, Lakewood, NJ.) according to manufacturer's instruction for a maximum of 4h with repeated resuspension. The suspension was strained through a 70- $\mu\text{m}$  filter (Greiner Bio-One, Alphen aan den Rijn, The Netherlands) and centrifuged at 800rpm for 5 minutes. The precipitate was resuspended in DMEM (Gibco by Thermo Fisher, Landsmeer, The Netherlands) supplemented with 10% Fetal Bovine Serum (Sigma Aldrich, Zwijndrecht, The Netherlands) in order to inactivate collagenase II activity and centrifuged at 800rpm for another 5 minutes. After cell counting using the Countess cell counter (Invitrogen by Thermo Fisher, Landsmeer, The Netherlands) cells were seeded on plastic surfaces in 6-well plates (Costar, Sigma Aldrich, Zwijndrecht, The Netherlands) at a concentration of  $10^5$  cells/mL in DMEM supplemented with 10% Fetal Calf Serum and 1% Penicillin/Streptomycin (Sigma Aldrich, Zwijndrecht, The Netherlands) as basal medium. Mesenchymal cellular populations were selected on their ability to adhere to plastic. Cells were passaged when they reached 70-80% confluence.

### Adipogenic differentiation assay

Adipogenic differentiation potential was assessed by exposing the adventitial mesenchymal cells to an adipogenic medium.<sup>16</sup> In short, adhering cells were cultured to 70-80% confluence and serum starved for 48h. Next, cells were exposed to an 'adipogenic' induction medium, consisting of DMEM, 10% Fetal Calf Serum, 1700nM insulin (Sigma Aldrich, Zwijndrecht, The Netherlands), 1 $\mu\text{M}$  dexamethasone, and 500 $\mu\text{M}$  IBMX (Sigma Aldrich) for 72h. For the remainder of the experiment, cells were maintained in 'adipocyte nutrition medium' containing 2% Fetal Calf Serum, 1700nM insulin and 1 $\mu\text{M}$  dexamethasone. Cells were briefly formalin-fixed and fat accumulation was visualized using Oil Red O (Sigma-Aldrich, Zwijndrecht, The Netherlands) staining. Fat accumulation uptake was quantified as % of the total cell surface area covered by Oil Red O staining.

### Statistical analysis

Differences in adventitial adipose tissue content were compared using the  $\chi^2$  test. Results of qPCR, Western Blotting and *in vitro* data were analyzed with Student's T-test or Wilcoxon-Mann-Whitney U-test to compare the different groups. Statistical significance was accepted at  $P < .05$ . All analysis were performed using the SPSS 23.0 software package (IBM Corp, Armonk, NY).

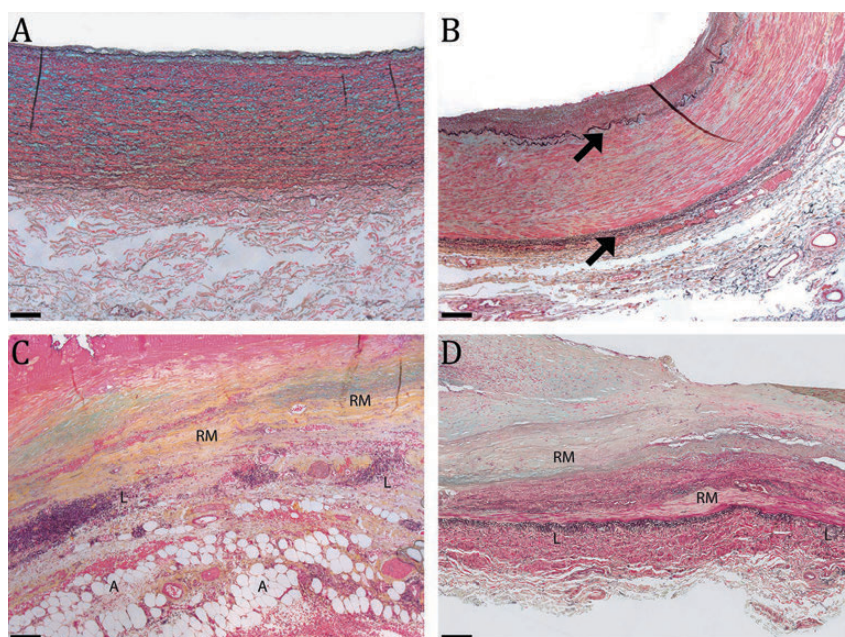
## RESULTS

Clinical characteristics are shown in table 1.

The histology of a normal infrarenal aorta and popliteal artery (Figure 1A, 1B, atherosclerotic aortic tissue as reference in Supplemental Figure 1A-E) illustrate the 3-layered vessel wall architecture with distinctive intima, media, and a loose collagenous adventitia.

**Table 1.** Patient Characteristics. Characteristics of patients from whom tissue was used in this study. No significant differences were present between the groups.

	Control Aorta	AAA	Control Poplitea	PAA
No. of subjects	11	31	10	15
Mean age (years)	66.36	70.94	53.60	67.80
Male gender (%)	45.45	87.10	60.00	93.33
Mean aneurysm diameter (cm)	N/A	6.32	N/A	3.19
Smoking (%)	63.64	74.19	10.00	73.33
Statin use	2	11	Unknown	2
ACE-inhibitor	1	8	Unknown	1



**Figure 1.** Histological overview of normal (non-atherosclerotic) aorta and popliteal artery and their respective aneurysms (Movat's pentachrome staining). A) healthy control aorta and B) normal popliteal artery. Multiple, parallel elastic laminae (black in Movat staining) in the normal aorta are characteristic for an elastic artery. The distinct inner and outer elastic laminae (**arrow**) of the popliteal artery characterize this artery as a muscular artery. C and D in the lower panel show representative examples of an aortic (C) and popliteal aneurysm (D). Both aneurysms show by extensive medial degradation. Prominent adventitial involvement (leucocyte infiltration (**L**), extensive matrix remodeling (**RM**) and presence of large adipocyte clusters (**A**)) is characteristic for AAA. Legend to the Movat staining: Black: elastin, yellow: collagen, blue: proteoglycans, mucin, red: smooth muscle cells and fibrin, and violet: nuclei. Various shades of green reflect co-localization of collagen (yellow) and proteoglycans (blue). Mag 50x, scale bars represent 200µm. More details of the degenerative and inflammatory processes are presented in Supplemental Figure 1.

AAA wall is characterized by profound *transmural* changes with loss of the normal 3-layer architecture (Figure 1C, Supplemental Figure 1F-I). The disease is hallmarked by extensive medial elastolysis and coagulation of the medial and adventitial structures. Both layers are transformed into a condensed collagen-rich matrix, with the circular band of vasa vasora marking the former outer medial zone. The former adventitial zone contains most of the inflammatory infiltrates that characterize the disease.<sup>1</sup>

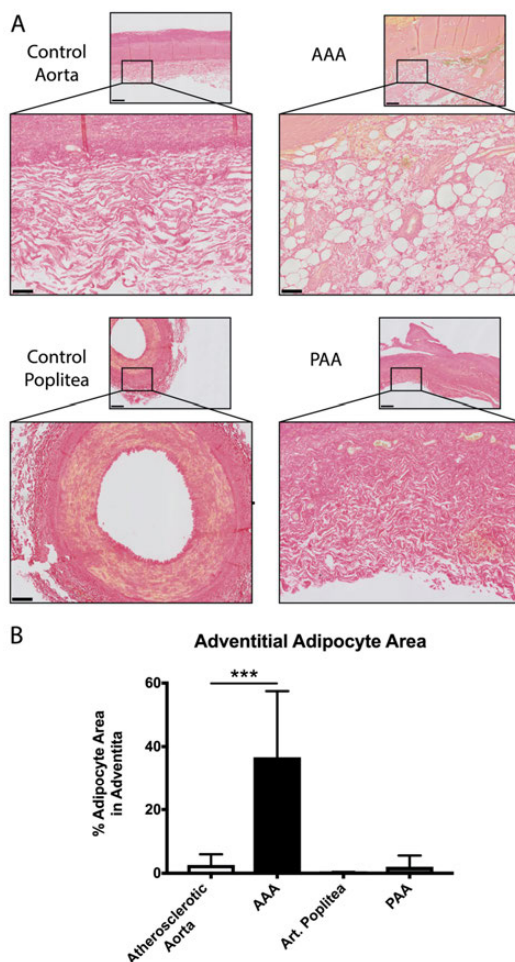
In sharp contrast to the AAA the 3-layer architecture is preserved in PAA. Changes appear mainly limited to the medial layer and include disruption of elastin laminae, loss of VSMCs, collagen accumulation, increased number of vasa vasora, and leucocyte infiltration. The adventitial layer appears largely preserved (Figure 1D). These observations point to adventitial involvement as the main discriminative feature between AAA and PAA.

Examination of the former adventitial zone in AAA disease revealed presence of ample adipocyte clusters in the former adventitial zone (Figure 1C, Figure 2A; Additional examples are shown in Supplemental Figure 2), illustrating that presence of adventitial adipocyte clusters is a common and extensive feature of AAA disease ( $P < 0.0001$ ). Although small adventitial adipocyte clusters are sporadically observed in normal aorta and PAA, the vast extent of these clusters is exclusive to AAA disease ( $P < 0.001$ , Figure 2B). We did not observe a correlation between AAA diameter and the adventitial adipocyte mass ( $r: -0.103$ ,  $P =$  not significant).

Emergence of these adventitial clusters of adipocytes, along with extensive adventitial fibrotic changes is consistent with the process of fatty degeneration. This phenomenon links to impaired tissue regeneration and is thought to involve transdifferentiation of resident mesenchymal cells into adipocytes.<sup>17,18</sup> The process of transdifferentiation critically depends on the collaborative action of transcription factors of the C/EBP family and KLF-5, and relies on PPAR- $\gamma$  activity for terminal differentiation and maintenance of the adipocyte phenotype. qPCR data (Figure 3) indicates active transcription of these factors with increased in KLF-5 and PPAR- $\gamma$  expression in AAA vs atherosclerotic control aorta ( $P < 0.000$  and  $P = 0.032$  respectively). Histological analysis (Figure 4) confirms abundant C/EBP $\beta$ , KLF-5 and PPAR- $\gamma$  protein expression in AAA, and shows these factors essentially, but not exclusively localize in the adventitial adipocyte clusters and mesenchymal cells, particularly those in close proximity to vasa vasora. Yet, double staining also showed expression of these factors in subsets of CD68+ macrophages. Results for Western blotting (results not shown) confirmed the above findings for C/EBP $\alpha$  ( $P = 0.027$ ) and KLF-5 ( $P = 0.039$ ). Results for PPAR- $\gamma$  did not reach significance ( $P = 0.073$ ).

All in all, these observations imply a pro-adipogenic environment in AAA disease. The adipogenic potential of AAA and control adventitial mesenchymal cells was tested by culturing these cells in adipogenic culture medium. This *in-vitro* test indicated a significantly higher adipogenic potential of AAA-derived adventitial mesenchymal cells compared control aorta to cells ( $P < 0.000$ ; Figure 5).

An obvious next question is whether the phenomenon of fatty degeneration associates with rupture. To test the latter we compared the gene expression and the gene expression-signature of ruptured ( $n=17$ ) and non-ruptured ( $n=31$ ) AAA wall samples. The differential expression data for non-ruptured vs ruptured AAA shows that 5 out of the 11 most prominently upregulated, differently expressed genes in ruptured AAA are adipocyte-related (Table 2). Moreover, Ingenuity-based pathway analysis identified the adipogenesis and PPAR signaling ( $P = 0.0003$  resp.  $0.0026$ ) pathways among the top-8 differentially activated canonical pathways in ruptured versus non-ruptured AAA.

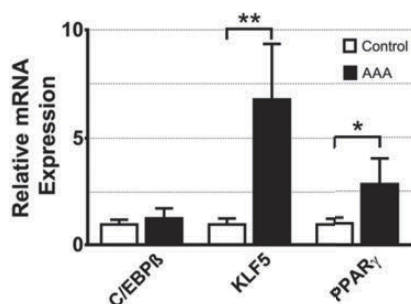


**Figure 2.** Adventitial adipocyte abundance is an exclusive for AAA. A) Representative images (Sirius Red) illustrating the medial-adventitial transition zone for normal aorta and popliteal artery, and their respective aneurysms. Adventitial adipocyte abundance is an exclusive feature of AAA (overview image: mag 20x, scale bar 500 $\mu$ m; adventitial inserts: mag 100x, scale bar 100 $\mu$ m). Of note: the control aorta shown is classified as end-stage atherosclerotic disease (Fibrous Calcified Plaque, Virmani classification<sup>15</sup>). B) Quantification of adventitial adipocyte abundance (expressed as the relative adventitial area (%) covered by adipocytes). Adventitial adipocyte abundance is an exclusive feature of AAA, \*\* P<0.001.

## DISCUSSION

This study identifies adventitial fatty degeneration as a thus far overlooked feature of AAA disease. This observation, along with the earlier recognized fibrotic changes, typifies larger AAA as a degenerative “dystrophic” condition. Enrichment of adipocyte-related genes and pathways in ruptured AAA versus non-ruptured controls implies an association between the extent of fatty degeneration and AAA rupture.

The apparent paradox between preclinical successes of anti-inflammatory/anti-proteolytic strategies in the context of AAA disease and clinical reality implies a translational gap.<sup>10</sup> This gap



**Figure 3.** Aortic wall mRNA expression of adipogenic transcription factors. Relative mRNA (control aorta = 1) expression for C/EBPβ, KLF-5 and PPAR-γ in control aorta and AAA. \* P<0.05, \*\* P<0.01; %. (semi-quantitative real-time PCR, control n=10; AAA n=10).

between available models of AAA disease and the actual disease is further illustrated by failure of the experimental models to progress to rupture (of note: while rupture is a common feature of the angiotensin model, it is now clear that this model reflects a process of aortic dissection rather than of AAA disease<sup>19,20</sup>).

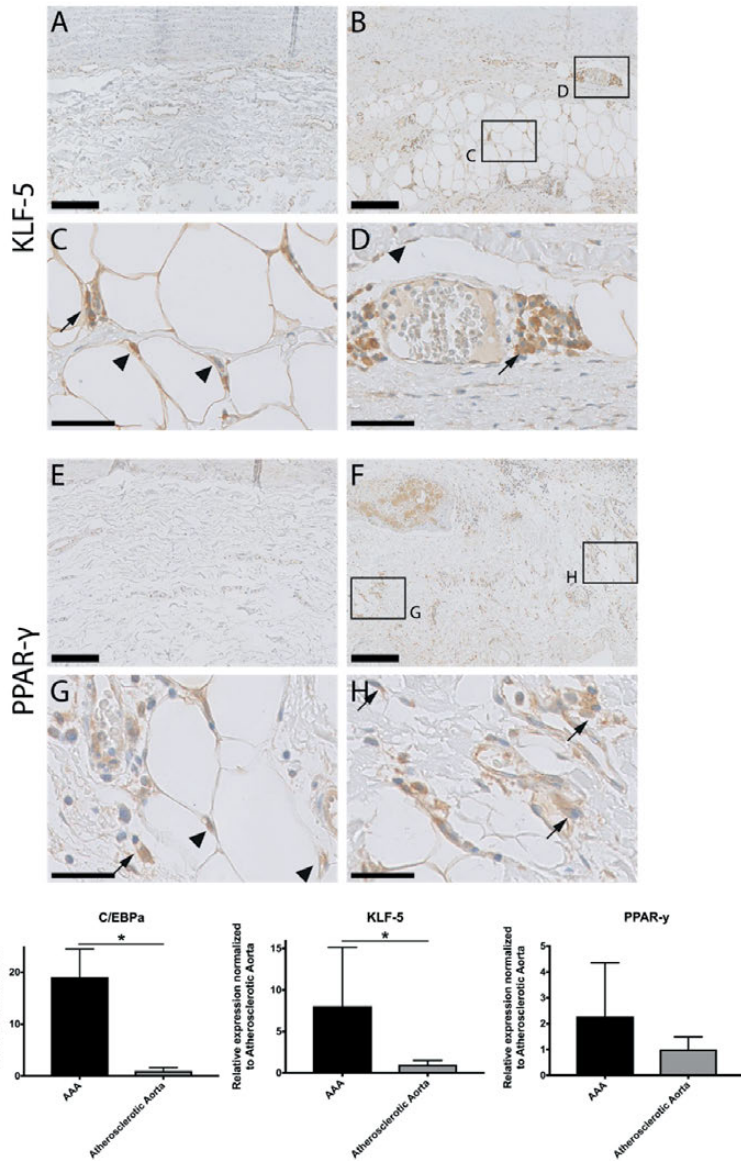
Apparent failure of the experimental models to progress to rupture implies the chain of events leading up to rupture as a distinct entity within the disease process. Such a concept is supported by the divergence of several risk factors for AAA growth and rupture, as well as contrasting rupture risks of AAA and PAA.<sup>13, 21, 22</sup> In search of auxiliary processes contributing to (terminal) AAA wall weakening, we considered a re-evaluation of histo-pathological aspects of abdominal aortic and popliteal aneurysms relevant. Particular attention was paid to the adventitial structures since Hurks et al. identified extensive adventitial involvement in AAA as a clear contrasting feature between AAA and PAA.<sup>23</sup>

Our re-evaluation confirms preservation of adventitial structures in PAA,<sup>23</sup> and extensive transmural remodeling and adventitial involvement in AAA disease. A remarkable, and to the best of our knowledge, novel observation for AAA disease is the abundance of isolated adipocyte islands within the former adventitial zone. Although isolated clusters of adipocytes are occasionally found in PAA and atherosclerotic control aortas, the extent of the phenomenon is unique to AAA.

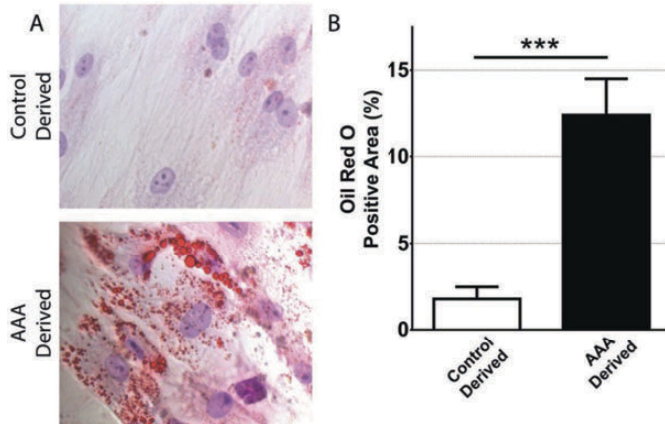
Adventitial adipocyte abundance in AAA could be the consequence of a passive process in which vanished adventitial matrix structures are replaced by peri-aortic adipose tissue. Such a phenomenon would imply continuity of the adipose islands with the per-aortic adipose tissue, which is clearly not the case. Moreover, strict confinement of the process to the former adventitia, the large adipocyte cell volume, and intertwining strands of matrix in between the adipocyte clusters are not consistent with a simple passive replacement mechanism.

An alternative explanation for the adipocyte abundance is the process of fatty (adipogenic) degeneration. Fatty degeneration is a well-known phenomenon in chronic degenerative conditions such as severe limb ischemia, muscle wasting dystrophies, and recurrent rotator cuff lesions.<sup>17, 18, 24</sup> The phenomenon is thought to reflect dysregulated repair processes that also include (trans) differentiation of mesenchymal cells into adipocytes.<sup>18, 25</sup>

The process of adipogenic transdifferentiation relies on the availability of transcription factors from the C/EBP and KLF-family families and presence of the nuclear receptor PPAR-γ<sup>26-28</sup> with



**Figure 4.** Tissue distribution (immunohistochemistry) of pro-adipogenic transcription factors in AAA. Immunohistochemical staining for KLF-5 and PPAR- $\gamma$ . A) Minimal KLF-5 expression in control aorta. B,C,D) Abundant KLF in AAA in adipocytes (arrow heads) and in mesenchymal cells (arrows in C and D) in the vicinity of vasa vasora and near the adipocyte clusters. E) minimal PPAR- $\gamma$  expression in control aorta. F,G,H) PPAR- $\gamma$  expression follows a similar expression pattern to KLF-5 being expressed in adipocytes (arrowheads) and 'mesenchymal' cells (arrows G and H). A,B,E,F mag 50x, scale bar 200 $\mu$ m; C,D,G,H mag 400x scale bar 50 $\mu$ m. I) Relative C/EBP $\alpha$ , KLF-5 and PPAR- $\gamma$  protein expression (data normalized to  $\alpha$ -actin) in AAA (solid bars) and atherosclerotic control aorta (grey bars). \* $P=0.027$  (C/EBP $\alpha$ ) resp. 0.039 (KLF-5).



**Figure 5.** Increased adipogenic potential of AAA-derived mesenchymal cells. A) Representative images (Oil-Red-O) illustrating fat accumulation in cultured aortic mesenchymal cells after 14 days of in-vitro culture. Minimal accumulation is seen in control aorta-derived mesenchymal cells (upper panel). Extensive fat accumulation is seen in AAA- cells (lower panel) (Mag 400x). B) Quantitative analysis of Oil-red-O staining. Significantly higher fat accumulation in AAA-derived mesenchymal cells (\*  $P < 0.000$ ; Control derived (n=6); AAA derived (n=6)).

**Table 2.** Microarray analysis of elective and ruptured AAA. Adipocyte-associated genes dominate the top 11 most differentially upregulated genes in ruptured (n=17) vs. stable AAA (n=31).

Gene	Fold Up Array	P Value Array	Rank in top 11	P Value PCR-validation
C7orf68	412	<0.000	1	<0.000
ANGPTL4	226	<0.000	2	<0.000
ADAMTS9	126	<0.000	5	0.0006
SLC39A14	107	<0.000	7	0.092*
SRPX2	74	<0.000	11	0.0029

\*Number of transcripts just above the detection limit of the PCR

sustained expression of PPAR- $\gamma$  as prerequisite for maintaining the adipocyte phenotype.<sup>26, 28</sup> We confirmed abundant C/EBP $\beta$ , KLF-5 and PPAR- $\gamma$  presence in the mesenchymal cells of the outer media and adventitia of the aneurysm wall, showing the transcriptional machinery required for fatty degeneration is present within mesenchymal cells of the AAA wall and co-localizes with the adipocyte clusters.

The process of fatty degeneration further depends on the ability of the resident mesenchymal cell population to undergo trans-differentiation into adipocytes. *In vitro* differentiation assays confirmed their adipogenic potential and indicated a significantly higher adipogenic potential for AAA-derived mesenchymal cells. This phenomenon implies intrinsic differences between control and AAA-derived mesenchymal cells. The persistence of the intrinsic differences *in vitro* ('priming' phenomenon) may hint at epigenetic reprogramming of AAA mesenchymal cells secondary to micro-environmental changes such as matrix properties and the growth factor/cytokine milieu.<sup>26, 29</sup>

Extensive adventitial fatty degeneration appears unique to AAA, and discriminates AAA from PAA disease. Reported associations between adventitial triglyceride content and AAA diameter suggest an association between the extent of fatty degeneration and disease progression.<sup>30</sup> As such it may contribute to the debilitation processes leading up to rupture.<sup>1</sup> To test the latter we analysed the data from a genome wide analysis of genes expressed in sized-matched stable and ruptured AAA. The clear enrichment of adipocyte-specific genes and the molecular pathways related to adipose tissue in ruptured AAA is consistent with increased adipogenic degeneration in ruptured AAA.

It came to our attention that quantifying the extent of fatty degeneration could contribute to an improved rupture risk estimation in patients with larger AAA. Unfortunately, although MRI provides an excellent distinction between adipose and surrounding tissue, we concluded that the infra-renal abdominal aorta is not easily accessible for the high-resolution level of imaging required for quantifying the extent adventitial fatty degeneration.

A possible role of a fatty degeneration in terminal aorta weakening may have major implications for the use of PPAR- $\gamma$  agonists in AAA patients. Recent studies propose PPAR- $\gamma$  activation as a means of attenuating AAA growth and preventing rupture.<sup>31,32</sup> Indeed, PPAR- $\gamma$  agonists quench vascular inflammation in both humans and small animals, and reduce AAA growth in different animal models.<sup>31, 33, 34</sup> Moreover, it has been suggested that PPAR- $\gamma$  signaling is crucial for the integrity of elastic fibers in mice protecting against aortic dilatation.<sup>32</sup> Yet, as fatty degeneration is not a feature of these animal models, potential negative aspects of PPAR- $\gamma$  activation may be missed. Over and above, our data implies abundant PPAR- expression in AAA wall samples, an observation consistent with comprehensive endogenous activation of the pathway. It is thus questionable whether additional exogenous PPAR- $\gamma$  activation will beneficially influence inflammation in and the progression of AAA.

Our observations rely on the availability of human AAA material. As a consequence all conclusions are based on surgical samples representing end-stage human disease. As adipogenic degeneration is not a feature in animal models of the disease, we were unable to examine the timing and role of adipogenic degeneration during AAA progression. A re-evaluation of histological images published by other groups also revealed the phenomenon of fatty degeneration; showing adventitial adipogenic degeneration is a universal phenomenon of AAA disease (Supplemental figure 4).<sup>23, 35-37</sup> Moreover, our studies rely on the availability of surgical specimens. In era of EVAR dominance for elective repair, wall surgical specimens are getting rare. As such our study is based on older material and the level of statin use is below current standards.

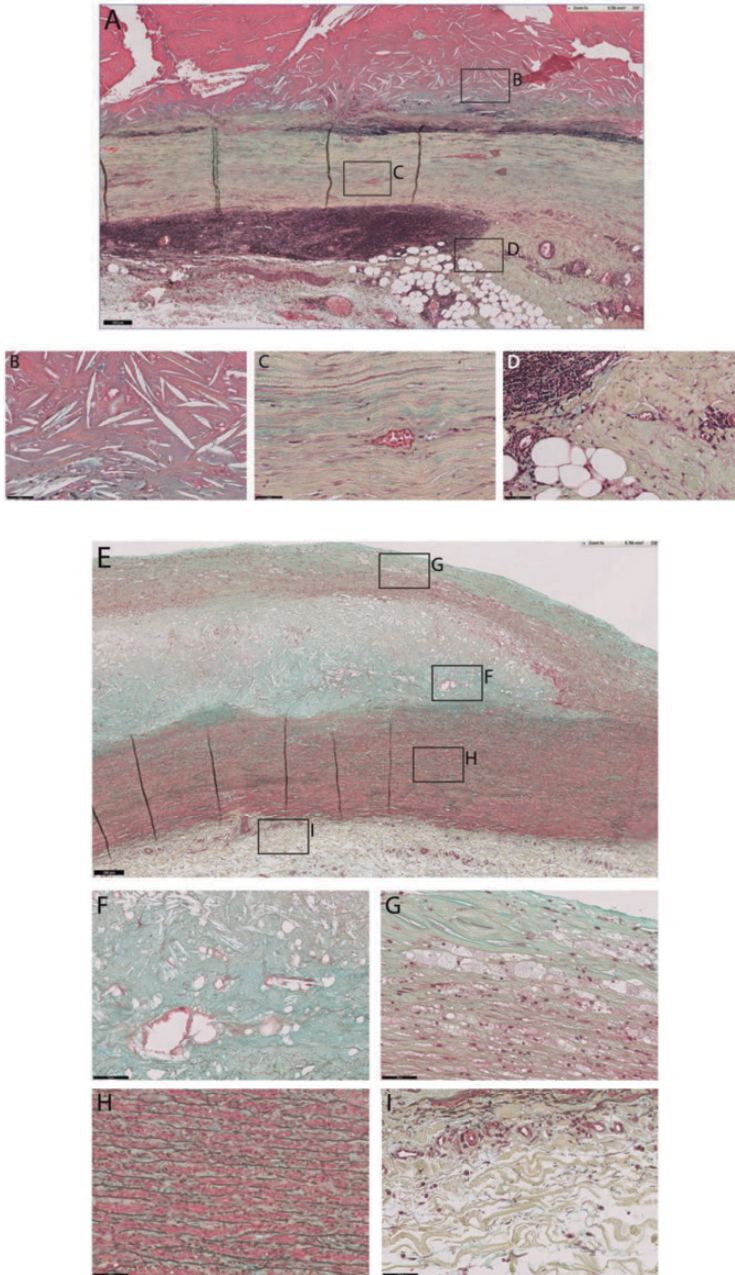
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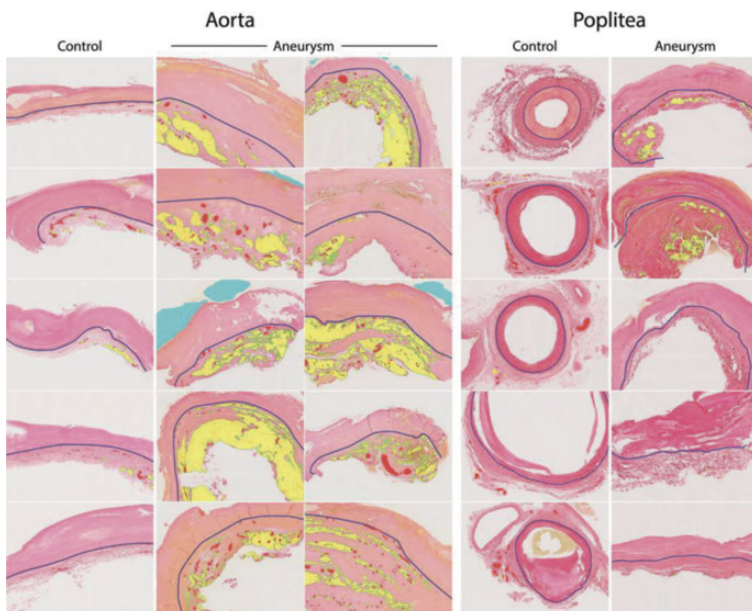
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## SUPPLEMENTAL DATA

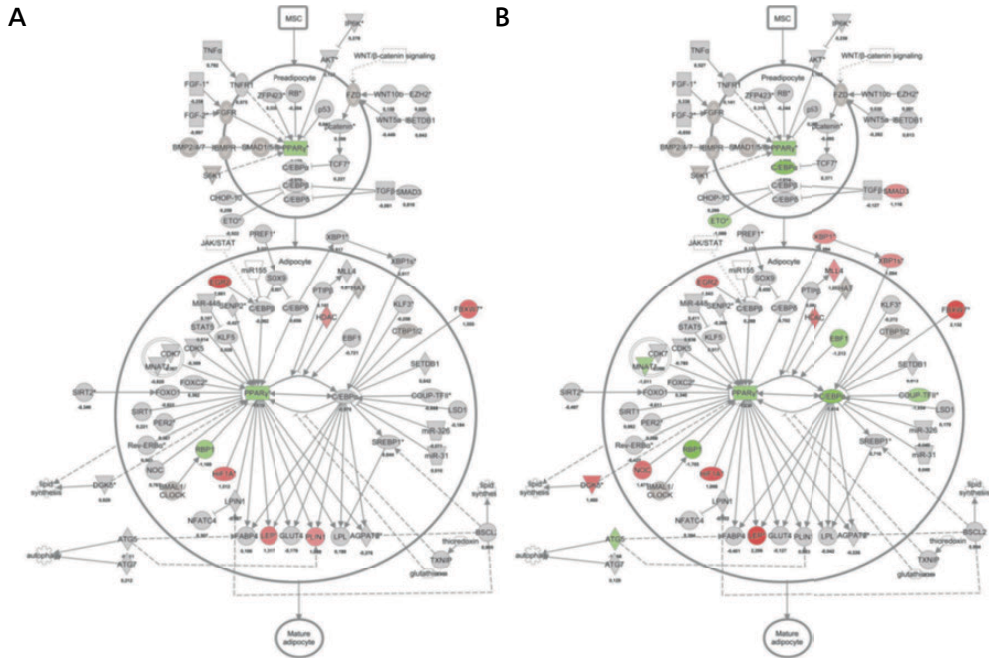


**Supplemental Figure 1.** Movat's pentachrome staining illustrating the distinctive histomorphologies of atherosclerotic control abdominal aorta (A) and AAA tissue (B). (A) The intimal layer of atherosclerotic aorta tissue is hallmarked by the presence of an atherosclerotic lesion (presented lesion: late fibroatheroma (Virmani classification<sup>15</sup>)). The lesion is characterized by presence of necrotic core with cholesterol crystals (arrow) (A-I). ▶

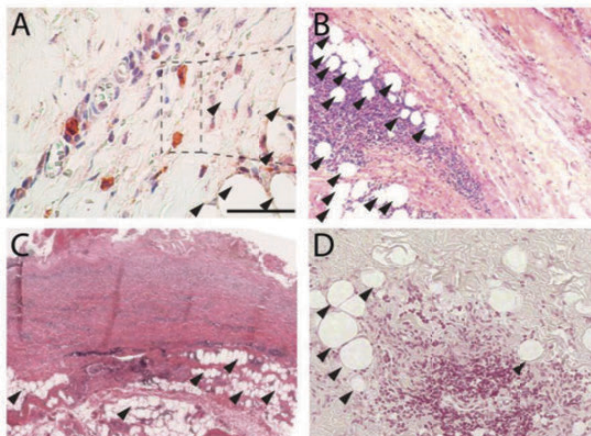
- ▶ The core is covered by a so called fibrous cap (A-II). Foam-cells present in the cap are marked (\*). A detail of the intact media underlying the atherosclerotic lesion is shown in A-III. The medial layer consists of multiple, uninterrupted lamellar units of elastin sheets with interspersed vascular smooth muscle cells. The adventitia (A-IV) consists of a loose collagenous network and harbors a vascular plexus (vasa vasora, indicated by the open triangles) at the medial-adventitial border. (B) shows the histo-morphology of an AAA. Note loss of typical 3-layer structure. The intima is covered by remnants of the luminal thrombus (fibrin is stains red in the Movat staining). Of note: the large basal cholesterol clefts (indicated by the arrows in B-I) at the basis of the thrombus presumably reflect cholesterol accumulation from trapped erythrocyte membranes. In the AAA, the intimal and medial layers are transformed into a condensed collagenous, vascular smooth muscle cell/proteoglycan-poor matrix ("fibrotic matrix", B-II). The zone harboring the vasa vasora (indicated by triangles in overview and detail B-II) remains as a landmark, marking the former medial-adventitial border zone (bz). The former adventitia (B-III) shows areas of fibrosis inter-dispersed with adipocyte clusters and is home to tertiary follicles (tf).



**Supplemental Figure 2.** Overview of adipocyte distribution within the adventitial layer. All images oriented with luminal side up (unless the whole lumen is visible). Adventitia is defined as area under the blue line with the yellow overlay representing all adventitial adipocytes and the red overlay representing all adventitial vasa vasora. Light blue overlay indicates adhering luminal thrombus. Mag 20x.



**Supplemental Figure 3.** Comparison of adipogenesis pathway on microarray between ruptured and elective AAA. Pathways analysis (Ingenuity Pathway Analysis) showing activation of the adipogenesis network in respectively ruptured (sFigure 3A; n=17) and stable (electively operated, (sFigure 3B; n=31)) aneurysm wall samples using atherosclerotic controls (n=10) as reference. Green overlay = downregulation; Red overlay = upregulation.



**Supplemental Figure 4.** Overview of histological images from previous publications showing adventitial fatty degeneration in AAA samples. Fatty degeneration can also be appreciated in earlier publications: A) Hinterseher, *Int J Mol Sci* 2015<sup>35</sup>; B) Golledge, *ATVB* 2007<sup>36</sup>; C) Hurks, *Atherosclerosis* 2014<sup>37</sup>; D) Hurks, *JVS* 2014<sup>23</sup>. Arrowheads denote adipocytes within the matrix of human AAA, illustrating adventitial adipogenic degeneration is a common phenomenon in AAA.



# Chapter

SUMMARY AND GENERAL DISCUSSION

# 10



## SUMMARY AND GENERAL DISCUSSION

Abdominal aortic aneurysms (AAAs) are potentially lethal due to rupture. Rupture occurs mainly in AAA greater than 55mm and acute repair still results in mortality over 30%. Although the results of elective treatment have significantly improved over the years and mortality is low (<3%), there is a considerable risk of morbidity. AAAs are prevalent mostly in elderly patients and generally only progress slowly in size. Therefore, treatment that slows aneurysm growth would allow patients to avoid aneurysm repair, in particularly elderly patients. Insight into the pathophysiology of the disease has improved over the past few years and continuing research has led the focus towards finding pharmaceutical means to inhibit or even abrogate aneurysm growth.

The aim of this thesis was to identify new possible targets for pharmacological treatment of AAAs and to apply this insight to the development of new therapies in a preclinical setting. Besides, understanding the cause of AAA progression can help identify secondary prevention strategies aimed at slowing down expansion.

The pathophysiological development of AAA is currently thought to arise through a complex interaction among the structural properties of the infrarenal aortic wall and various risk factors. Several risk factors for AAA disease have been described in epidemiological screening studies. Amongst others, a history of high plasma cholesterol, hypertension and smoking history, have been associated with AAA disease. These associations have led to various clinical studies evaluating the potential of pharmaceutical strategies, such as anti-hypertensive agents and statins, to inhibit AAA growth rate. A systematic review on current conducted human studies evaluating these pharmaceutical strategies is described in **Chapter 2**. This chapter highlights lack of high quality human studies evaluating the potential of pharmacological inhibition of AAA growth. The majority of the studies had little scientific value because of the retrospective design and small sample size. Moreover, the interpretation of the studies was hindered by lack of standardized measurements and inappropriate statistical analysis. Currently, no pharmaceutical therapy can be recommended for the stabilization of AAA disease.

Chapter 2 also describes the effect of pharmacological cardiovascular risk management on AAA growth. It has long been thought that AAA disease is closely related to atherosclerosis disease. Both diseases are found in the arteries, they have both similar predisposing risk factors and they exhibit similar immune cells at the lesion site. However, after the unsuccessful testing of cardiovascular risk management for human AAA disease the resemblance is less alike than initially thought. Beside the apparent stenotic and occlusive nature in atherosclerosis and dilatation in AAA disease there are more differences. Only 10% of the AAA cases reveal atherosclerotic plaques at the side of AAA formation and aneurysms rarely occur at atherosclerotic prone arteries. It is proposed that the risk factors of both diseases can promote atherosclerosis and AAA disease separately, but the risk factors cannot be defined as causative. For example smoking has a much stronger promoting effect on AAA disease than on atherosclerosis<sup>1</sup>.

In a large population screening study smoking accounted for >70% of all AAAs<sup>2</sup>. The strong association between smoking and AAA has been described as early as 1958<sup>3</sup> and underlined by Lederle in 2011 who described a possible specific link between declining age adjusted AAA mortality and a reduction in annual adult per capita cigarette consumption<sup>4</sup>. Therefore, smoking cessation should be pursued regardless of other therapies.

Another potential risk factor for AAA disease is a history of pulmonary emphysema. Several parallels have been observed between AAA disease and chronic obstructive pulmonary disease (COPD). Reduced lung function occurs commonly in AAA patients and is associated with an increase in risk of AAA rupture<sup>5</sup>. In both diseases there is a strong association with smoking and this might explain the association between lung function and aneurysm development and growth. However, **Chapter 3** reveals that the increased prevalence of COPD in AAA patients is independent from smoking. This suggests that this relationship only reflects a common susceptibility. This is supported by the appearance of increased inflammation in both diseases with in particular converging pathways including the matrix-metallo protease 9 (MMP9) and neutrophil elastase pathway<sup>6,7</sup>. In aneurysm disease, increased expression of pro-inflammatory cytokines, such as MMP9, induces degradation of the extracellular matrix and therefore enhances vascular dilatation<sup>8</sup>. Higher levels of circulating pro-inflammatory cytokines, causing e.g. proteolysis, have been found in AAA patients compared to controls without AAA disease<sup>9,10</sup>. Besides, pathological studies of end stage human AAA biopsies and those performed in animal models have demonstrated the importance of inflammation, proteolysis and vascular smooth muscle cell loss in AAA<sup>11-13</sup>. Therefore, insight into immune responses in AAA disease is important for the discovery and application of new therapies.

Several studies identify the vitamin D receptor (VDR) as a potent immunoregulatory factor<sup>14</sup>. Reports on the potential of VDR antagonists are diverse. There are several reported mechanisms such as quenching NFkB, MAPK and interference with macrophage activation. In **Chapter 4** it is demonstrated that in AAA disease the effects of VDR activation through a vitamin D analogue are restricted to the T-helper cell content (CD4+) and the expression of cysteine proteases cathepsin K and L. It is unclear if the effects of VDR activation on T-helper cell content will influence AAA disease. The role of T-helper cells in AAA disease remains unclear because results from animal studies are inconclusive and clinically accelerated aneurysm progression during intensive immune suppression has been reported<sup>15-18</sup>. Also the effect of inhibiting cysteine proteases on AAA growth remains uncertain. Collagen is the primary matrix component of the media and adventitia of the aortic wall and a prominent target of cysteine proteases. Therefore, interference with cysteine proteases has great potential in pharmaceutical stabilization of AAA growth. In **Chapter 5** the importance of cysteine proteases by quantitative collagen degradation assays assessing the general cysteine protease and MMP mediated collagen degradation is established. Although the cysteine proteases: cathepsin K, L and S have separately been positively linked to AAA progression, inhibiting selectively cathepsin K and S with statins did not result in effective growth reduction or stabilization of AAAs<sup>8,19-21</sup>. Due to this, in Chapter 5 the effect of a broader spectrum, multiple cysteine proteases inhibitor (E64) on AAA growth was tested. The inhibition of cysteine proteases with the E64 effectively blocked aneurysm formation and preserved collagen in two different murine models of AAA disease. Neutrophils play a crucial role in the vascular inflammation seen in AAA disease<sup>22,23</sup>. They appear to be responsible for reduced cystatin C levels, the primary endogenous inhibitor of several cysteine proteases. They are also known to produce several serine proteases as well as MMP8 and MMP9, all known to promote degradation of the extracellular matrix. One of the main activators of neutrophils is interleukin 8 (CXCL8). Abundant expression of CXCL8 is a hallmark of human AAA. It is responsible for the ongoing inflammatory response in the aortic wall<sup>6</sup>. Through binding of the CXCR1/2 receptors CXCL8 causes chemotaxis and activation of neutrophils. Moreover, CXCL8 is also the putative receptor for CXC-mediated angiogenesis, another key feature of human

AAA disease<sup>24;25</sup>. **Chapter 6** describes the CXCL8/neutrophil axis in human AAA and the effect of blocking the neutrophil receptors on the aneurysm formation in the elastase model. Interference with the CXCL8/neutrophil axis resulted in fully abrogating experimentally treated mice compared to control mice. Currently phase 2 trials for several CXCR2 antagonists are being conducted. The first, a phase 2 trial on the effect of a CXCR2 antagonist in COPD is highly promising and there is a significant improvement in lung function and a reduction in inflammation. There has been no negative effect on bone marrow functions in healthy subjects and no increase in general infections. This suggests that blocking neutrophil chemotaxis and activation has great potential in reducing human AAA growth<sup>26;27</sup>. Currently the way to impair neutrophil chemotaxis, is through colchicine. This compound has been used over decades in the treatment of gout. **Chapter 7** addresses the inhibiting effect of colchicine on aneurysm formation in the murine elastase model of AAA disease. Chapter 7 also proposes two different mechanisms in which colchicine has an effect on human neutrophils in the aneurysm wall. The first direct inhibition of neutrophil chemotaxis is by inhibiting the effect of TNF $\alpha$  and by reducing adhesion of neutrophils to the endothelial cells. Furthermore colchicine has an indirect effect mediated via the NALP3 inflammasome. The NALP3 inflammasome activates several interleukines via caspase-1 and activates the production of e.g. CXCL8. We describe the prominent activation of the NALP3 inflammasome and its pathway in the human AAA tissue. Several mechanisms leading to the activation of the inflammasome have been proposed. One of the most plausible explanations is the activation through the mitochondrial reactive oxygen species (ROS) induced by smoking; a major risk factor of AAA disease. There are ongoing concerns about the therapeutic margin between the side-effects of colchicine. A recent study on colchicine preventing the post-pericardiotomy syndrome in patients after cardiac surgery and a study on colchicine preventing pericarditis revealed no difference in side-effects between placebo and colchicine. However, the effect in AAA patients still needs to be investigated. Another inflammatory hallmark of AAA disease, besides abundant CXCL8 expression, is increased expression of interleukin 6 (IL6) in the human AAA wall. Also a polymorphism in the IL6 gene has been identified as a remarkable strong and independent risk factor for AAA disease<sup>28</sup>. For years, IL6 has been described as a pro-inflammatory cytokine<sup>29-31</sup>. However, recent studies also prescribe IL6 anti-inflammatory properties with roles in tissue protection and regeneration<sup>32-34</sup>. The two roles of IL6 are thought to reflect two different signaling routes: the *classical* IL6 receptor route being responsible for the anti-inflammatory/tissue regenerative activities, and the non-classical, *trans-signaling* route via the soluble IL6 receptor being responsible for the pro-inflammatory activities<sup>29;31;35</sup>. A role for IL6 in AAA disease is investigated in **Chapter 8**. The IL6 receptor CD126 was modestly present in human AAA tissue samples in contrast to the soluble receptor, which was abundantly present in both atherosclerotic samples and AAA samples. The phosphorylation of STAT 3 in AAA human tissue samples indicates activity of the IL6/STAT3 axis in aneurysm disease. However, this does not differentiate between the pro- or anti-inflammatory routes. Therefore the properties of anti-IL6 treatment in the murine elastase model were tested. Unexpectedly, inhibiting interleukin 6 with an anti-IL6 antibody resulted an aneurysm rupture in ~50% of the treated animals. Delayed treatment did not result in a decrease in aneurysm formation. These observations suggest a protective role for IL-6 in the acute phases of injury. However, with modest expression of the classical signaling route, the observations do not indicate a major role for IL-6 in aneurysm diseases.

Strong clinical and molecular associations exist between AAA and popliteal artery aneurysms (PAA). Yet, while the natural history of AAA is that of rupture, the primary concern in PAA's is thrombosis and rupture of PAA is rare<sup>36</sup>. A patho-histological (re-)examination of human AAA samples and popliteal aneurysm wall samples, in **Chapter 9**, revealed adipogenic degeneration of the adventitial layer of the AAA wall. Moreover, enrichment of adipocyte-related genes and pathways in ruptured AAA versus non-ruptured controls implied an association between the extent of fatty degeneration and AAA rupture.

## CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis underlines the importance of chronic inflammation and proteolytic activity in AAA formation. Investigating human aneurysm tissue and using the elastase mouse model of aneurysm disease, several potential targets for pharmacologically stabilizing aneurysms were revealed.

Mouse models of human disease pathogenesis have become a central part of biomedical research. This is because the laboratory mouse provides the most experimentally accessible mammalian model, sharing e.g. organ systems and systemic physiology with humans.

Currently the translation from the murine models to the clinical trials is challenging. This might question the validity of the existing mouse models of AAA disease. Differences in physiology, as well as variations in homology of molecular targets between mice and humans, may lead to translational limitations. Therefore, the clinical impact of the pharmacological targets investigated in this thesis remains unclear. An ironic shortcoming of the ability to assess the usefulness of the murine models is the lack of knowledge of the biochemical and cellular characteristics of initiating factors in the human disease. Nonetheless, the pathological studies of end stage human biopsies have demonstrated several similarities between the cellular and biochemical characteristics of the mouse models and human AAA disease. Besides, the mouse models, as described in Chapter 1, reproduce adequately the hallmark features of human AAA disease. Consequently, can mimic the disease pathology and be utilised to provide a genuine insight into the pathogenesis of AAA. In particular the elastase model of AAA disease has enabled more detailed investigations on the cellular and molecular mechanisms of the disease in a controlled manner. Even though murine models remain a unique source of *in vivo* information, in the near future other emerging translational alternatives will complement or may eventually replace the link between *in vitro* studies and clinical applications. In the past years a wide range of alternatives to animal-based preclinical research has emerged. These include epidemiological studies, autopsies, computer modeling and phase 0 studies. These approaches towards clinical disease are promising and might have a great impact on the field of human AAA disease.

It has been generally accepted that the natural history of an AAA is progressive aortic wall degradation, ultimately culminating in loss of structural integrity and aortic rupture. The segmental weakening of the artery and profound matrix changes, with increased intra-molecular collagen cross linking in the AAA wall, are a hallmark of AAA disease. A complete loss of normal collagen architecture was found. Besides, the connections that normally allow the tissue to behave as a coherent network are missing in AAA disease<sup>36</sup>. These changes in the aortic wall do not necessarily reflect the primary cause of AAA formation. More likely, these changes reflect inappropriate

collagen deposition in a setting that is characterized by ongoing inflammation and activation of multiple proteolytic pathways.

This thesis highlights the importance of chronic inflammation in the abdominal aortic aneurysm disease and designates multiple inflammatory pathways as potential targets for pharmaceutical stabilization of the expansion of the vessel wall. Especially the neutrophil-receptor pathway has been indicated as great value in the formation of AAA. The surprising complete abrogation of aneurysms in the elastase mouse model with the neutrophil-receptor inhibitor, DF2156A, together with the high expression of the CXCL8-axis components in human AAA disease, indicates a great significance of this pathway. Since human AAA development is typically an inflammatory process diffused over a large number of years, disrupting the ongoing inflammatory response by blocking the activation loop in this pathway yields highly promising prospects for clinical stabilization of the aneurysm wall. DF2156A is currently under investigation in phase 2 trials for COPD. Therefore, the translation into a phase 2 trial investigating the effect of DF2156A on AAA disease should be feasible in the near future. The results of this trial are highly awaited.

Besides DF2156A, this research has yielded several other new therapeutic targets applications (such as colchicine and E64) that could be translated into the clinic. Since colchicine is a registered drug for gout, the translation into a clinical trial to treat AAA disease could be relatively quick and easy. E64, the broad-spectrum cathepsin inhibitor, still needs additional investigation into its efficacy and safety profiles before clinical application could be initiated.

Currently, as depicted in Chapter 2, the number of randomized controlled trials is low and the scientific quality of the available literature is limited. Patient drug compliance is difficult to assess and a major challenge. Another challenge for randomized controlled trials is the slow growth rate of the AAA. The mean aortic diameter increases between 1-2.5 mm/year and these changes are within the inter-observer measurements error reported for ultrasound<sup>377</sup>. Therefore, careful planning, standardization of measurements and quality control of the clinical trials needs to be ensured. Finally, it is becoming more and more apparent that AAA disease is highly multi-factorial, with several risk factors, genetic predisposition and actual mechanical difference of the aneurysm itself. Therefore, a number of very large, carefully planned RCTs is required that correct for the large amount of potential bias.

This thesis investigates the pathophysiology of abdominal aortic aneurysms and proposes several pharmaceutical inflammatory targets to manipulate its growth. Hopefully, the data will elicit further research and translation of the compounds to clinical trials. Ultimately, to get closer to pharmaceutically stabilizing AAA growth.

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# Chapter

NEDERLANDSE SAMENVATTING

# 11



## NEDERLANDSE SAMENVATTING

Het aneurysma van de abdominale aorta (AAA) is een veel voorkomend en potentieel levensbedreigend fenomeen door de kans op ruptuur van de vaatwand. Ruptuur van het AAA komt voornamelijk voor in AAAs groter dan 55mm en de acute behandeling heeft een hoge mortaliteit (>30%). Ondanks dat de resultaten van een electieve behandeling significant verbeterd zijn over de jaren en de mortaliteit laag is (<3%), is het morbiditeitsrisico nog steeds hoog. AAAs komen het meest voor bij oudere patiënten en de diametergroei van de aorta is over het algemeen langzaam. Daarom zou een therapie gebaseerd op het verminderen van de groei van het aneurysma de operatieve electieve behandeling kunnen uitstellen en bij oudere patiënten zelfs onnodig kunnen maken. In de afgelopen jaren is er steeds meer duidelijk geworden over de pathofysiologie van het AAA waardoor meer onderzoek zich is gaan richten op het vinden van een farmacologische strategie om de groei van het aneurysma te verminderen of zelfs helemaal te stoppen.

Het doel van dit proefschrift is het identificeren van nieuwe aangrijppunten voor farmacologische behandeling van het AAA en om de nieuwe inzichten om te zetten naar nieuwe therapieën en deze te testen in een preklinische setting. Daarnaast kunnen de nieuwe inzichten die verkregen zijn in dit proefschrift bijdragen aan het ontwikkelen van nieuwe secundaire preventieve maatregelen die gericht zijn op het vertragen van de AAA groei. Op dit moment wordt gedacht dat het AAA zich ontwikkelt door een serie van complexe interacties tussen de structurele eigenschappen van de AAA vaatwand en verschillende risicofactoren. Een aantal van deze risicofactoren zijn beschreven in epidemiologische studies. Een hoog plasma cholesterol, hypertensie en roken zijn onder andere geassocieerd met AAAs. Deze verbanden hebben geleid tot verschillende klinische studies die hebben gekeken naar potentiële farmacologische strategieën, zoals anti-hypertensiva en statines, om de groei van het aneurysma te remmen. Een systematisch overzicht van al deze klinische studies wordt gegeven in **Hoofdstuk 2**. Dit hoofdstuk belicht het gebrek aan kwaliteitsstudies naar het effect van potentiële farmacologische stabilisatie van AAA groei. De meerderheid van de studies was van weinig wetenschappelijke waarde door een verkeerde studieopzet en te weinig deelnemers. Daarnaast wordt de interpretatie van de studies bemoeilijkt door gebrek aan gestandaardiseerde metingen en verkeerde statistische analyses. Momenteel kan er geen farmacologische therapie voor de stabilisatie van de groei van het AAA worden aanbevolen. Hoofdstuk 2 beschrijft ook het effect van farmacologisch cardiovasculair risicomanagement op AAA groei. Lange tijd is gedacht dat AAA sterk gerelateerd is aan arteriosclerose. Beiden worden gevonden in arteriën, ze hebben gelijke predisponerende risicofactoren en in beide ziekten komen gelijke immuuncellen tot expressie in het aangedane weefsel. Desalniettemin, na het tevergeefs testen van cardiovasculair risico management voor AAAs, lijkt de gelijkenis tussen beide ziekten minder groot dan in eerste instantie werd gedacht. Naast het voor de hand liggende verschil van stenose en dilatatie van de aandoeningen zijn er meer verschillen. Slechts 10% van de abdominale aneurysma's laat daadwerkelijk een arteriosclerotische plaque aan de kant van het aneurysma zien. Daarnaast komen de aneurysma's niet tot nauwelijks voor in de arteriën die het meeste vatbaar zijn voor arteriosclerose. Men denkt dat de risicofactoren van beide ziekten zowel arteriosclerose en AAA kunnen bevorderen, echter deze risicofactoren kunnen niet als oorzakelijk worden gezien. Roken heeft bijvoorbeeld een veel sterkere relatie met AAA dan met arteriosclerose. In een

groot bevolkingsonderzoek verklaart roken ongeveer 70% van alle aneurysma's. Deze sterke relatie tussen roken en AAA is al voor het eerst beschreven in 1958 en wordt onderstreept door Lederle in 2011 die een specifiek verband beschrijft tussen vermindering in AAA mortaliteit en een afname in jaarlijkse sigaretten consumptie. Daarom moet worden opgemerkt dat stoppen met roken altijd moet worden nagestreefd, naast andere behandelingen.

Een andere potentiële risicofactor voor AAA is een medische voorgeschiedenis met longemfyseem. Verschillende parallellen zijn opgemerkt tussen AAA en chronische obstructief longlijden (COPD). Verminderde longfunctie komt vaak voor in AAA patiënten en is geassocieerd met een verhoogd risico op AAA ruptuur. Bij beide ziekten is een sterke associatie gevonden met roken en dit zou het verband tussen longfunctie en AAA groei kunnen verklaren. Echter, **Hoofdstuk 3** laat zien dat de verhoogde prevalentie van COPD in aneurysma patiënten onafhankelijk is van roken. Dit suggereert dat deze relatie een gemeenschappelijke gevoeligheid laat zien. Dit wordt ondersteund door verhoogde ontstekingsmarkers in beide ziekten met gelijke activeringsroutes zoals de matrix-metallo protease 9 (MMP9) en neutrophil elastase ontstekingsroute. In AAA veroorzaakt verhoogde expressie van pro-inflammatoire cytokines, zoals MMP9, de afbraak van de extracellulaire matrix en vergroot zo de vasculaire dilatatie. Verhoogde niveaus van circulerende pro-inflammatoire cytokines, die zorgen voor proteolyse, zijn gevonden in AAA patiënten en niet in aorta's zonder aneurysma. Daarnaast laten pathologische studies van eind stadium humane aneurysma bipten en de diermodel studies het belang van ontsteking, proteolyse en het verlies van gladde spiercellen in de vaatwand zien. Hierdoor is inzicht in de immunologische reacties in AAA van groot belang voor het ontdekken en toepassen van nieuwe therapieën. Verschillende studies hebben de vitamine D receptor (VDR) geïdentificeerd als een potentiële factor in de immunoregulatie. De verslaglegging over vitamine D receptor antagonisten is divers. In de literatuur wordt een aantal mechanismen beschreven zoals het beïnvloeden van NFκB, MAPK en macrofaag activering. **Hoofdstuk 4** laat zien dat in AAA het effect van VDR activering door een vitamine D analoge beperkt blijft tot het aantal T helper cel (CD4+) en de expressie van cysteine proteasen K en L. Het is onduidelijk of deze T helper cellen een effect hebben op AAA. Deze rol blijft onduidelijk omdat resultaten van dierstudies niet doorslaggevend zijn en ook omdat klinische rapporten zijn gepubliceerd waarin versnelde AAA groei werd waargenomen in patiënten behandeld met immuunsysteem onderdrukkende medicatie. Daarnaast is het effect van het remmen van cysteine proteasen in AAA niet bekend. Collageen is het primaire bestanddeel van de matrix van de media en adventitia van de aorta vaatwand en een belangrijk doelwit van cysteine proteasen. Daarom zou het effect van het beïnvloeden van de cysteine proteases op de AAA vaatwand gunstig kunnen zijn in het remmen van de AAA groei. In **Hoofdstuk 5** wordt het belang van cysteine proteasen in AAAs onderstreept door kwalitatieve collageen degradatie experimenten die het effect van cysteine proteasen en matrix metallo proteasen op collageen laten zien. Ondanks dat de cysteine proteasen: cathepsine K, L en S allemaal onafhankelijk al gekoppeld zijn aan AAA progressie, heeft het selectief remmen deze factoren met statines in klinische studies niet geleid tot het remmen van de AAA groei. Daarom is in hoofdstuk 5 het effect van een breed spectrum cysteine protease remmer (E64) op AAA groei getest. Het remmen van cysteine proteasen met E64 heeft in twee verschillende muismodellen voor AAA geleid tot het blokkeren van AAA formatie en het behoud van collageen in de vaatwand. Neutrofielen spelen een cruciale rol in

de vasculaire ontsteking in AAA. Zij lijken onder andere verantwoordelijk te zijn voor verminderd cystatine C, wat de primaire endogene remmer is van cysteine proteasen. Daarnaast produceren de neutrofielen verschillende serine proteasen, MMP8 en MMP9. Factoren die allemaal bekend staan om het bevorderen van de extracellulaire matrix afbraak in de vaatwand. Een van de belangrijkste activatoren van neutrofielen is interleukine 8 (CXCL8). Een overvloed aan CXCL8 expressie is een kenmerk van AAA. CXCL8 is verantwoordelijk voor de voortdurende ontsteking in de aorta vaatwand. Door binding met de CXCR1/2 receptoren zorgt CXCL8 voor activering van de neutrofielen. Daarnaast zorgt CXCL8 ook voor CXC-gestuurde angiogenese wat ook een kenmerk is van AAAs. **Hoofdstuk 6** beschrijft de CXCL8/neutrofiel-as in humane AAAs en het effect van het blokkeren van de neutrofiel receptoren op aneurysma formatie in het elastase muismodel. Het beïnvloeden van de as resulteerde in volledige blokkade van aneurysma formatie in het muismodel. Op dit moment zijn er fase 2 studies naar het klinische effect van de neutrofiel receptor remmers gestart. De eerste, een fase 2 studie naar het effect van neutrofiel receptor remmers in COPD die veelbelovend is, laat een significante verbetering in longfunctie en verminderde ontsteking zien. Daarnaast is er in de eerste studies geen negatief effect beschreven van de neutrofiel receptor remmer op beenmerg functies en ook werd er geen toename gezien in het voorkomen van algemene infecties. Bij elkaar genomen, suggereert dit, dat het blokkeren van neutrofiel chemotaxis en neutrofiel activering zeer veel belovend is in het klinisch remmen van AAA groei. De huidige manier om neutrofiel chemotaxis te remmen is door colchicine. Dit is een geneesmiddel dat reeds jaren wordt gebruikt in de behandeling van jicht. **Hoofdstuk 7** beschrijft het effect van colchicine op aneurysma ontwikkeling in het elastase muismodel. Daarnaast beschrijft het ook twee verschillende mechanismen waarop colchicine een effect heeft op neutrofielen in de aneurysma vaatwand. Het eerste is het direct remmen van neutrofiel chemotaxis door het beïnvloeden van TNF $\alpha$  en door het verminderen van de adhesie van de neutrofielen aan de endotheel cellen. Daarnaast heeft colchicine een indirect effect, gestuurd door het NALP3 inflammasome. Dit inflammasome activeert verschillende interleukines via caspase-1 en zo ook de productie van onder andere CXCL8. In hoofdstuk 7 wordt verhoogde activering van het NALP3 inflammasome en zijn activeringsroute in humaan AAA weefsel beschreven. Verschillende mechanismen voor activering van het NALP3 inflammasome zijn in de literatuur beschreven. Een van de meest aannemelijke verklaringen voor de activering van dit inflammasome in AAA is de activering door mitochondriale reactieve zuurstof deeltjes (ROS), geïnduceerd door roken; een zeer belangrijke risicofactor voor AAA. Er zijn belangrijke bezwaren over de therapeutische marge tussen colchicine en de bijwerkingen van het medicijn. Recente studies naar het effect van colchicine op de preventie van het post-pericardiotomie syndroom in patiënten na hartoperaties en een studie naar de preventie van pericarditis lieten geen verschil zien in bijwerkingen tussen placebo en colchicine. Desalniettemin moet het effect van colchicine in AAA patiënten nog worden onderzocht. Een ander kenmerk van de ontsteking gezien in AAA, naast CXCL8, is een toename in de expressie van interleukine 6 (IL6) in de aneurysma vaatwand. Daarnaast is een polymorfisme van het IL6 gen beschreven als sterke en onafhankelijke risicofactor voor AAA. Jarenlang is IL6 beschreven als pro-inflammatoir cytokine. Echter, recente studies beschrijven IL6 als een anti-inflammatoir cytokine met verschillende functies in weefsel protectie en regeneratie. Het wordt gedacht dat de verschillende functies van IL6 worden gestuurd door twee verschillende signaleringsroutes:

de klassieke route via de IL6 receptor die verantwoordelijk wordt gehouden voor de anti-inflammatoire en weefsel regeneratieve activiteiten en de niet-klassieke, trans-signaling route die verantwoordelijk wordt gehouden voor de pro-inflammatoire activiteiten. Een rol voor IL6 in AAA wordt onderzocht in **Hoofdstuk 8**. De IL6 receptor CD126 was matig aanwezig in arteriosclerotische vaatwand en in aneurysma vaatwand. Dit in tegenstelling tot de oplosbare receptor, die in beide type vaatwand overvloedig aanwezig is. De fosforylatie van STAT3 in humaan AAA weefsel laat de activiteit van de IL6/STAT3 as in AAA zien. Echter, dit differentieert niet tussen de verschillende activeringsroutes. Daarom werd het effect van anti-IL6 in het elastase muismodel onderzocht. Geheel onverwacht werd gevonden dat het remmen van IL6 met een anti-IL6 antilichaam resulteert in aneurysma ruptuur in bijna 50% van de behandelde dieren. Vertraagde behandeling met anti-IL6 resulteerde niet in een vermindering van AAA formatie. Dit suggereert een beschermende rol voor IL6 in de fase van acute schade. Echter, met milde expressie van de klassieke signaleringsroute, wijzen deze bevindingen op een marginale rol voor IL6 in AAA.

Er is een sterke klinische en moleculaire relatie tussen AAA en aneurysmata van de arteria poplitea (PAA). Echter, de groei van het AAA resulteert uiteindelijk in ruptuur, terwijl ruptuur in PAAs zeldzaam is. Een her-evaluatie van de humane AAA en PAA vaatwand, in **Hoofdstuk 9**, laat zien dat er vervetting van de vaatwand plaats vindt in de AAA vaatwand in tegenstelling tot in de PAA vaatwand. Daarnaast wijst een toename van de vetcel gerelateerde genen en hun pathways in geruptureerde AAAs in vergelijking met niet geruptureerde AAAs op een link tussen de vervetting van de AAA vaatwand en het voorkomen van ruptuur.

## CONCLUSIES EN TOEKOMST PERSPECTIEVEN

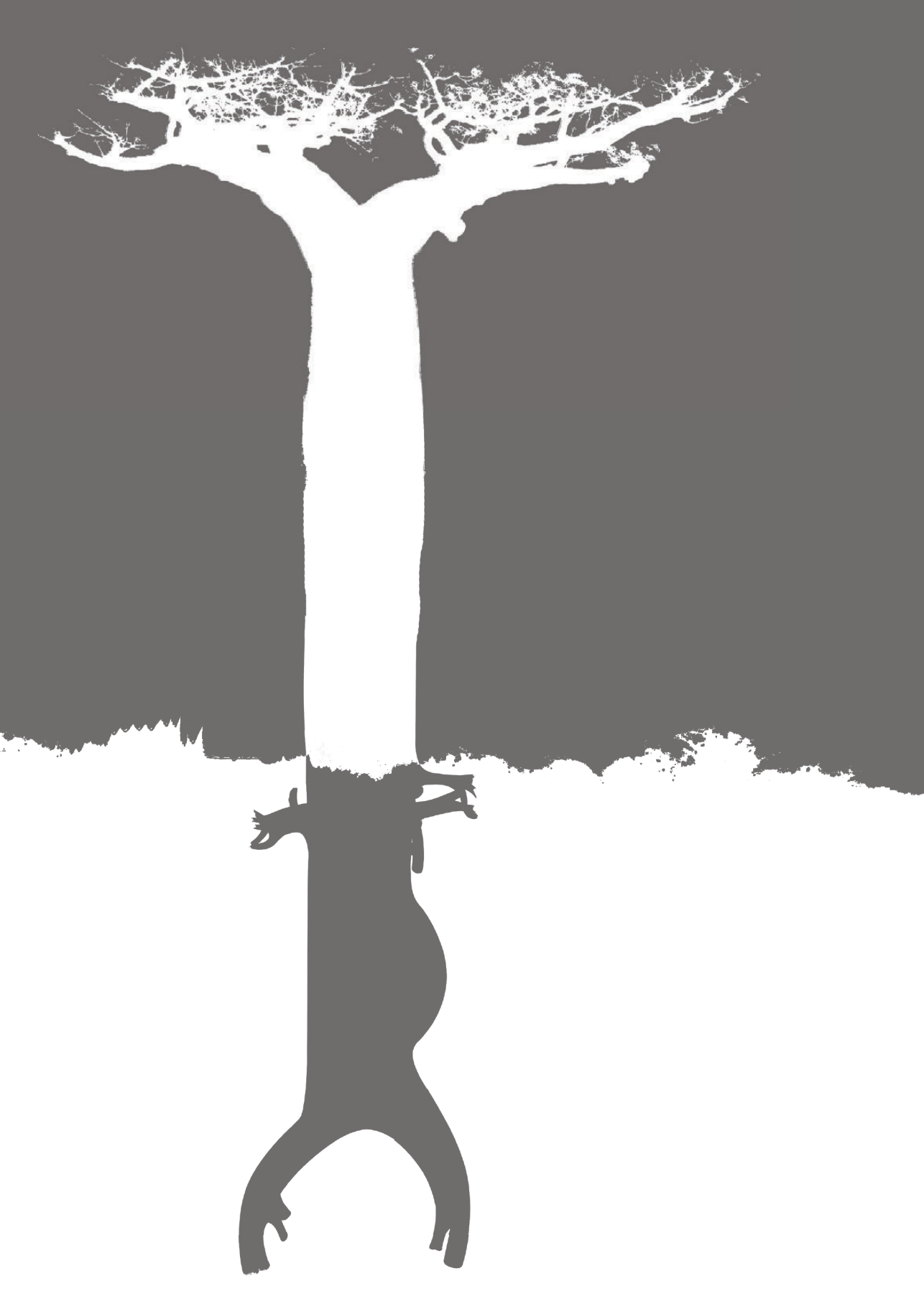
Dit proefschrift onderstreept het belang van chronische ontsteking en proteolytische activiteit in aneurysma formatie. Het onderzoeken van humaan aneurysma weefsel en het gebruik van het elastase muismodel heeft geleid tot verschillende potentiële aanknopingspunten voor farmacologische stabilisatie van het AAA. Muismodellen van menselijke ziekten zijn een deel geworden van biomedisch onderzoek. Dit komt omdat de laboratorium muis het meest toegankelijke experimentele zoogdiermodel is dat onder andere orgaansystemen en systemische fysiologie met de mens deelt. Op dit moment is de vertaling van muismodellen naar klinische trials moeizaam. Dit kan leiden tot het in twijfel trekken van de validiteit van de beschikbare muismodellen. Verschillen in fysiologie en variaties in de homologie van de moleculaire doelwitten tussen mens en muis kunnen leiden problemen. Daarom is de klinische impact van de onderzochte farmacologische doelwitten in dit proefschrift onduidelijk. Een ironische tekortkoming van het gebruiken van muismodellen is het gebrek aan kennis over de biochemische en cellulaire karakteristieken van de uitlokkende factoren in AAAs. Desalniettemin, de pathofysiologische studies van eindstadium humane weefsel biopten heeft een groot aantal overeenkomsten tussen de cellulaire en biochemische karakteristieken laten zien tussen de muismodellen en menselijke ziekte. Daarnaast reproduceren de muismodellen, zoals beschreven in Hoofdstuk 1, de belangrijkste kenmerken van AAAs. Hierdoor kunnen de modellen een deel van de pathofysiologie imiteren en worden zo gebruikt om een beter inzicht te krijgen in de humane situatie. In het bijzonder heeft het elastase muismodel er op een gereguleerde manier voor gezorgd dat er veel meer gedetailleerde kennis beschikbaar is over de cellulaire en moleculaire mechanismen van AAA. Ondanks dat muismodellen een unieke

bron van informatie, worden alternatieven gezocht om het gat tussen experimenteel onderzoek en de klinische situatie te verkleinen. In de afgelopen jaren is een grote reeks aan alternatieven voor dier experimenteel onderzoek opgekomen zoals epidemiologische studies, autopsie studies, computer modelstudies en fase 0 studies. Deze nieuwe methoden voor het onderzoek naar de klinische ziekten zijn veel belovend en hebben wellicht grote invloed op het veld van humaan AAA onderzoek. Het is algemeen geaccepteerd dat het natuurlijk beloop van AAA wordt gekenmerkt door progressieve vaatwand degradatie, leidend tot verlies van de integriteit van de vaatwand en aorta ruptuur. De segmentale verzwakking van de arterie en de duidelijke matrix veranderingen, met toegenomen intra-moleculaire collageen crosslinking in de AAA vaatwand zijn karakteristiek voor AAA. In een eerdere studie is een compleet verlies van de normale collageen architectuur in AAA gevonden. Daarnaast werd gezien dat de connecties die zorgen dat het weefsel zich gedraagt als een coherent netwerk niet aanwezig zijn in AAA. Deze veranderingen in de aortawand laten niet direct de primaire oorzaak van het aneurysma zien. Waarschijnlijker is het dat de veranderingen een verkeerde collageen afzetting laten zien in een situatie die wordt gekenmerkt door chronische ontsteking en activering van meerdere proteolytische routes.

Dit proefschrift haalt het belang naar boven van de chronische ontsteking van de vaatwand in AAA en belicht verschillende ontstekingsroutes als mogelijke doelwitten voor farmacologische stabilisatie van de vaatwand. In het bijzonder de neutrofiel receptor activeringsroute blijkt een belangrijke doelwit. De verrassende complete blokkade van de aneurysma formatie in het elastase muis model met de neutrofiel receptor remmer DF2156A, samen met de hoge expressie van de CXCL8-as in humaan AAA weefsel wijst op een belangrijke rol voor deze route. Aangezien het ontstekingsproces gezien in het aneurysma in de meeste gevallen verspreid is over de jaren, zou het doorbreken van de cirkel van activering in deze route zeer veelbelovend kunnen zijn in de klinische stabilisatie van het AAA. DF2156A wordt op dit moment onderzocht in fase 2 studies voor COPD. Daarom zal de vertaalslag naar een klinische trial in AAA patiënten reeds mogelijk kunnen zijn in de aankomende jaren. De resultaten van deze studie wordt met spanning afgewacht. Naast DF2156A heeft het onderzoek in dit proefschrift ook andere potentiële medicijnen aangewezen voor de farmacologische behandeling van het AAA (zoals colchicine en E64). Aangezien colchicine al een geregistreerd medicijn is zou de vertaling naar een klinische trial met dit middel ook relatief snel en gemakkelijk moeten gaan. E64, de breedspectrum cathepsine remmer behoeft echter nog additioneel onderzoek naar het effect en veiligheid voor dat een klinische trial gestart kan worden. Zoals beschreven in Hoofdstuk 2, is het aantal huidige gerandomiseerde klinische studies laag en de wetenschappelijke waarde en kwaliteit van de beschikbare literatuur is beperkt. Medicatie gebruik en therapietrouw is moeilijk te meten in deze studies en daarom een grote uitdaging. Een andere uitdaging voor gerandomiseerde klinische studies is de lage groeisnelheid van het AAA. De gemiddelde diameter van de aorta groeit tussen de 1 en 2.5 mm per jaar en deze verschillen in de diameter zitten in de range van de onderzoeksvariatie die is beschreven voor echografiemetingen. Daarom moet een goed studie design, het standaardiseren van de meting en de kwaliteitscontrole van klinische trials worden gewaarborgd. Tenslotte wordt het meer en meer duidelijk dat AAA een zeer multifactoriële aandoening is met verschillende risicofactoren, genetische predispositie en grote mechanische verschillen in de verschillende AAAs. Daarom is een groot aantal, goed opgezette, grote klinische trials nodig om te corrigeren

voor alle mogelijke bias. Dit proefschrift onderstreept het belang van chronische ontsteking in de AAA vaatwand en biedt een aantal potentiële doelwitten aan voor farmacologische behandeling van de groei van het AAA. Hopelijk draagt dit proefschrift bij aan het inspireren van toekomstig onderzoek en de vertaling naar klinische trials. Dit alles met als doel uiteindelijk dichterbij het farmacologisch stabiliseren van het AAA te komen.



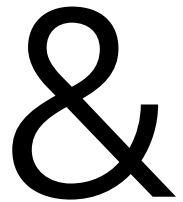


# Appendix

LIST OF PUBLICATIONS

DANKWOORD

CURRICULUM VITAE





## LIST OF PUBLICATIONS

### **Editor's Choice - Pharmaceutical Management of Small Abdominal Aortic Aneurysms: A Systematic Review of the Clinical Evidence.**

Kokje VB, Hamming JF, Lindeman JH.

Eur J Vasc Endovasc Surg. 2015 Dec;50(6):702-13. doi: 10.1016/j.ejvs.2015.08.010. Epub 2015 Oct 9. Review.PMID:26482507.

### **An association between chronic obstructive pulmonary disease and abdominal aortic aneurysm beyond smoking: results from a case-control study.**

Meijer CA, Kokje VB, van Tongeren RB, Hamming JF, van Bockel JH, Möller GM, Lindeman JH.

Eur J Vasc Endovasc Surg. 2012 Aug;44(2):153-7. doi: 10.1016/j.ejvs.2012.05.016. Epub 2012 Jun 15. PMID: 22705161.

### **Activation of the vitamin D receptor selectively interferes with calcineurin-mediated inflammation: a clinical evaluation in the abdominal aortic aneurysm.**

Nieuwland A, Kokje VB, Koning OH, Hamming JF, Szuhai K, Claas FH, Lindeman JH.

Lab Invest. 2016 Jul;96(7):784-90. doi: 10.1038/labinvest.2016.55. Epub 2016 May 30. PMID:27239732.

### **Inhibition of cysteine protease activity reduces aneurysm expansion through decreased matrix degradation.**

Kokje VB, Tammaro A, Hibender S, Xu B, Everts V, Kuiper J, van Berkel ThJC, Dalman RL, de Waard V, Lindeman JH.

Submitted for publication.

### **CXCL8 hypersignaling in the Aortic Abdominal Aneurysm: the oral CXCR2 antagonist DF2156A fully abrogates experimental aneurysm formation.**

Kokje VB, Gäbel G, Dalman RL, Koole D, Northoff BH, Holdt LM, Hamming JF, Lindeman JH.

Submitted for publication.

### **Colchicine inhibits aortic aneurysm formation in a rodent model.**

Kokje VB, Nieuwland A, Hamming JF, Lindeman JH.

Submitted for publication.

### **IL-6: A Janus-like factor in abdominal aortic aneurysm disease.**

Kokje VB, Gäbel G, Koole D, Northoff BH, Holdt LM, Hamming JF, Lindeman JH.

Atherosclerosis. 2016 Aug;251:139-46. doi: 10.1016/j.atherosclerosis.2016.06.021. Epub 2016 Jun 11. PMID:27318834

### **Adventitial Adipogenic Degeneration is an Unidentified Contributor to Aortic Wall Weakening in the Abdominal Aortic Aneurysm.**

Doderer S, Gäbel G, Kokje VB, Koole D, Northoff Holdt B, Hamming JF, Lindeman JH.

Accepted for publication in the Journal of Vascular Surgery.



## DANKWOORD

Het tot stand komen van dit proefschrift is een team effort geweest van het begin tot aan het einde. Iedereen bedankt die ervoor heeft zorg gedragen dat dit proefschrift tot stand heeft kunnen komen. Jullie samenwerking, begeleiding, gezelligheid, inspiratie en steun heeft er voor gezorgd dat ik dit onderzoek heb kunnen doen. Met het besef hier niet geheel volledig in te kunnen zijn, wil ik ook graag een aantal mensen speciaal bedanken.

Mijn promotor, prof. J.F. Hamming, wil ik van harte bedanken voor de kans die mij geboden is op de afdeling Heelkunde van het LUMC. Bedankt voor alle begeleiding en adviezen, ik heb veel geleerd.

Mijn copromotor, dr. J.H.N. Lindeman. Jan, bedankt voor het vertrouwen dat je me hebt gegeven na het keuzevak om te starten met onderzoek. Bedankt voor de samenwerking de afgelopen jaren.

Prof. R.L. Dalman, Monica, Geoff, Noriyuki, Julie. Thank you so much for the opportunity to work in your laboratory and to learn the elastase mouse model. I had a wonderful time.

Adri: bedankt voor alle hulp en begeleiding in het lab.

‘De Quax groep’: Paul, Yael, Teun, ChunYu, Margreet, Sabine, Rob, Reggie, Erna, Jacco, Mark, Zeen, en alle anderen: Bedankt dat jullie me hebben geadopteerd in de groep. De congressen, bakkies, bbqs, lunches om 12.15, etentjes en uitjes zijn hoogtepunten geweest in de LUMC jaren.

Alle co-auteurs die hebben bijgedragen aan het uitvoeren en (her)schrijven van de hoofdstukken opgenomen in dit proefschrift.

Mijn kamergenoten van D6-35: Dorrotya, Mark, Sabine, Leonie, Stephanie, Arend-Jan, Kirsten, Chantal. Bedankt voor al jullie gezelligheid en collegialiteit!

Mijn paranimfen: Lieve Joost, lieve broer, niet alleen vieren we samen onze verjaardag maar delen we alles. Wij zijn er altijd voor elkaar. Dank je wel. Een betere broer kan ik niet wensen.

Lieve Eva, al meer dan 12 jaar ben je mijn vriendinnetje. We hebben zoveel samen meegemaakt, je staat altijd voor me klaar. Ik kan je niet genoeg bedanken voor je vriendschap.

Lieve vrienden en vriendinnen: Hugoline, Maarten, Marcella, Dieuwertje, Pauline, Iris, Mira, Zsuzsa, Bibi, Emilie, Marije, V16 huisgenootjes, Claire, Marta, Antoine, Chris & Shahab. Bedankt voor jullie vriendschap, steun en gezelligheid.

Lieve Oma, we zijn twee handen op één buik. Ik ben blij dat je zo dichtbij me staat. Ik ben trots op je en weet dat jij ook trots op mij bent. Ook de rest van onze (samengestelde) familie bedankt voor jullie interesse en steun.

Papa & Mama: Dit proefschrift draag ik aan jullie op. Jullie zijn mijn rots in de branding.

Lieve Boyan, bedankt dat je er altijd voor me bent.

## CURRICULUM VITAE

Vivianne Kokje was born on August 6<sup>th</sup>, 1987, in Delft, The Netherlands. After graduating (with honors) from the athenaeum Stebo in The Hague, she started medical school at the Leiden University in 2005. During her studies, she worked as a medical assistant at both the Ear, Nose and Throat department as well as the Intensive Care Unit at the Leiden University Medical Center (LUMC). In addition, she did volunteer work in different hospitals in Nepal, Ghana and Uganda.

In 2008, when she participated in an 'Aneurysm and Rupture' elective course, she was given the opportunity to conduct her scientific internship with Dr. J.H.N. Lindeman at the Surgery department of the LUMC. This internship was extended in 2009 into a PhD program, for which she spent several months working at the laboratory of Dr. R.L. Dalman at Stanford University (USA). The results of her research under the supervision of Prof. Dr. J.F. Hamming and Dr. J.H.N. Lindeman are depicted in this thesis.

After completing her clinical rotations and graduating 'cum laude' from medical school in 2016, Vivianne started working as a General Surgery resident at the Medical Center Haaglanden in The Hague. The year after, she worked as a Plastic and Reconstructive Surgery resident at the LUMC. She moved to Geneva, Switzerland in 2016 to work as a post-doctoral researcher at the Ear, Nose and Throat department of the University Hospital of Geneva (HUG) under the supervision of Dr. P. Senn.

Currently, Vivianne is an Ear, Nose and Throat resident, under the supervision of Prof. C. Simon, at the Lausanne University Hospital (CHUV), Switzerland. She combines her clinical work with research in both the hospital in Lausanne (CHUV) and Geneva (HUG).



